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HIGHLIGHTS IN PEDIATRIC NEPHROLOGY: 2021

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Febrile Proteinuria in Hospitalized Children: Characterization of Urinary Proteins

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Background: Transient proteinuria during febrile illness is a common phenomenon. Recent studies have re-examined the pathophysiology of proteinuria and new urinary markers to characterize it, including B7-1 (CD80), which is expressed also in glomerular podocytes and influences the glomerular barrier.

Aim: To investigate the pattern of proteinuria in febrile non-renal diseases, including B7-1.

Methods: We prospectively analyzed urine samples of 44 febrile children and 28 afebrile controls for different protein components: albumin (glomerular marker), β2-microglobulin (tubular marker), uromodulin (Tamm Horsfall protein-THP, a renal endogenous protein) and B7-1. Febrile illness was characterized as focal bacterial vs. viral. Exclusion criteria were underlying renal disease, steroid treatment or urinary tract infection.

Results: Elevated urine albumin (64.5 ± 10.3 vs. 17.8 ± 4 mg/g, mean ± S.E.M., $p = 0.0009$) and β2-microglobulin (1.44 ± 0.34 vs. 0.182 ± 0.03 mg/g, mean ± S.E.M., $p = 0.005$] and decreased uromodulin (10.5 ± 1 vs. 26.7 ± 2.2 Arbitrary units, mean ± S.E.M., $p = 0.0001$) excretion were found during febrile illness vs. controls. Urine B7-1 was also increased in the febrile group (0.27 ± 0.05 vs. 0.07 ± 0.01 ng/ml, mean ± S.E.M., $p = 0.001$), and was the only marker which was significantly higher in bacterial vs. viral disease.

Conclusions: Febrile proteinuria is not generalized: while proteins of both glomerular and tubular origin increase, uromodulin decreases. Urine B7-1 is increased during fever, more significantly in bacterial infections. Thus, urinary B7-1 may be used as an additional marker to differentiate between febrile states of bacterial vs. viral origin.

Keywords: β2-microglobulin, uromodulin, CD80 (B7-1) protein, albuminuria, proteinuria, fever

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INTRODUCTION

Normal glomerular barrier prevents the filtration of high molecular weight proteins into the Bowman's capsule. Subsequently, proximal tubular reabsorption retrieves proteins of lower molecular weight that escaped glomerular barrier, leading to a minimal amount of protein of circulation origin in urine. In addition, uromodulin, or Tamm Horsfall protein is synthesized

and partly secreted by the distal tubule and found in urine in normal states (1, 2). Transient proteinuria is a common finding in several illness states. It has been described during fever, sepsis, trauma and anaphylaxis, without relation to renal disease (3). Several studies in the past have described transient proteinuria during febrile illness and whether its origin was glomerular or tubular (4, 5). However, no studies have been performed on the role of urinary uromodulin in this process. Recent studies have reported new evidence on the pathophysiology of infection related proteinuria, including the role of B7-1(CD80) protein, expressed on both circulating leucocytes as well as on glomerular epithelial cells (podocytes). This protein was shown to modulate the podocyte cytoskeleton and influence on glomerular filtration barrier in different proteinuric states (6, 7). No studies have been performed to simultaneously examine the involvement of markers of different renal compartments (glomerular, tubular, and endogenously synthesized), including B7-1, in this common phenomenon. We hypothesized that glomerular B7-1 may be upregulated also in transient febrile proteinuria. Therefore, in this study we characterized the type of urinary proteins (albumin, β2-microglobulin, uromodulin, and B7-1) in children with febrile proteinuria.

PATIENTS AND METHODS

The research protocol of the study has been approved by the local Helsinki committee. Study consent was signed by patients' parents. In this prospective study, children with febrile illness (temperature above 38°C) of infectious origin, hospitalized in pediatric wards were enrolled. The exclusion criteria were underlying renal disease, signs of urinary tract infection, or corticosteroid treatment. Children without fever who were hospitalized for elective surgical procedures served as controls. Clinical, laboratory and imaging data were recorded for each patient and the child was finally diagnosed as having a viral [no specific focus of infection, normal peripheral blood leucocyte (WBC) count] or a specific bacterial disease.

TABLE 1 | Demographic characteristics.

	Febrile	Afebrile	*P* value
Number	44	28	
Age (mean ± S.E.M.)	6.6 ± 0.79	4.9 ± 0.72	0.12
Male (%)	27 (60%)	21 (75%)	NS
Background illness	17 (39%)	3 (11%)	0.02
Previous hospitalization	18 (41%)	8 (29%)	NS
Temperature on admission (°C), (mean ± S.E.M)	38.6 ± 0.9		
Maximal temperature (°C), (mean ± S.E.M)	39 ± 0.74		
Hours of fever prior to admission (mean ± S.E.M)	31 ± 4		
Suspected bacterial disease (%)	23 (52%)		

Urine samples in both study and control groups were collected at the time of admission. Urine samples were checked with Multistix® urinalysis strips for white blood cells, nitrites, protein, blood and specific gravity. Then urine samples were biochemically tested for total protein, albumin (as glomerular injury marker), β2-microglobulin (tubular marker), uromodulin (Tamm Horsfall protein-THP, a renal endogenous protein), and B7-1 (CD-80) protein. Urine protein response on the sticks was determined using a semi quantitative determination (+1 to +4) method by photometric color test. Urine creatinine was determined using kinetic color test (Jaffé method). Urine albumin was determined by immune turbidometric test. All these tests were performed using an Olympus Life &[[Inline Image]]Material Science system. Urinary β2-microglobulin was determined using Microparticle Enzyme Immunoassay (MEIA)(Abbott Ax SYM, Germany). Tamm Horsfall protein (THP)/uromodulin concentration was analyzed by Western blot analysis using rabbit anti THP antibody (Santa Cruz). Protein expression was quantified densitometrically using *Image J* software and expressed as arbitrary units (AU). Urine B7-1 (CD80) protein concentration was analyzed using an enzyme-linked immunosorbent assay kit (Human sCD80 Instant Elisa Kit, eBioscience, Affymetrix, North America), according to the test protocol. Briefly, after a 3 h incubation of 50 μl urine samples at room temperature, the microwell strips were washed 3 times with approximately 400 μl wash buffer per well. Then 100 μl of TMB substrate solution was pipetted to all wells and the microwell strips were incubated again at room temperature for 10 min. The substrate reaction was stopped by quickly pipetting 100 μl of Stop Solution, and then immediately read for absorbance at 450 nm using a spectro-photometer (SpectraMax Paradigm Multi-Mode Microplate Reader, SoftMax Pro Software, 2014). A standard curve was created by plotting the mean absorbance for each standard concentration on the ordinate against the measured sCD80 concentration on the abscissa.

Group comparison was performed using standard statistical tests: *t*-test for continuous variables, chi square for categorical values and ANOVA test for comparison of more than 2 continuous variables. Analyzing receiver operating characteristic (ROC curves) was assessed using *pROC*, an open-source package (8).

RESULTS

Fifty-six febrile children admitted to the hospital were approached for consent. 12 patients were excluded, 11 because of insufficient amount of urine and one patient because of positive urine culture. Finally 44 febrile children age 2 months−17.7 years, (6.6 ± 0.79 years, mean ± S.E.M.) were enrolled and compared with 28 controls age 3 months−16.5 years (4.9 ± 0.72 years, mean ± S.E.M., $p = 0.12$). There were 27 (60%) males in febrile group vs. 21 (75%) ones in the control group. Seventeen patients (39%) in febrile group vs. 3 patients (11%) in the control

group had some background illness, 18 (41%) in febrile group vs. 8 (29%) in the control group were previously hospitalized (**Table 1**). Temperature on admission in febrile group was 38.6 ± 0.9°C, maximal temperature was 39 ± 0.74°C, the fever duration prior to admission was similar for both bacterial and viral groups and averaged 31 ± 4 h (mean ± S.E.M). In the study group febrile disease was of bacterial origin in 52% of cases and of viral origin in 48%. Bacterial diagnoses included: pneumonia (9), rickettsiosis (2), dysentery (4), cellulitis with abscess (1), mastoiditis (1), acute otitis media (1), and occult bacteremia (1). The diagnosis of pneumonia was based on positive findings on chest X-Ray examinations (lobar infiltrate). In one patient blood serologic test was positive for Mycoplasma Pneumonia. Blood cultures were negative in all the patients with pneumonia except one which was positive for Pneumococcus Pneumonia. All patients with pneumonia except two had elevated leucocyte count (24 ± 2.6*10^3/ul, mean ± S.E.M.). In the patient with rickettsiosis, the diagnosis was based on clinical and laboratory findings and confirmed serologically. Mastoiditis was diagnosed based on clinical findings and leukocytosis (17*10^3/μl). In the patient with dysentery, stool cultures were positive for shigella (2), salmonella (1), and campylobacter (1). In the patient with an abscess and cellulitis, positive culture for Staph aureus was obtained from the pus. Otitis media was diagnosed clinically, but this patient was also diagnosed with pneumonia, confirmed by a chest X-ray. The diagnosis of occult bacteremia was made based on fever and leukocytosis (20*10^3/μl). Febrile patients without specific focus of infection and normal peripheral blood leucocyte count were diagnosed as having viral infections.

Urine samples were obtained 31 ± 4.1 h (mean ± S.E.M.) from the beginning of fever. The assessment of different urinary proteins is summarized in **Table 2**. Multistix® urinalysis strips were positive for protein (in those samples with specific gravity of more than 1010) in 13 out of 43 febrile patients (30%) and only in 1 patient from the control group (3.7%) (two-tailed $\chi^2 = 0.005$). In the febrile group urine protein/creatinine ratio was above normal (200 mg/g) in 32 cases (72.2%) vs. 5 cases (18%) in the control group ($\chi^2 = 0.001$). In the febrile group 27 patients (60%) had a urine albumin\creatinine ratio above 30 mg/g (upper limit of norm) vs. 3 patients in the control group ($\chi^2 < 0.005$). Urinary albumin\creatinine ratio in the febrile group was 64.5 ± 10.3 mg/g, mean ± S.E.M. Of note, the albumin fraction in urine was less than half of the total protein. There was a wide distribution in urine β2-microglobulin excretion in the study group: 1.44 ± 0.34 mg\g, mean ± S.E.M. In the control group, urine β2-microglobulin was significantly lower (0.182 ± 0.03 mg/g, mean ± S.E.M, $p = 0.01$) (**Table 2**). Tamm-Horsfall Protein (THP) excretion in urine was significantly decreased in febrile children vs. control (10.5 ± 1 vs. 26.7 ± 2.2 arbitrary units, mean ± S.E.M, $p = 0.0001$).

The study group comparison was further subdivided into three groups: afebrile children, patients having febrile disease of bacterial origin (according to above mentioned criteria) vs. non-bacterial origin. As mentioned, total protein excretion was higher in febrile children vs. controls. However, there was no difference between bacterial and nonbacterial febrile groups. Albumin excretion was also elevated in febrile children vs. control without a clear difference between nonbacterial and bacterial groups (**Table 2**). There was no significant difference in urine

TABLE 2 | Characteristics of proteinuria.

Urine values (mean ± S.E.M)	Normal value	Afebrile ($n = 28$)	Febrile		*P*-value AF vs. F	*P*-value Bacterial vs. Viral
Protein/creatinine (mg/g)	<200	150 ± 17	Total ($n = 44$)	430 ± 54.5	0.0001	
			Bacterial ($n = 23$)	480 ± 83.3		NS
			Viral ($n = 21$)	380 ± 65.3		
Albumin/creatinine (mg/g)	<30	17.8 ± 4	Total ($n = 44$)	64.5 ± 10.3	0.0009	
			Bacterial ($n = 23$)	44.7 ± 8.85		NS
			Viral ($n = 21$)	117.9 ± 33.2		
β2mgl/creatinine (mg/g)	≤0.132	0.182 ± 0.03	Total ($n = 44$)	1.44 ± 0.34	0.005	
			Bacterial ($n = 23$)	1.218 ± 0.35		NS
			Viral ($n = 21$)	1.693 ± 0.58		
B7-1 (ng/ml)	NA	0.07 ± 0.01	Total ($n = 25$)	0.27 ± 0.05	0.001	
			Bacterial ($n = 11$)	0.4 ± 0.07		0.002
			Viral ($n = 14$)	0.14 ± 0.04		
Tamm-Horsfall protein (AU)	NA	26.7 ± 2.2	Total ($n = 44$)	10.5 ± 1	0.0001	
			Bacterial ($n = 23$)	7 ± 1		0.14
			Viral ($n = 21$)	9.5 ± 1.3		

AF-afebrile, F-febrile, NA-not available, NS-not significant, AU-arbitrary units. AF-afebrile, AU-arbitrary units, β2mgl-beta-2 microglobulin, F-febrile, NA-not available, NS-not significant.

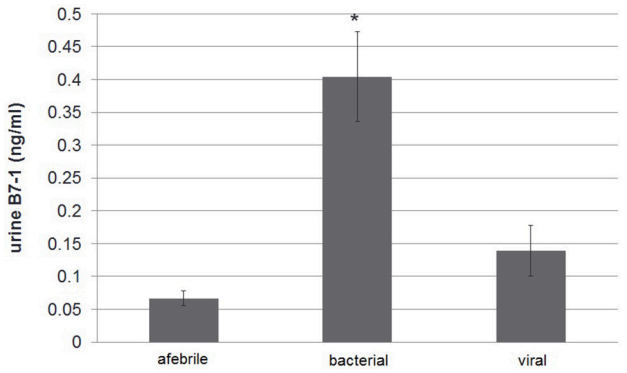

FIGURE 1 | Urine B7-1 excretion in febrile disease of bacterial vs. viral origin and vs. control afebrile group: urine B7-1 concentration was significantly higher in cases of bacterial disease vs. viral and in comparison with the control group $^*p < 0.0001$.

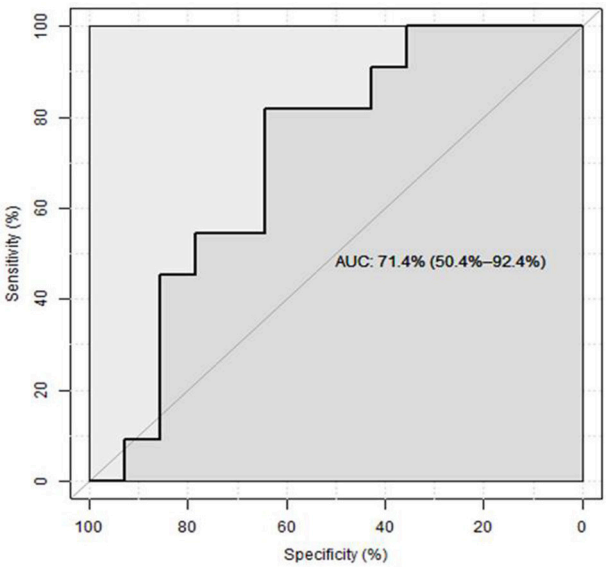

FIGURE 2 | ROC curve assessing the predictive value of urine B7-1 for bacterial infection in febrile children (8).

β2-microglobulin excretion between bacterial and nonbacterial groups but its excretion was significantly elevated in each of these groups vs. control ($p < 0.05$). Uromodulin (THP) excretion was lower in the bacterial vs. nonbacterial group, but this difference was not significant ($p = 0.14$).There was a significantly higher excretion of B7-1 in urine in the febrile group vs. afebrile controls (0.27 ± 0.05 vs. 0.07 ± 0.01 ng/ml, mean ± S.E.M., $p = 0.0001$). Urine B7-1 concentration was also significantly higher in cases of bacterial disease vs. nonbacterial (0.4 ± 0.07 vs. 0.14 ± 0.04 ng/ml, mean ± S.E.M., $p < 0.002$) (**Table 2**) (**Figure 1**). An ROC curve that tests the performance of urine B7-1 to differentiate between viral and bacterial infections yielded an AUC value (±S.E.M) of 0.85 ± 0.08 (95% CI: 0.71–0.99, $p = 0.003$) (**Figure 2**). The optimal mathematical point along the curve is a urine B7-1 value of 0.132, providing a sensitivity of 91.7% and a specificity of 61.5%. Thus, there was no significant difference in urine excretion of any of the checked proteins in the cases of bacterial vs. viral infection except for urine B7-1 that was significantly higher in the cases of bacterial infections.

DISCUSSION

In this study we first characterized the type of urinary proteins in children with febrile proteinuria. Elevated urinary total protein (in the mild-non-nephrotic range), urine albumin and β2-microglobulin excretion were found in febrile children. Urine B7-1 excretion also was higher in the cases of febrile states. Not all urinary proteins showed a pattern of transient elevation during febrile illness. For example, uromodulin (Tamm-Horsfall protein), that is excreted in urine in normal states, was actually significantly decreased during fever. This protein is secreted by the kidney and is thought to play role in water\electrolyte balance and kidney innate immunity. Studies in *Umod* knockout mice showed that uromodulin has a defensive role against urinary tract infection (UTI) (10, 11), since this protein binds to pathogens of the urinary tract, such as type 1-fimbriated *E. coli*, and interferes with their binding to uroplakins on the urothelium (12). Several *in vitro* studies showed that THP is able to bind to immunity-related molecules, such as immunoglobulin G, complement 1q, and tumor necrosis factor-α (9, 13, 14). Uromodulin also acts as a chemoattractant (15) and as a proinflammatory molecule. It interacts with monocytes, neutrophils and myeloid dendritic cells leading to activation of components of the immune system via toll-like receptor 4 (16). The physiological role of this process is still unclear. Saemann et al. hypothesize that uromodulin is released into kidney parenchyma in cases of tubular damage and serves as a signal to activate local immune response to prevent bacterial invasion (16). This unexpected decreased urinary THP excretion during fever can be explained by its excessive excretion into blood, as Prajcer et al. showed that inflammatory damage in thick ascending limb leads to decreased urinary and increased blood uromodulin level (17). Medullary cystic kidney disease type 2 (MIM 603860) and familial juvenile hyperuricemic nephropathy (MIM 162000) are autosomal dominant tubulointerstitial kidney diseases that are due to UMOD gene mutations and are collectively called uromodulin-associated kidney disease (UAKD). Decreased urine uromodulin excretion in these diseases leads to nephropathy, interstitial nephritis, hyperuricemia, renal stone formation and renal insufficiency. In this study, in spite of elevated albumin and β2-microglobulin urine excretion during febrile disease, most of urine protein was neither albumin nor β2-microglobulin. The increase in urinary albumin excretion suggests that additional high molecular weight proteins may also be excreted, most probably due to a transient disturbance in glomerular barrier. Recent studies have reported new evidences on the pathophysiology of proteinuria of glomerular origin, including the role of B7-1. This protein is expressed in glomerular epithelial cells (podocytes) and influences glomerular anatomical barrier in different proteinuric states. B7-1 influences the actin

cytoskeleton of podocytes and slit diaphragm organization leading to proteinuria. Both genetic aberrations (e.g., deletion of α3 integrin or nephrin), toxic stimuli (such as PAN induced reactive oxygen species), or direct stimulation of the TLR-4/CD14 receptor on the podocyte can cause B7-1 induction. B7-1 then induces the podocyte's foot process effacement and disruption of the slit diaphragm complex, leading to proteinuria. Reiser et al. (6) also showed rapid upregulation of B7-1 in podocytes and nephrotic-range proteinuria by *in vivo* exposure to low-dose LPS in wild type and SCID mice. B7-1 knockout mice were protected from this LPS induced nephrotic range proteinuria, suggesting a role of podocyte (and not white blood cell) B7-1 expression in the pathogenesis of proteinuria. Podocyte's B7-1 is upregulated in patients with certain glomerular diseases. Positive B7-1 immunostaining was observed in biopsy specimen from patients with recurrent focal segmental glomerulosclerosis (FSGS) a disease associated with severe proteinuria. Yu et al. showed the resolution of nephrotic range proteinuria after B7-1 inhibition with Abatacept (CTLA-4–Ig) treatment in patients with rituximab-resistant recurrent FSGS and in patients with glucocorticoid-resistant primary FSGS (18), suggesting that B7-1 can be a target for the treatment of proteinuria. However, these findings have recently been challenged by Baye et al. (19). These new observations led us to explore the possible role of B7-1 in febrile children. As mentioned, B7-1 was found to be the only urine biomarker that was not just significantly higher in febrile disease vs. controls, but also higher in febrile cases of bacterial disease vs. viral origin.

Activation of toll-like receptors (TLRs) is basic in the initiation of innate immunologic response (20, 21). For example, exposure to lipopolysaccharide (LPS) induces B7-1 expression via TLR-4 in podocytes, leading to reorganization of its cytoskeleton, foot process effacement and proteinuria. B7-1 acts as a costimulatory molecule in this process, as previously mentioned (6). We suppose that in the cases of bacterial infections toxins or other bacterial components lead to proteinuria via similar mechanism and B7-1 acts as a co-stimulatory molecule in this process, being upregulated and eventually found in urine.

LIMITATIONS OF THE STUDY

The study was performed on a relatively small sample of patients, due to budget limitations. Thus, the negative statistically significant differences found between the bacterial and viral groups may become significant in larger study groups. In addition, only a limited number of urinary excreted proteins were analyzed in this study, and the correlation between blood level of these proteins and their excretion was not assessed.

In summary, in this first study we have shown that febrile proteinuria is not a generalized nonspecific phenomenon: while proteins of both glomerular and tubular origin increase, uromodulin decreases. Urine B7-1, a possible marker of increased glomerular permselectivity is increased during fever, more significantly in bacterial infections. Thus, if verified by larger studies, urinary B7-1 may be used as an additional marker to differentiate between febrile states of bacterial and viral origin.

ETHICS STATEMENT

The research protocol of the study has been approved by the Soroka University Medical Center local Helsinki committee. Study consent was signed by patients' parents.

AUTHOR CONTRIBUTIONS

EG and EI recruited the patients. EI wrote a preliminary report. EG wrote the manuscript's first draft. YS performed the laboratory analyses and reviewed the manuscript. DL conceived the study design and finalized the manuscript's last version.

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Genetic Testing for Steroid-Resistant-Nephrotic Syndrome in an Outbred Population

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Background: Steroid-resistant nephrotic syndrome (SRNS) is a leading cause of end-stage kidney disease in children and young adults. Despite advances in genomic science that have led to the discovery of >50 monogenic causes of SRNS, there are no clear guidelines for genetic testing in clinical practice.

Methods: Using high throughput sequencing, we evaluated 492 individuals from 181 families for mutations in 40 known SRNS genes. Causative mutations were defined as missense, truncating, and obligatory splice site variants with a minor allele frequency <1% in controls. Non-synonymous variants were considered pathogenic if determined to be deleterious by at least two *in silico* models. We further evaluated for differences in age at disease onset, family history of SRNS or chronic kidney disease, race, sex, renal biopsy findings, and extra-renal manifestations in subgroups with and without disease causing variants.

Results: We identified causative variants in 40 of 181 families (22.1%) with SRNS. Variants in *INF2*, *COL4A3*, and *WT1* were the most common, accounting for over half of all causative variants. Causative variants were identified in 34 of 86 families (39.5%) with familial disease and 6 of 95 individuals (6.3%) with sporadic disease (χ^2 $p < 0.00001$). Family history was the only significant clinical predictor of genetic SRNS.

Conclusion: We identified causative mutations in almost 40% of all families with hereditary SRNS and 6% of individuals with sporadic disease, making family history the single most important clinical predictors of monogenic SRNS. We recommend genetic testing in all patients with SRNS and a positive family history, but only selective testing in those with sporadic disease.

Keywords: focal segmental glomerulosclerosis, genetic testing, monogenic disease, podocyte, steroid-resistant nephrotic syndrome

BACKGROUND

Nephrotic syndrome (NS) is a clinical syndrome characterized by massive proteinuria, hypoalbuminemia, peripheral edema, and hyperlipidemia. It may be complicated by venous thromboembolism, cardiovascular disease, and increased risk of infections due to loss of immunoglobulins (1, 2). With an annual incidence of 2–7 per 100,000 and a prevalence rate of 16 per 100,000, NS is the most common glomerular disease in children and adults (2–4).

The initial response to corticosteroid treatment is the most important clinical predictor of long-term prognosis (5, 6). The majority of patients with steroid-resistant nephrotic syndrome (SRNS) will progress to end-stage kidney disease (ESKD) within 5–10 years of diagnosis (7, 8). In addition, SRNS accounts for >10% of all cases of children with ESKD and is the most prevalent diagnosis among children receiving maintenance dialysis (1, 9, 10).

The two most common histopathologic changes seen on renal biopsy are minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) (1). MCD is the most common histopathological finding in children, but the prevalence of FSGS is increasing (11). Under light microscopy, there are no notable changes in MCD, but electron microscopy demonstrates microvillus transformation and impaired podocyte foot process adherence to the glomerular basement membrane (12). Patients with FSGS have segmental scarring of the glomeruli that progresses over time. While 95% of patients with MCD achieve remission after 8 weeks of corticosteroid treatment, only 20% with FSGS have a complete response to steroids (2).

The precise molecular pathophysiology of SRNS remains unclear, but it is understood to be caused by podocyte dysfunction or loss, leading to dysfunction of the charge- and size-selective glomerular filtration barrier (3, 12). The glomerular filtration barrier consists of three layers- a fenestrated capillary endothelium, the glomerular basement membrane, and podocytes with interdigitating foot processes connected by a slit diaphragm. In healthy tissue, the podocyte excludes the filtration of albumin and other macroproteins (13). Changes in the podocyte architecture lead to increased permeability and the resultant proteinuria seen in NS. MCD is characterized by reversible changes to the podocyte architecture without podocyte loss (12). Conversely, FSGS is characterized by podocyte depletion; 20-40% podocyte loss leads to segmental scarring, which in turn causes glomerular enlargement and further podocyte depletion (12).

Early studies of congenital NS led to the discovery of mutations in *NPHS1* (which encodes the podocyte protein nephrin) as a cause of early onset SRNS (14). Advances in genomic technologies over the past 20 years, including the advent of next-generation sequencing, have led to the discovery of >50 genes associated with SRNS (10, 15). The vast majority of these genes code for proteins located in the glomerular filtration barrier, specifically within the podocyte and slit diaphragm, hence SRNS is regarded as a podocytopathy (3, 15).

Single-gene (monogenic) causes of kidney disease account for a minority of cases, with an estimated prevalence between 5 and 30% (7, 15–18). Despite technological advances allowing for easier and more cost-effective detection of monogenic kidney disease, the likelihood of identifying a genetic cause decreases with increasing age of disease onset (19, 20). Mutations in autosomal recessive genes tend to present early in childhood. Autosomal dominant genes tend to have varying degrees of penetrance and severity and typically present later in childhood or into adulthood (4, 8, 21).

The clinical utility of genetic testing in NS is evolving, but there are no clear guidelines for clinical practice. Recommended testing criteria have included testing all patients with SRNS vs. only those with early-onset SRNS, patients with extra-renal manifestations, patients with a family history of NS or consanguineous background or all patients with NS prior to biopsy or completion of steroid treatment (4, 8, 9, 17, 21, 22). Many recommendations are based on studies of cohorts enriched for monogenic disease, such as those with congenital or infantile NS or cohorts with high rates of consanguinity. In this study, we sought to use high throughput sequencing to determine the prevalence of mutations in 40 known SRNS genes and to identify which clinical characteristics were associated with high probability of identifying genetic SRNS in a heterogeneous population with low rates of consanguinity.

METHODS

Human Participants

This study was approved by the institutional review board of Duke University Medical Center and the institutional review boards of all collaborating sites. Written informed consent was obtained from study participants and from the parents of participants under the age of 18. Patients were enrolled from 1998 to 2017 after obtaining informed consent. Subjects in this international cohort were enrolled from multiple centers in the United States, Canada, New Zealand, United Kingdom, Nigeria, and Sri Lanka. Eligible participants in this study included patients with a clinical diagnosis of nephrotic syndrome defined as proteinuria >40 mg/m^2/h, hypoalbuminemia, and edema or patients with biopsy-proven FSGS or MCD. Clinical records of all subjects were reviewed for age at diagnosis of disease, biopsy reports, race, sex, full family history, and presence or absence of extra-renal manifestations suggestive of syndromic disease. Additional data were gathered on treatment course, including response to initial corticosteroid therapy and history of recurrence of NS following kidney transplant.

Preliminary Genomic Sequencing

Eligible families were analyzed by direct sequencing of candidate genes, linkage analysis, and next generation sequencing methods including whole-exome sequencing (WES) and podocyte-exome sequencing. Sequencing and variant analysis were performed as previously described (23–29).

Targeted Sequencing of Custom Amplicons

DNA from the probands of families who had not undergone genetic sequencing prior to April of 2017 and those in whom no causative variant was identified by prior methods

were analyzed using targeted sequencing of custom amplicons (TSCA). One hundred eighty-one families with SRNS in whom no causal variant was previously identified and four control subjects with a known causative mutation (positive controls) were analyzed using TSCA. Using Illumina Design Studio (Illumina Inc., San Diego, CA), 1,528 amplicons 250 bp in length were designed and selected to cover all coding regions and 5′ untranslated regions of 45 known SRNS genes and NS risk genes. The library preparation was performed at the Duke Molecular Physiology Institute using Illumina TrueSeq kit. Genomic sequencing was performed by the Duke Center for Genomic and Computational Biology on the Illumina NextSeq 500 platform, mid-output, with 150 bp paired-end reads according to the manufacturer's guidelines. The average genomic coverage was 932×. Sequence reads were aligned to the GRCH37/hg19 human reference genome using BWA-MEM. Using internal quality control metrics for each sample, variants with poor quality were removed. Effects were predicted using SNPEff and the Ensembl Variant Effect Predictor, and variants with an effect of "INTRON," "INTRAGENIC," "UTR_3_PRIME," and "UTR_5_PRIME," or no effect reported were removed.

Causative Variant Calling

Five sequenced genes (*PLCG2, APOL1, MYH9, HLA-DQA1,* and *COL4A5*) were excluded from this analysis; four are risk alleles (*PLCG2, APOL1, MYH9, HLA-DQA1*) and one (*COL4A5*) is associated with X-linked disease. We evaluated TSCA data for causative variants in the remaining 40 known SRNS genes (**Supplementary Table S1**, **Figure 1**). We removed all variants with a minor allele frequency ≥1%, synonymous variants, and intronic variants except for those at obligatory splice site regions. The remaining variants included nonsynonymous variants, truncating variants, and obligatory splice site variants.

For the ten autosomal dominant genes screened by TSCA (*INF2, COL4A4, COL4A3, ACTN4, ANLN, TRPC6, WT1, CD2AP, ARHGAP24,* and *LMX1B*), we filtered for heterozygous variants. For the remaining 30 autosomal recessive genes, we filtered first for homozygous variants. In patients with potential compound heterozygous variants (i.e., two or more heterozygous variants in a single gene) further variant analysis was performed only on those in whom parental DNA was available.

All potential disease-causing variants identified by WES, candidate gene sequencing, and TSCA were confirmed by Sanger sequencing. All sequence variants were analyzed using Sequencher™ software (Gene Codes Corp., Ann Arbor, MI, United States). For patients with potential compound heterozygous variants identified by TSCA, Sanger sequencing was also performed on the parents to determine whether variants were present on a single chromosome (cis) or alternate chromosomes (trans).

The confirmed variants were evaluated using three *in silico* software models [PolyPhen-2 (30), SIFT (31), and MutationTaster (32)] to determine the effect of amino acid changes. Those found to be deleterious by at least two models were considered to be pathogenic.

Statistical Analysis

Continuous variables are expressed as median and interquartile range. Comparisons between categorical variables were made using the chi-square test, and $p < 0.05$ was considered significant.

RESULTS

Cohort Description

Our study cohort was comprised of 492 patients from 181 families with SRNS with a low prevalence of consanguinity (**Table 1**). The majority of the families (150 of 181, 82.9%) were enrolled from the United States.

Eighty-six families (47.5%) had a family history of SRNS or chronic kidney disease (CKD). Sixty-five of these families (75.6%) had an autosomal dominant pattern of inheritance, defined as ≥2 affected individuals in two or more generations. Twenty-one families (24.4%) had an autosomal recessive pattern of inheritance, defined as ≥2 affected individuals in a single generation. Ninety-five individuals were enrolled who had no family history of SRNS or CKD. These individuals were classified as having sporadic disease.

The male-to-female ratio of our cohort was 1.5:1. The median age at diagnosis was 12 years (range 1–61 years). Fifty-eight percent of the cohort were white non-Hispanic, and 25.4% were black. Renal biopsy results were available for the probands of 153 families. FSGS was the most common histological finding and was present in 109 cases. MCD was present on 26 renal biopsies.

Identification of Causative Variants

We evaluated results from whole-exome sequencing, candidate gene sequencing, and targeted sequencing of custom amplicons to identify variants in 40 genes known to cause SRNS. We detected causative variants in 40 of 181 families (22.1%) in 12 known SRNS genes (**Supplementary Table S2**). Variants in *INF2* (12 families), *COL4A3* (5 families) and *WT1* (4 families) were the most common and accounted for 55% of all identified causative variants.

We detected 38 distinct variants in 12 of 40 known SRNS genes (**Supplementary Table S2**). Nineteen of these variants have been previously reported and can be found in public databases. Thirteen novel variants were first discovered in this cohort and have been reported by our group (23–29). We identified eight likely disease-causing variants by TSCA, including six novel variants in five known SRNS genes (*WT1, ACTN4, INF2, TRPC6,* and *NPHS2*) (**Table 2**, **Figure 2**). There were no compound heterozygous variants confirmed by TSCA.

Clinical Factors Associated With Mutation

Causative variants were identified in 34 families (39.5%) with a family history of disease, compared to only six individuals (6.3%) with sporadic disease (**Table 3**). This difference was statistically significant (χ^2 28.93, $p < 0.0001$). The difference in the mutation detection rate between patients with an autosomal dominant pattern of inheritance (29 of 65 families) and those

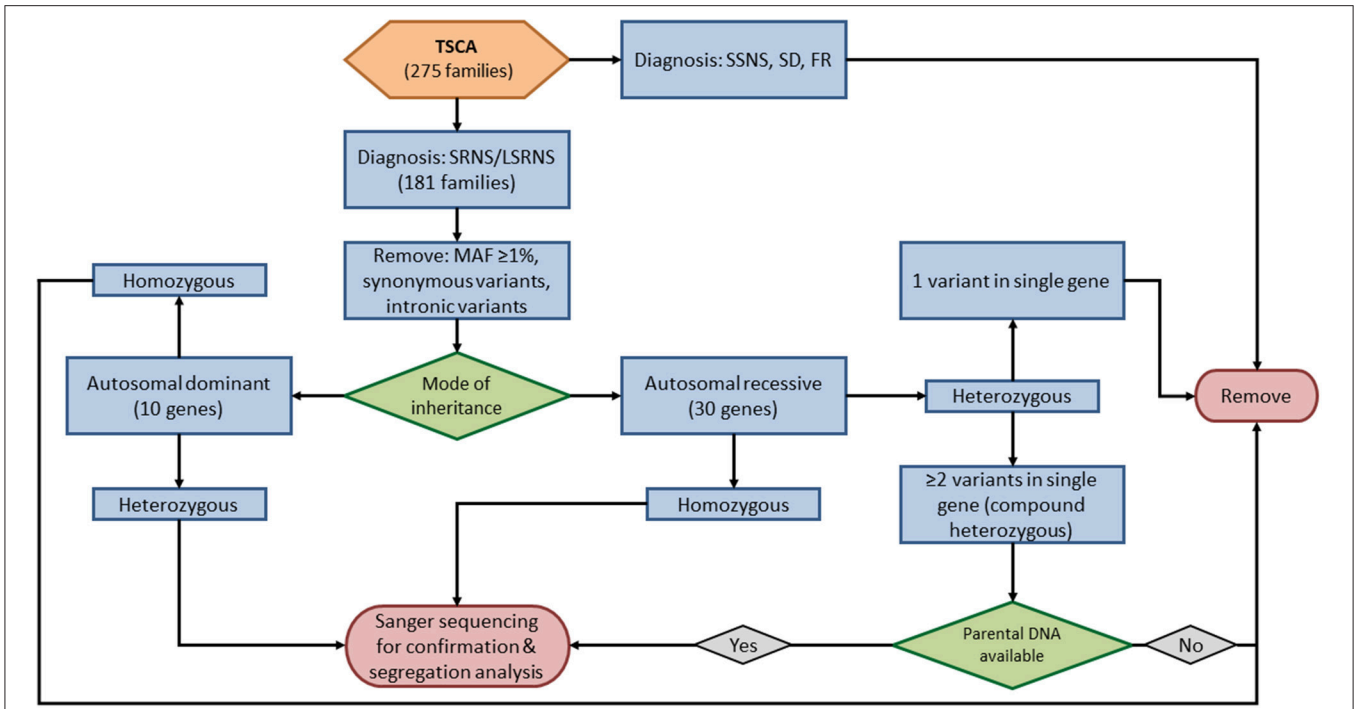

FIGURE 1 | Filtering parameters for variants identified by targeted sequencing of custom amplicons.

TABLE 1 | Demographics of 181 families with familial and sporadic SRNS.

Characteristic	Autosomal dominant	Autosomal recessive	Sporadic	Full cohort
Age at onset (median in years) [interquartile range]	23 [15]	20 [19]	6 [10]	12 [19.5]
SEX				
Male (%)	22 (33.8)	15 (71.4)	55 (57.9)	92 (50.8)
Female (%)	43 (66.2)	6 (28.6)	40 (42.1)	89 (49.2)
RACE				
White, Non-hispanic	41	17	48	106 (58.6)
Black	17	3	26	46 (25.4)
Hispanic	6	0	14	20 (11.0)
Other	1	1	7	9 (5.0)
HISTOLOGY				
FSGS	39	14	56	109 (60.2)
MCD	4	1	21	26 (14.4)
Other	1	1	16	18 (10.0)
No biopsy (includes ESKD)	21	5	2	28 (15.5)
US sample (%)	48 (73.8)	15 (71.4)	87 (91.6)	150 (82.9)

Age at onset and race are defined by the proband in each family.

with an autosomal recessive inheritance (5 of 21 families) was not significant (χ^2 2.87, $p = 0.09$). There was no significant difference in mutation detection rate based on race (white vs. non-white χ^2 2.77, $p = 0.10$), country of origin (US vs. non-US χ^2 0.41, $p = 0.52$), sex (χ^2 1.43, $p = 0.23$), or renal biopsy findings (FSGS vs. MCD χ^2 0.25, $p = 0.61$).

DISCUSSION

Due to a lack of population-based studies, the prevalence of monogenic SRNS in children with SRNS is unknown.. However, data from different cohorts suggest that the prevalence varies between 5 and 30%, depending on the population being studied; higher prevalence rates are generally found in populations with high rates of consanguinity and populations with founder mutations in different genes (33–35).

Prior investigations into the prevalence of monogenic disease have been performed on cohorts selected to enhance the likelihood of identifying monogenic NS. In a large international study of patients with SRNS that showed a 30% prevalence of monogenic NS, fewer than 20% of families were from the United States, and many were from regions with a high degree of consanguinity (16). A more recent study from the longitudinal Nephrotic Syndrome Study Network (NEPTUNE) identified an overall prevalence of only 4.2% in US patients with sporadic disease (33).

Our study was designed to provide guidelines for clinical practice in the United States by screening a cohort that is generalizable to the US population. More than 80% of the 181 families enrolled in this study were from the US, with a low rate of consanguinity. There was no significant difference in the rate of mutation detection between the US and non-US subgroups. Furthermore, family history was collected from all participants

TABLE 2 | Six novel variants in known SRNS genes identified by targeted sequencing of custom amplicons.

Family	Race	Country	Gene	Protein change	Inheritance	Age at onset (yr)	Renal biopsy	Diagnosis
6,511	White	USA	*WT1*	p.T416A	AD	31–35	FSGS	SRNS
6,586	White	USA	*WT1*	p.C393Y	AD	46–50	FSGS	SRNS
6,725	White	USA	*NPHS2*	p.Y162X	Sporadic	1–5	FSGS	SRNS
34,262	White	USA	*ACTN4*	p.D874N	Sporadic	6–10	FSGS	SRNS
34,462	Black	USA	*INF2*	p.E249X	AD	6–10	FSGS	SRNS
40,015	Hispanic	USA	*TRPC6*	p.G39fsX41	Sporadic	6–10	MCD	SRNS

AD, autosomal dominant; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; SRNS, steroid-resistant nephrotic syndrome.

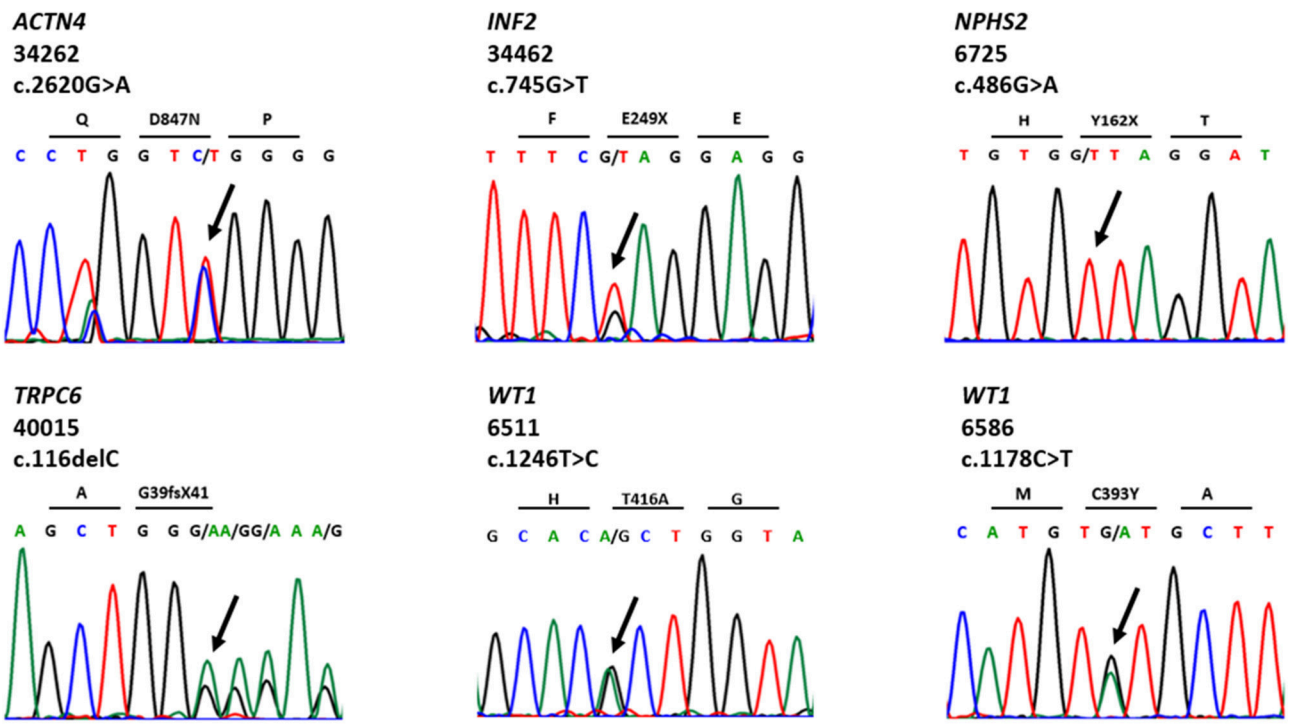

FIGURE 2 | Chromatograms for novel variants identified by targeted sequencing of custom amplicons.

of our study, thus allowing for analysis of patterns of inheritance and mutation detection rates in familial vs. sporadic disease.

We detected causative mutations in 22.1% of our study cohort (40 of 181 families), comprised of 38 distinct variants in 12 known SRNS genes. The majority of these were autosomal dominant genes, possibly due to the large proportion of families with autosomal dominant disease in our cohort or due to decreased prevalence of homozygous autosomal recessive mutations in an outbred population. Six of the variants identified by TSCA are novel, including three variants in exons not covered by most SRNS gene panels.

Genetic testing has the potential to provide extremely useful information when utilized in the correct context. There are currently no guidelines for the use of genetic testing for SRNS in clinical practice in the United States and other countries. Our study suggests that clinicians should consider genetic testing as they would any other diagnostic test; by determining if the test is likely to aid in clinical diagnosis, if the test would change the approach to therapy or if the test would provide clinicians with additional information to discuss the short and long term prognosis of the disease (22). Advantages of establishing molecular diagnosis in patients with SRNS include: (i) better disease definition, (ii) well-informed discussions of short- and long-term prognosis, (iii) selection of appropriate kidney donors to protect both the donor and recipient, (iv) better prediction of post-transplant outcomes, and (v) improved interpretation of clinical trials as they apply to a given patient.

Although the advent of high throughput sequencing has accelerated the pace of gene discovery in SRNS, the amount of data produced requires analytical resources that are often outside the scope of reasonable clinical practice. Consequently, there is a need for guidelines for the use of next-generation sequencing strategies in clinical practice. Typical gene panels are limited to a few genes and often do not include all coding regions of

TABLE 3 | Clinical characteristics of 40 families in whom disease-causing mutations were identified compared to those in whom no causative mutation was found.

Characteristic	Mutation *n*(%)	No mutation *n*(%)	*p*-value
Sex			0.23
Male	17 (18.5)	75 (81.5)	
Female	23 (25.8)	66 (74.2)	
Race			0.09
White, non-hispanic	28 (26.4)	78 (73.6)	
Other	12 (16.0)	63 (84.0)	
Family history			<0.00001
Yes	34 (39.5)	52 (60.5)	
No	6 (6.3)	89 (93.7)	
Histopathology			0.61
MCD	5 (19.2)	21 (80.8)	
FSGS	26 (23.9)	83 (76.1)	
Sample origin			0.52
US	32 (21.3)	118 (78.7)	
Non-US	8 (26.7)	22 (73.3)	

these genes. A benefit of massively parallel high throughput sequencing such as that employed in this and other recent studies is an increased chance of detecting a causative mutation. Whole-exome sequencing covers all regions of the genome and allows for the identification of known and novel mutations as well as the possible identification of new candidate genes. However, it produces a massive amount of data, identifying on average 2,000–4,000 non-synonymous variants with a MAF <1%. This amount of data requires significant effort to interpret results (9). Targeted high throughput sequencing such as TSCA limits the volume of data compared to WES, while still allowing for sequencing of all coding regions of candidate genes.

For clinical decision making, clinicians must certainly consider their own patient population. It has been demonstrated that patients with NS who live in regions with higher rates of consanguinity show a higher prevalence of monogenic disease due in part to the increased likelihood of homozygous variants in autosomal recessive genes (36). Studies conducted outside of the US on the large PodoNet Registry cohort (www.podonet.org) have demonstrated the prognostic value of genetic testing in this population (34, 35). Parameters for genetic testing should therefore be different for different patient populations (36).

In order to provide clinical guidelines for genetic testing in US patients with SRNS, we evaluated our cohort for characteristics that were associated with the identification of a monogenic cause of SRNS. We identified causative mutations in almost 40% of families with a family history of SRNS or CKD compared to only 6% of patients with SRNS and no family history, a difference that was highly significant and makes a positive family history of SRNS or CKD the single most important clinical predictor of mutation amongst patients with SRNS. Although more variants were identified in patients with presumed autosomal dominant disease (45%) compared to autosomal recessive disease (24%) this difference was not statistically significant. We acknowledge that, because there were more families with dominant inheritance in this cohort, it is possible that there was some bias leading to an increased number of detected mutations in autosomal dominant genes and families with dominant inheritance. There were no other clinical factors associated with mutation identification in this cohort. We therefore conclude that a positive family history of SRNS or CKD, regardless of the pattern of inheritance, should be a primary consideration in determining whether genetic testing is warranted.

One limitation to our study was the identification of compound heterozygotes through TSCA. Current methods do not identify on which chromosome a variant is located, meaning that compound heterozygous traits cannot be classified as cis or trans, and thus requiring sequencing of parental DNA. For this reason, it is possible that some compound heterozygous mutations were missed in our cohort. New sequencing technologies are in development that will allow for this distinction in future high throughput targeted sequencing and may increase findings. In addition, because this is a retrospective study, some components of the history and follow-up were limited for some patients. Information regarding age of disease onset or diagnosis was missing for some families, as were details of extra-renal manifestations suggestive of syndromic disease. Statistical analysis could therefore not be performed for these two clinical parameters.

In conclusion, our data show that a family history of SRNS or CKD is the single most important clinical predictor of mutation in patients in a US population. We therefore recommend genetic testing in all patients with SRNS and a family history of SRNS or CKD. In patients with no family history (i.e., those with sporadic NS), steroid-resistance is insufficient as a sole criterion for genetic testing and we do not recommend universal testing in these patients. There is still a need for a larger population-based study to develop a robust algorithm for genetic testing in patients with SRNS.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Review Board of Duke University with written informed consent from all adult subjects and from the parents of all subjects under 18 years of age. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of Duke University and the Institutional Review Boards of all collaborating sites.

AUTHOR CONTRIBUTIONS

JV gathered clinical data, performed experiments, analyzed data, and prepared the manuscript. RG and DH conceived, designed, and supervised the study. RG and GH edited the manuscript. MC-S and AM performed experiments, and MC-S analyzed genomic data. DH, RG, GW, BL, and GH provided laboratory support and conceptual advice. RG, CE, AS, AA, TH, and JL gathered clinical data and biological samples. All

authors discussed results and commented on the manuscript and provided approval for this paper.

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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.2018.00307/full#supplementary-material

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Psychosocial Assessment of Candidates for Transplantation (PACT) Score Identifies High Risk Patients in Pediatric Renal Transplantation

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Background: Currently, there is no standardized approach for determining psychosocial readiness in pediatric transplantation. We examined the utility of the Psychosocial Assessment of Candidates for Transplantation (PACT) to identify pediatric kidney transplant recipients at risk for adverse clinical outcomes.

Methods: Kidney transplant patients <21-years-old transplanted at Duke University Medical Center between 2005 and 2015 underwent psychosocial assessment by a social worker with either PACT or unstructured interview, which were used to determine transplant candidacy. PACT assessed candidates on a scale of 0 (poor candidate) to 4 (excellent candidate) in areas of social support, psychological health, lifestyle factors, and understanding. Demographics and clinical outcomes were analyzed by presence or absence of PACT and further characterized by high (≥3) and low (≤2) scores.

Results: Of 54 pediatric patients, 25 (46.3%) patients underwent pre-transplant evaluation utilizing PACT, while 29 (53.7%) were not evaluated with PACT. Patients assessed with PACT had a significantly lower percentage of acute rejection (16.0 vs. 55.2%, $p = 0.007$). After adjusting for HLA mismatch, a pre-transplant PACT score was persistently associated with lower odds of acute rejection (Odds Ratio 0.119, 95% Confidence Interval 0.027–0.52, $p = 0.005$). In PACT subsection analysis, the lack of family availability (OR 0.08, 95% CI 0.01–0.97, $p = 0.047$) and risk for psychopathology (OR 0.34, 95% CI 0.13–0.87, $p = 0.025$) were associated with a low PACT score and post-transplant non-adherence.

Conclusions: Our study highlights the importance of standardized psychosocial assessments and the potential use of PACT in risk stratifying pre-transplant candidates.

Keywords: renal transplantation, pediatrics, kidney, adherence, psychosocial factors

INTRODUCTION

Renal transplantation is the treatment of choice for end-stage renal disease in children and adolescents. Identification of psychosocial factors that can negatively impact post-transplant care is important to ensure successful clinical outcomes, which include adherence to medications, freedom from rejection and long-term patient and allograft survival. For children with end-stage renal disease, psychosocial stressors associated with increased morbidity and mortality include lower socioeconomic status and limited parental support (1, 2). Currently, there is no standardized approach to determine psychosocial readiness in pediatric kidney transplantation.

Multiple pre-transplant assessment tools have been developed for adults with scant data in pediatrics. These include the Stanford Integrated Psychosocial Assessment for Transplant (SIPAT), the Structured Interview for Renal Transplantation (SIRT), and the Transplant Evaluation Rating Scale (TERS) (3–5). The Pediatric Transplant Rating Instrument (P-TRI) attempted to address this gap in pediatric transplantation; however, follow-up studies have shown inconsistency with poor inter-rater reliability (6, 7). Other studies have simply used clinical judgement without a tool to risk-stratify patients (8).

The most widely used tool, the Psychosocial Assessment of Candidates for Transplantation (PACT), was developed in the 1980s as a standardized pre-transplant psychosocial evaluation tool (9). Since its development, PACT has shown good inter-rater reliability, improved clinical ease of use, and is a uniform framework for pre-transplant evaluation across all organ systems (9–11). As PACT has shown clinical utility and reliability in adult solid organ transplantation (12–15), we hypothesized that PACT could identify high risk pediatric transplant recipients and potentially improve transplant outcomes. In this pilot study, we aimed to assess the effect of implementing a standardized assessment such as PACT on clinical outcomes.

MATERIALS AND METHODS

Study Design

In this retrospective analysis, eligible patients for inclusion were <21 years of age at time of transplantation, had continued post-transplant follow-up, and underwent renal transplantation for end-stage renal disease from January 1, 2005 to December 31, 2015 at Duke University Medical Center. Patients were excluded for missing transplant data or not receiving their primary transplantation or follow-up at our institution. The Duke University Institutional Review Board (IRB Protocol #0078991) approved this study.

Study Outcomes

The primary endpoint of our study was biopsy proven acute rejection graded by Banff criteria within 3 years post-transplantation (16). Secondary endpoints included post-transplant dialysis, length of hospital stay, 30-day readmission, missed post-transplant appointments per year, non-adherence, renal allograft survival, and patient death. Non-adherence was determined by patient self-report, undetectable drug levels, or missed appointments (17, 18).

Pact Score and Psychosocial Analysis

The PACT assessed candidates on 4 domains including social support, psychological health, lifestyle factors, and understanding of transplant and follow-up. PACT contained 8 subsection items: family support, family availability, personality factors, risk for psychopathology, ability to sustain change, drug and alcohol abuse, medical adherence, and relevant knowledge. The social support domain included evaluation of family support and family availability with an emphasis on relationships. The psychological health domain incorporated assessment of personality factors/psychiatric disorders and risk for psychopathology, which focused on coping mechanisms and family history of psychiatric disorders. The lifestyle factors domain evaluated dietary/exercise habits and the ability to change unhealthy behaviors, drug and alcohol use, and compliance with medications/medical advice. Finally, the understanding of transplant and follow-up domain encompassed relevant transplant knowledge and receptiveness to education. (19). Each category was assessed by scale of mild, moderate, or severe (9, 20). An overall final transplant candidacy score from 0 (contraindication to transplant) to 4 (excellent candidate) was assigned by the social worker. A low PACT score was defined as a score $\leq$2 and a high PACT score was defined as $\geq$3 (21). The psychosocial assessment was incorporated into the decision to transplant and patients with low PACT score were monitored more closely after transplant.

The psychosocial assessment was one of the initial steps for assessing transplant candidacy and was completed 1–2 months prior to listing for transplant. Trained licensed hospital social workers who specialize in pre-transplant evaluations performed our psychosocial assessments using language suitable for a 5th grade reading level. All psychosocial assessments were incorporated into multidisciplinary meetings to help evaluate the readiness of potential candidates alongside other concerns such as medical comorbidities. Preceding the PACT, all patients received a pre-transplant psychosocial analysis by a licensed social worker from 2005 to 2010. However, the pre-transplant psychosocial rating before the implementation of the PACT was not standardized, subject to variability in content (home life, finances, transportation, school, and adherence) depending on the evaluator and differed in the criteria for candidate suitability. The PACT was implemented to standardize pediatric renal pre-transplant assessments in 2010. All pre-transplants assessments were completed using PACT from 2010 to 2015. Final subsection scores represented the abilities and risk factors present in the primary caregivers and the child, starting at the developmental age of 12. Patients were listed regardless of PACT score. Increased pre-transplant visits with social worker intervention were initiated for patients with a low PACT score and transplanted only for score of >1. Patients with psychiatric illnesses were referred to outpatient psychiatry for titration of medications and counseling pre-transplant and not transplanted until issues controlled. Patients with low PACT scores defined as <2, were monitored more closely post-transplantation by medical team,

TABLE 1 | Demographic characteristics between recipients with and without a PACT assessment.

	PACT assessment	No PACT assessment	*p*
n	25	29	
Age at transplant (median [IQR])	14.80 [8.51, 17.08]	13.39 [4.12, 15.30]	0.249
Female (%)	9 (36.0)	8 (27.6)	0.711
Race (%)			0.766
African American	10 (40.0)	11 (37.9)	
Caucasian	10 (40.0)	14 (48.3)	
Other	5 (20.0)	4 (13.8)	
BMI at transplant (median [IQR])	22.00 [12.00, 35.00]	25.00 [12.00, 37.00]	0.952
HLA Mismatch [mean (sd)]	1.00 (1.55)	2.52 (1.70)	0.001
Class I PRA > 20% (%)	1 (4.0)	1 (3.4)	0.999
Class II PRA > 20% (%)	2 (8.0)	4 (13.8)	0.809
Pre-transplant dialysis (%)	19 (76.0)	17 (58.6)	0.289
Induction (%)			0.443
Anti-thymocyte Globulin	8 (33.3)	6 (21.4)	
IL-2 inhibitor	11 (45.8)	18 (64.3)	
None	4 (16.7)	2 (7.1)	
Other	1 (4.2)	2 (7.1)	
MAINTENANCE IMMUNOSUPPRESSION			
Calcineurin inhibitors	25 (100.0)	26 (89.7)	0.29
Steroids	25 (100.0)	26 (89.7)	0.29
Anti-metabolites	25 (100.0)	26 (89.7)	0.809
EBV immune	11 (44.0)	11 (37.9)	0.861
CMV immune	7 (28.0)	10 (34.5)	0.828
Diagnosis (%)			0.449
Dysplasia	5 (20.0)	12 (41.4)	
FSGS	8 (32.0)	8 (27.6)	
Glomerulonephritis	3 (12.0)	1 (3.4)	
Obstructive Uropathy	3 (12.0)	3 (10.3)	
Other	6 (24.0)	5 (17.2)	
Re-transplant (%)	1 (4.0)	2 (6.9)	0.999
Living donor (%)	6 (24.0)	16 (55.2)	0.041
Total ischemia (hours, median [IQR])	11.75 [2.18, 15.23]	1.50 [1.00, 16.00]	0.339
Follow-up time [years, mean (sd)]	2.87 (2.30)	8.34 (4.12)	<0.001

social workers, and when appropriate by psychiatrist and/or psychologists. In cases with multiple psychosocial assessments over time, the PACT score closest to the time of transplant was used.

Statistical Analysis

Patients were stratified based on presence or absence of a PACT assessment. Of those with a PACT assessment, sub-analysis by low vs. high PACT score was performed. Baseline characteristics and unadjusted outcomes were compared using the Kruskal-Wallis test for continuous variables and Pearson χ^2 test for categorical variables. For acute rejection, a multivariable linear model was used adjusting for HLA mismatch. A model examining probability of freedom from acute rejection was generated using the Kaplan-Meier method.

A p-value of less than 0.05 was deemed statistically significant. Statistical analysis was performed using R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

TABLE 2 | Clinical outcomes in recipients with a PACT assessment and with no PACT assessment.

	PACT assessment	No PACT assessment	*p*
n	25	29	
Post-transplant dialysis (%)	9 (36.0)	12 (42.9)	0.819
Patient death [mean (sd)]	1.00 (0.00)	1.07 (0.26)	0.188
Missed appointments per year [mean (sd)]	0.44 (0.78)	0.17 (0.30)	0.108
Non-adherence (%)	7 (28.0)	16 (55.2)	0.082
Graft failure (%)	0 (0.0)	3 (10.3)	0.29
30-day readmission (%)	5 (20.0)	3 (10.3)	0.541
Hospital stay (days, median [IQR])	7.00 [5.00, 9.50]	7.00 [6.00, 10.00]	0.506
Acute rejection [mean (sd)]	4 (16.0)	16 (55.2)	0.007
Delayed graft function (%)	6 (25.0)	3 (10.3)	0.295

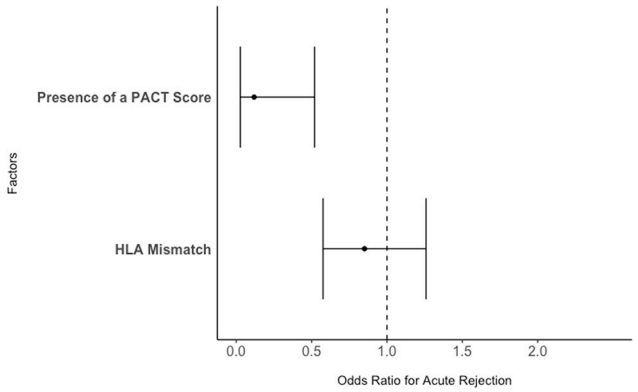
RESULTS

Demographics

A total of 54 pediatric patients who underwent renal transplantation at Duke University from 2005 to 2015 met the inclusion criteria. Of these, 25 (46.3%) patients had a PACT assessment while 29 (53.7%) patients had no PACT assessment. Demographics and clinical characteristics between the two groups are shown in **Table 1**. Recipients with a PACT assessment were better matched (HLA mismatch 1.00, 2.52 $p = 0.001$), received a lower percentage of their allografts from living donors (24 vs. 55.2%, $p = 0.04$), and had less follow-up time (2.87 vs. 8.34 years, $p < 0.001$) when compared to their no PACT assessment counter parts. In PACT assessment recipients, the median PACT score was 3 (IQR 3.00–4.00). Additionally, 4 patients met the definition of a low PACT score (≤ 2) while 21 patients had a high PACT score (≥ 3) (21). Otherwise, there were no significant differences in gender, race, BMI, PRA, pre-transplant dialysis, PRA, immunosuppression regimen, viral serology status, diagnosis, re-transplant, or cold ischemia time between the two groups (**Table 1**).

Clinical Outcomes

Unadjusted clinical outcomes between PACT assessment and no PACT assessment patients are displayed in **Table 2**. Patients with PACT assessment had a significantly lower percentage of acute rejection within the first 3 years post-transplant (16.0 vs. 55.2%, $p = 0.007$) and a lower percentage of non-adherence (28.0 vs. 55.2%, $p = 0.08$). After adjustment for HLA mismatch, lower odds of acute rejection remained associated with having a



Variables	Odds ratio	*p*-value
Presence of a PACT score	0.119 (95% Confidence Interval 0.027–0.52)	0.005
HLA mismatch	0.851 (95% CI 0.576–1.259)	0.42

FIGURE 1 | The effect of PACT score and HLA mismatch on the odds of acute rejection in pediatric kidney transplantation. The presence of a PACT score remained significantly associated with lower odds of rejection within 3-years post-transplant even after adjusting for HLA mismatch.

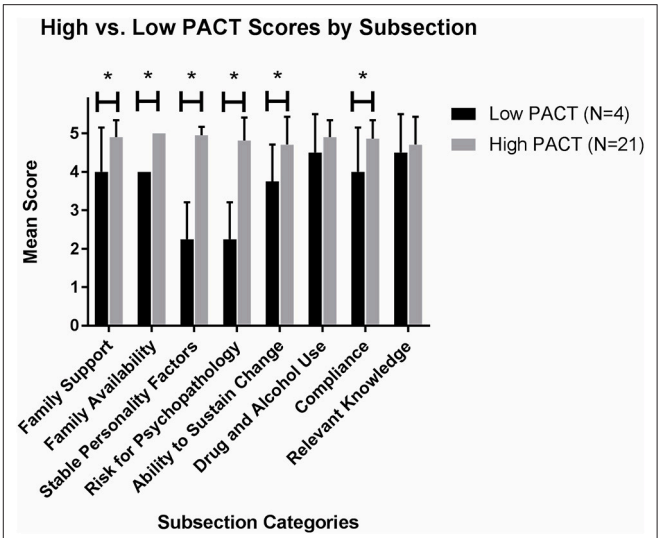


FIGURE 2 | Differences in PACT subcategories between candidates with high vs. low PACT scores. Patients with a low PACT score ≤2 had less family support, family availability, stable personality factors, ability to change and compliance and increased risk for psychopathology than those with high PACT score ≥3. *denotes significance ($p < 0.05$).

TABLE 3 | Clinical outcomes between high PACT (≥3) and low PACT scores (≤2) in recipients with a PACT assessment.

	Low PACT	High PACT	*p*
Post-transplant dialysis (%)	3 (75.0)	6 (28.6)	0.228
Patient death [mean (sd)]	0 (0.0)	0 (0.0)	
Missed appointments per year [mean (sd)]	1.22 (0.68)	0.32 (0.74)	0.062
Non-adherence (%)	3 (75.0)	4 (19.0)	0.094
Graft Failure	0 (0.0)	0 (0.0)	
30-day readmission (%)	0 (0.0)	5 (23.8)	0.682
Hospital stay (days, median [IQR])	14.00 [5.50, 22.25]	7.00 [5.00, 9.00]	0.64
Acute rejection in 3 years of transplant [mean (sd)]	0 (0.0)	4 (19.0)	0.835
Delayed graft function (%)	1 (25.0)	5 (25.0)	0.999

TABLE 4 | Odds of non-adherence by PACT subsection in recipients with a PACT assessment.

Variable	Odds ratio	95% confidence interval	*p*-value
Family support	0.38	0.11–1.41	0.15
Family availability	0.08	0.01–0.97	0.047
Personality factors	0.37	0.13–1.03	0.056
Risk for psychopathology	0.34	0.13–0.87	0.025
Ability to sustain change	1.03	0.34–3.06	0.97
Drug and alcohol abuse	4314	0.00–Inf	0.99
Medical adherence	0.44	0.13–1.53	0.2
Relevant knowledge	1.1	0.32–3.74	0.88

PACT score (Odds Ratio 0.119, 95% Confidence Interval 0.027–0.52, $p = 0.005$) but not HLA mismatch (OR 0.851, 95% CI 0.576–1.259) (**Figure 1**).

When comparing High PACT (≥ 3) vs. Low PACT Scores (≤ 2), the high PACT mean was 3.52 (SD 0.51) while the low PACT group mean was 1.75 (SD 0.50). The low PACT group had a higher percentage of non-adherence and increased rate of missed appointments per year than the high PACT group (**Table 3**). Compared to the high PACT group, patients with lower PACT scores had less family support ($p = 0.01$) and family availability ($p < 0.0010$), more unstable personality factors ($p < 0.001$), increased risk for psychopathology ($p < 0.001$), less ability to sustain change ($p = 0.03$), and less medical adherence ($p = 0.02$) (**Figure 2**). Overall, non-adherence in the low PACT group was associated with less family availability (Odds Ratio 0.08, 95% Confidence Interval 0.01–0.97, $p = 0.047$) and increased risk for psychopathology (OR 0.34, 95% CI 0.13–0.87, $p = 0.025$; **Table 4**).

DISCUSSION

Our study was the first to examine the clinical utility of PACT in a pediatric renal transplantation. Our overall goals were to understand the impact of introducing a standardized psychosocial assessment such as PACT and to elucidate any differences in the clinical outcomes between patients with low and high PACT scores. Similar to adult transplant studies in BMT, lung, and liver recipients, our pilot investigation demonstrated that assessment with PACT was associated with modest improvement in clinical outcomes. Previous studies have shown PACT to be associated with lower in-hospital

mortality, shorter length of stay and readmission duration (10, 12, 13, 22, 23). Similar to prior studies, we demonstrated an association between using a standardized assessment, such as PACT, and lower post-transplant morbidity, specifically less acute rejection and non-adherence. Compared to a non-standardized assessment, incorporating PACT into the pre-transplant approach was helpful to identify high risk patients that required more frequent post-transplant monitoring by the medical team including monthly labs, social worker visits, and psychiatry/psychology appointments when appropriate.

The need for pre-transplant psychosocial evaluation has been known for decades, however, standardization of tools or criteria remain insufficient in modern solid organ transplantation (20). Adult measures such as The Stanford Integrated Psychosocial Assessment for Transplant (SIPAT), the Structured Interview for Renal Transplantation (SIRT), and the Transplant Evaluation Rating Scale (TERS) (3–5) have not been studied in pediatrics. PACT has been shown to correlate with outcomes and is among the most studied in adult transplantation. In order to provide one unifying pre-transplant assessment for both pediatric and adults, we evaluated PACT in transplanted children. Our study bridges a gap in the PACT literature and provides important information regarding its potential utility in pediatric renal transplantation to identify those at risk for rejection and non-adherence. Pediatric measures such as the Pediatric Transplant Rating Instrument (P-TRI) and the Psychosocial Assessment Tool (PAT) (24), adapted for pediatric kidney transplantation, suffer from inter-rater variability and did not correlate with clinical outcomes (6, 7, 25).

While outcomes by overall PACT score have been examined, few studies have examined differences in subscales. In bone marrow transplantation (BMT), several subscales were correlated with better outcomes. Foster et al found that adherence was associated with lower in-hospital mortality, shorter length of stay and readmission duration, and faster engraftment. Additionally, higher scores on family availability and on relevant knowledge/receptiveness to education were associated with decreased mortality (12). While our pilot study was not powered for mortality analysis, our results showed that the difference between a high and low PACT score in this pediatric population was driven by family support, innate personality factors, and adherence, similar to what was reported for the BMT population. Additionally, a low PACT score was found in patients with missed appointments and non-adherence. Of those with a PACT score, non-adherence was more likely found in candidates with a higher risk of psychopathology and lack of family support.

Although our pilot study highlights the potential utility of PACT in children, there are limitations. Retrospective analyses are affected by selection and indication biases. By adjusting for HLA mismatch in our analysis, we attempted to minimize significant differences between recipients with no PACT assessment and a PACT assessment. Despite the known association of *de novo* donor specific antibodies (DSA) and non-adherence, baseline DSA and *de novo* DSA were not routinely screened in all patients at our institution until recently (17, 26, 27). Thus, it could not be included as a clinical outcome. Additionally, patients in the two cohorts were transplanted during two different time periods. Patients transplanted between 2005 and 2010 were not assessed with PACT, while those transplanted between 2010 and 2015 were evaluated utilizing PACT. Potential differences in pre- and post-transplant care between these time periods may have impacted our results. One notable difference due to time of transplant was percentage of living related donors in the PACT negative group, most likely due to these recipients undergoing transplant prior to the Share 35 kidney allocation policy. After this policy, which gave pediatrics priority for the best deceased donor allografts, the rate of deceased donor transplantation increased for children (28). We were unable to account for this difference in our final model, but a higher percentage of living donors would be predicted to decrease rates of acute rejection and not increase (29, 30). Lastly, patients without the PACT assessment had longer follow-up time and thus more time to develop poor clinical outcomes. To eliminate this bias, we only evaluated acute rejection episodes within the first 3 years post-transplantation. Although our sample size is small, and our study was not designed to be powered for mortality or graft failure analyses, we found significant findings that warrant further investigation.

In conclusion, renal transplantation requires significant attention to psychosocial factors for successful clinical outcomes. Currently, a standard psychosocial evaluation for transplant candidates across all centers is lacking. Our study highlights that implementation of a standardized measure, such as the PACT, can identify pediatric kidney transplant candidates at risk for poor outcomes, which can align with pre-transplant measures utilized in the adult setting. Thus, future prospective studies may be indicated prior to widespread use.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KF significantly contributed to the acquisition of data, analysis and interpretation of data for the work. He drafted the manuscript. VC contributed to the acquisition of data and interpretation of data. SN, AC, DW, JF, RG, and DV all contributed to the interpretation of data and critical revisions of the manuscript that significantly added to the scientific content. DC contributed to the analysis and interpretation of data that added to the content of the manuscript. EC contributed to the design of the study, interpretation of data, and drafting of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Developing a Research Mentorship Program: The American Society of Pediatric Nephrology's Experience

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Background: Most pediatric nephrologists work in academia. Mentor-mentee relationships provide support and guidance for successful research career. Mentorship program implementation is valuable in medical fields for providing research opportunities to young faculty.

Methods: The American Society of Pediatric Nephrology (ASPN) established a research mentorship program to (a) assist with matching of appropriate mentor-mentee dyads and (b) establish metrics for desirable mentor-mentee outcomes with two independent components: (1) the grants review workshop, a short-term program providing mentor feedback on grant proposals, and (2) the longitudinal program, establishing long-term mentor-mentee relationships. Regular surveys of both mentors and mentees were reviewed to evaluate and refine the program.

Results: Twelve mentees and 17 mentors participated in the grant review workshop and 19 mentees were matched to mentors in the longitudinal program. A review of NIH RePORTER data indicated that since 2014, 13 NIH grants have been awarded. Mentees in the longitudinal program reported that the program helped most with identifying an outside mentor, improving grant research content, and with general career development. Mentors perceived themselves to be most helpful in assisting with overall career plans. Email communications were preferred over phone or face-to-face communications. Mentees endorsed strong interest in staying in touch with their mentors and 100% of mentors expressed their willingness to serve in the future.

Conclusion: This mentorship program was initiated and supported by a relatively small medical society and has shown early success in cultivating mentoring relationships for a future generation of clinician-scientists.

Keywords: mentorship, research, society, grants, pediatrics

INTRODUCTION

Mentoring is a special partnership between two people based on common goals and expectations, focus, and mutual trust and respect (1). Successful mentoring requires commitment to this process by both mentors and mentees and it serves as an important career advancement mechanism for both (2). Mentors provide guidance and promote a mentee's achievement in any or all of the following: academic pursuits, clinical excellence, life/professional goals and work-life balance. Neely et al. wrote, "Development of an academic career easily follows a clinical course for which there are multiple role models; however, development of an academic research career involves few role models, and rarely do instructional guides reach out to the new faculty" (3). In addition to professional aptitude, two components appear essential for a successful research career: (1) a broad supportive infrastructure, and (2) a person- and time-specific continuous mentor-mentee relationship. Positive mentoring relationships require trust, respect, shared information, resources, and expectations as well as professional, enthusiastic, supportive, and collaborative problem-solving (4).

Although a number of papers and internet resources address mentorship programs (5–8), very few focus on development of an academic research career for physician-scientists (3, 9–12). These resources are mostly institution-based and oriented to students and/or graduate medical trainees (13–15). Two recent papers addressed more sponsorship-type programs instead of structural research mentorships (16, 17). Faculty development programs from medical societies are more oriented toward educational research. For example, the American Society of Hematology developed an educational scholarly project (18) and the American Society of Pediatric Hematology/Oncology supports a mentoring program for early career members (19). The Society for Maternal-Fetal Medicine expressed additional concern about forthcoming training of physician-scientists: uncertainties related to future administration of health care, federal support for research, attrition of physician-scientists, and an inadequate supply of new scholars (20). Furthermore, the Accreditation Council for Graduate Medical Education has increased the scholarly productivity requirements for residency programs, placing even more pressure on faculty to be productive in this realm.

Recognizing the need, the American Society of Nephrology (ASN) recently introduced an extensive set of electronic educational resources, presented in brief animations, podcasts, and other media for internal medicine nephrologists. These resources detail effective communication, identifying the right mentor or mentee, navigating mentorship challenges, and assessing a mentee's understanding of expectations (21). These resources developed by an adult medicine subspecialty professional society addressed universal challenges to academic medicine. Although virtually all ASPN members are also ASN members, gaps remained for those trained in pediatric nephrology.

As with other pediatric subspecialties, many pediatric nephrologists work in small divisions with as few as 1–3 faculty in a group. Thus, the lack of a local, experienced research mentor to guide research development and review early faculty progress is common. Recognizing that many junior investigators could benefit from the addition of established senior investigators to their mentoring team, the ASPN created a research mentorship program in 2014.

The specific goals of the ASPN mentorship program were (1) to provide junior investigators with successful research mentors outside their home institutions who have similar research interests, and (2) to provide mentorship training to more established investigators who are ready to become mentors. This program allowed mentors to support junior faculty career development, academic pursuits, and development of grant funding proposals. In this descriptive paper, we present the unique experiences and early data gathered during the ASPN research mentorship program development and implementation. Our experiences may serve as an example for other medical societies seeking to improve junior faculty research mentoring experiences.

METHODS

Development of the ASPN Mentorship Program

The ASPN Mentorship Program was conceived and developed by the ASPN Research Committee in 2014 to match mentors with self-identified mentees and establish an environment for desirable mentor-mentee research outcomes. The program was designed to be dynamic and open to modifications based on periodic surveys and evaluations by all participants. In 2016, a Quality Improvement (QI) framework was formalized with creation of an American Board of Pediatrics (ABP)-approved Maintenance of Certification (MOC) Part 4 program. The program was administered by the co-chairs of the Research Committee, who typically serve 3-year terms. ASPN senior leadership (Council of the Society) ensures management support and monitors step-by-step progress reports.

The mentoring program had two components: (a) the short-term grant review program, designed to help a mentee with a specific grant submission and (b) the longitudinal program, which facilitates the establishment of long-term mentor-mentee relationships to aid in career development. Mentors and mentees can choose to participate in one or both components. Furthermore, the mentee can participate in the grant review program as often as desired. One of the most important approaches to ensure success of the program is optimal matching of mentors and mentees.

Requirements for Mentors and Mentees

The ASPN Research Committee co-chairs selected potential mentors for each mentee among the existing research committee members, current members of the Society, or established non-Society mentors aligned within the field of pediatric nephrology. The selection process was iterative, with feedback from both mentees and potential mentors to ensure no conflicts of interest or conflicts in commitment. To participate in the program,

mentees need only to be ASPN members in good standing. ASPN mentees who entered in the longitudinal program perceived that mentors would help with their career and research development.

Grant Review Program

The grant review program consisted of an annual in-person grant review workshop held in conjunction with a large national meeting. These workshops were held in a "mock study section" format, with 3–4 mentors serving as primary and secondary reviewers for 2–3 grants presented orally by groups of mentees. The structure of these workshops was tailored to the needs of the mentees who were assigned to small groups based on research topic or methodology. Mentors are selected based on proven track record of grantsmanship sufficient for the mentees' stage of training. However, often mid-level and senior investigators were assigned as reviewers for the same career development grants in order to also provide experience for mid-level faculty on grant review. Mentees submitted drafts of their grant applications in advance of the in-person workshop, and the mentors were asked to complete a written review using a standard NIH-style review template. Mentees were required to submit a specific aims page at a minimum and were also encouraged to submit other grant sections (e.g., Research Strategy, Career Development). All participants were asked to keep the review process confidential.

Longitudinal Program

Mentees and mentors in the longitudinal program signed a Mentoring Partnership Agreement on enrollment to formalize the relationship and clarify individual expectations. These expectations included committing to regular meetings (in person at least annually; by phone or virtually at other time points), maintaining confidentiality, reviewing and revising the mentees' goals statements annually, providing each other with honest, direct, and respectful feedback, and providing program feedback to the Research Committee. Mentees were asked to write a goals statement, to be reviewed/updated annually with their mentors. Mentees were also encouraged to contact the mentor regularly via phone or electronic communication to discuss specific needs related to grant review, programmatic development, promotion, and tenure.

Mentors in the program are experienced, independently-funded physician scientists, who are able to work with mentees to develop goals with realistic expectations and individualized career development plans. Mentors who reviewed grants for mentees also participated in the grant review component were often selected to become a longitudinal mentor. It was important for mentors to commit to meeting routinely with the mentees to review progress, give feedback, provide recommendations for improvements, and guide academic career development (e.g., assist with grant proposals, manuscript submissions, and oral presentations). The Program leadership maintains Mentorship Partnership Agreements but does not review goals statements or drafts of grants/manuscripts, which are only shared between mentees and their mentors.

Role of the ASPN in Program Promotion and Support

The role of the ASPN in the mentorship program was to advertise the program, elicit participation, judiciously select and contact mentors for each new mentee in the program, review program feedback and outcome metrics regularly to improve the program organization, and develop education workshops. Research Committee members who participated in the QI project had to attend meetings to review survey data at least twice yearly for 2 years to be eligible to receive ABP MOC Part 4 credit.

Research Mentorship Program Evaluation

Surveys were sent to mentees and mentors using Survey Monkey®. Surveys were designed based on program metrics and questions were left unchanged from year-to-year to allow tracking of program efficacy over time in a standard fashion. Mentees and mentors in the longitudinal program were asked about methods and rates of contact with each other within the context of the program. Mentees and mentors in the grants review workshop were asked about what portions of their grants were specifically reviewed. Metrics selected to rate the quality of the program included expectations, number, and format of meetings between mentors and mentees, type of grants reviewed, number and content of sections of grants reviewed, and perceived helpfulness of mentee-mentor interactions in specific areas (i.e., improving grantsmanship). NIH grants are tracked using the RePORTER database (https://projectreporter.nih.gov/reporter.cfm). Metrics were collated by the ASPN Research Committee co-chairs and reviewed in aggregate at Committee meetings on a quarterly basis. Program improvements were implemented on an ongoing basis.

In response to participants' feedback that the program would benefit from more active oversight from the Research Committee, a mentorship program Oversight Subcommittee was formed in 2017. This Oversight Committee, which consists of former co-chairs of the Research Committee and other senior ASPN members, reaches out to mentees and mentors in the longitudinal program on a regular basis to ensure continued mentor-mentee contact and to provide educational resources.

RESULTS

Outcomes of the ASPN Mentorship Program

Since its inception, 12 mentees and 17 mentors have participated in the grant review program and 19 were matched as mentor-mentee pairs for the longitudinal component. Ten mentees participated in both programs, so there have been 21 unique mentees in the mentoring program. Of these, 16 were women and 5 were men. At the time of enrollment in the mentoring program, 17 mentees were junior faculty and 4 were in pediatric nephrology fellowship. Fellowship mentees were also supported by their local Scholarship Oversight Committees. Of the longitudinal program mentees, 8 have "graduated" from their formal mentoring relationship and were able to submit a grant. Mentees were considered to have "graduated" if they had reached

TABLE 1 | American Society of Pediatric Nephrology longitudinal research mentorship program: Mentees' perceptions on expectations and meetings with mentors over a 2-year period, *n* (%) (Questions were added in 2017, so 2016 data is not available).

Survey year	2017 *n* = 7	2018 *n* = 8
Question 1: What were your expectations going into the program?		
Submit R01	2 (29)	3 (33)
Submit career development grant	4 (57)	5 (56)
Submit institutional or foundation grant	0	0
Formulating a research career	1 (14)	2 (11)
Question 2: Has this program met your expectations?		
Yes	6 (86)	8 (100)
No	1 (14)	0
Question 3: Have you discussed expectations with your mentor?		
Yes	6 (86)	8 (100)
No	1 (14)	0
Question 4: In the past 6 months, how many times did you have contact with your mentor?		
None	2 (29)	1 (12.5)
1	1 (14)	4 (50)
2-3	4 (57)	2 (25)
4 or more	0	1 (12.5)
Question 5: In what manner did you communicate with your mentor?		
Email	2 (29)	3 (44)
Phone	3 (43)	2 (29)
In person	2 (29)	(29)
Video chat	0	0
Question 6: Do you have another meeting scheduled?		
Yes	1 (14)	4 (50)
No	6 (86)	4 (50)

TABLE 2 | American Society of Pediatric Nephrology longitudinal research mentorship program: Mentors' perceptions on expectations and meetings with mentees at the end of the represented year, *n* (%).

Survey year	2016 *n* = 5	2017 *n* = 8	2018 *n* = 6
Question 1: Do you think you were a good fit for your mentee's research focus?			
Yes	5 (100)	8 (100)	8 (100)
No	0	0	0
Question 2: In what manner did you communicate with your mentee?			
Email	5 (100)	8 (100)	6 (100)
Phone	1 (20)	5 (63)	4 (67)
In person	4 (80)	6 (75)	5 (83)
Video chat	1 (20)	0	1 (17)
Question 3: Number of times you met with your mentee?*			
None	–	1 (13)	3 (50)
1	–	3 (38)	2 (33)
2–3	–	3 (38)	0
4 or more	–	1 (13)	1 (17)
Question 4: Would you be willing to serve as a mentor in the future?			
Yes	4 (80)	8 (100)	6 (100)
No	1 (20)	0	0

*Mentors were surveyed after each year of the active program. *Question added to survey in 2017.*

TABLE 3 | American Society of Pediatric Nephrology longitudinal research mentorship program: Mentees' and mentors' reports of which sections of the grant were reviewed [*n* (%) of positive responses].

Grant sections reviewed	Mentees			Mentors		
Years	2016 *n* = 6	2017 *n* = 7	2018 *n* = 8	2016 *n* = 5	2017 *n* = 8	2018 *n* = 6
No comment	2 (33)	1 (14)	2 (25)	0	2 (25)	1 (17)
Concept/plan only	1 (17)	1 (14)	3 (38)	4 (80)	2 (25)	4 (67)
Specific aims	3 (50)	5 (71)	6 (75)	1 (20)	6 (75)	3 (50)
Significance	1 (17)	3 (43)	1 (13)	1 (20)	2 (25)	2 (33)
Innovation	1 (17)	4 (57)	1 (13)	1 (20)	2 (25)	1 (17)
Approach	2 (33)	5 (71)	2 (25)	2 (40)	5 (63)	2 (33)
Career development plan	0	1 (14)	1 (13)	0	1 (13)	0

their goals within the program, which were delineated at the time of program entry, and therefore chose to formally end their participation. From the 21 mentees who have participated in the mentoring program since 2014, review of NIH RePORTER data in October 2018 indicated 13 successful NIH grants following mentorship program participation: 5 R01s, 3 R03s, and 5 K awards. Although non-NIH funding (commercial, private foundations, institutional internal funds, etc.) represents a substantial source of research support this information was more difficult to track. We are planning to collect this information in our upcoming surveys.

Tables 1, **2** detail longitudinal program mentee and mentor responses, respectively, to survey questions regarding expectations, mentee-mentor fit, frequency of communication, future meeting plans. Sections of grant proposals reviewed in the longitudinal program are shown in **Table 3**. **Figure 1** represents satisfaction with the grant review (A) and longitudinal components (B) of the program in various areas.

In 2018, 100% of mentees in the longitudinal program agreed that the program met their expectations and that expectations were clearly communicated with the mentors. During a 6 month period, 50% of mentees met with their mentors face to face at least once, 25% met 2–3 times, and 12.5% met 4 or more times (**Table 1**). The vast majority of the longitudinal program mentees discussed the *Specific Aims* of their grants but interactions were less focused on their overall career development plan (**Table 3**). For the longitudinal component, email communications were preferred over phone or face-to-face communications.

While every mentor over the initial 3-year-period strongly believed that he/she was a good fit for their mentee, mentors' perceptions of the program differed from mentees' in key aspects. Mentors in both the grants review and longitudinal components

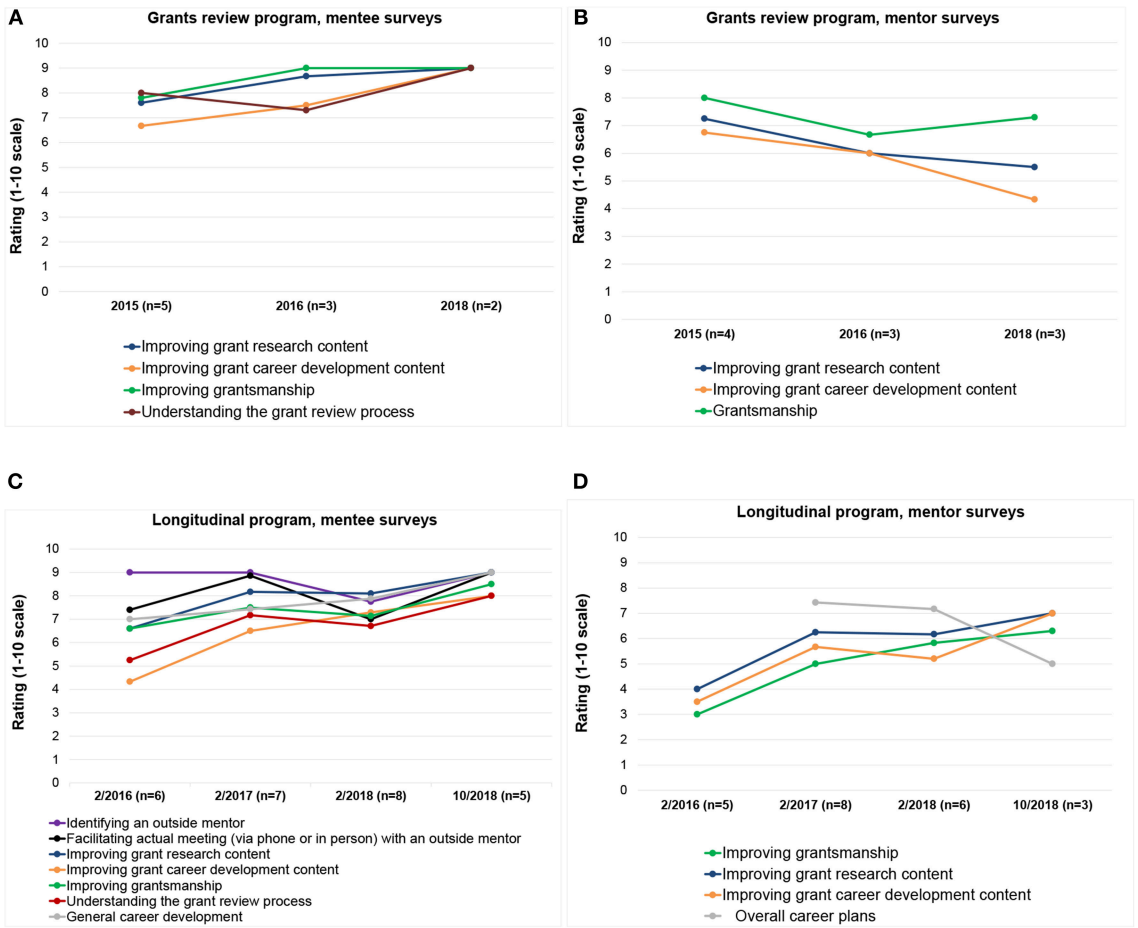

FIGURE 1 | American Society of Pediatric Nephrology research mentorship program: Mentees' ratings of the program's helpfulness in different areas, and mentors' ratings of their own helpfulness to their mentees. **(A)** Grant review mentees, **(B)** Grant review mentors, **(C)** Longitudinal program mentees, and **(D)** Longitudinal program mentors.

of the program perceived their overall helpfulness relatively low on a scale from 1 to 10 (**Figures 1B,D**), despite the mentees rating the program's helpfulness relatively highly (**Figures 1A,C**). Although no more than 14% of longitudinal program mentees reported reviewing long term career development plans with their mentors (**Table 3**), mentees rated the program relatively highly for helpfulness in general career development (score 7.0–7.9 out of 10, **Figure 1B**). Similarly, mentors also reported that they believed they were helpful to their mentees in "overall career plans" (score 7.2–7.4 out of 10, **Figure 1D**). As the program has evolved and improved over the years, 100% of mentors expressed their willingness to serve in the future (**Table 2**). During the course of this program, the longitudinal component demonstrated sustained improvement in satisfaction (**Figures 1C,D**).

DISCUSSION

We described the development and management of a junior faculty oriented research mentorship program within a pediatric medical subspecialty. The ASPN mentorship program experience demonstrates participant satisfaction, mentor-mentee stability, programmatic improvement over time, and notably a high yield of NIH grants during the project implementation period. Development of such a program within a relatively small pediatric society is challenging but essential. This model could serve as an example for other similar medical organizations.

The ASPN mentorship program addresses an important gap identified by academic nephrologists at the start of their careers. While some of the challenges experienced by new faculty in pediatric subspecialties are common with other aspiring physician scientists in other medicine careers, some are unique to those in pediatric medicine and nephrology. New faculty in pediatrics can become overwhelmed with the demands of juggling clinical practice with a research career. Initiating research—from conceptualization, proposal development, grant submission and ultimately to implementation and dissemination—is challenging, but extremely important for academic advancement.

The challenges in pediatric nephrology research are many, including a widespread array of pediatric nephrology divisions throughout the country that are small and with a paucity of locally available mentors experienced in multiple areas of investigation (22). In addition, nephrologists have several specific needs that are dependent on patients' unique psychosocial issues and physiology/pathophysiology of kidney disease (23); these issues are only more complicated in children. Several renal-related conditions are relatively uncommon in children, making identification and recruitment of potential clinical study participants extremely difficult.

The small work force in pediatric nephrology complicates the accessibility of young faculty to find research mentorship from established, independently-funded pediatric nephrologists. Through the country, with exception of few large centers, pediatric nephrology programs are represented by 2–5 clinical faculty (24). Moreover, many institutions do not have the infrastructure to support junior/mid-level investigators, which require networking and career guidance from outside mentors. The ASPN mentorship program addressed many of these obstacles by matching experts in the field willing to teach and mentor young investigators even outside their home institutions. Because of modern advances in communication, physical location and distance between mentors and mentees was not taken into consideration, rather their common research interest and desire to pursue the same scientific goals.

Lessons Learned From the ASPN Mentorship Program

Oversight by the ASPN ensured the success of this program and collection of outcome measurements of the program was critical. Governing the program through the ASPN research committee proved to be a viable option. Customized pairing of mentees with mentors based on academic and research expertise was a high priority for participants. We believe that requesting drafts of mentees' grant applications in advance of the in-person workshop, and requesting that mentors complete a written review, substantially increased the success rate of the program because it ensured that participants were highly motivated.

Mentees highly regarded the ability to receive individualized feedback from established investigators on their research grant proposals. Although not employed by ASPN, similar programs might use an "intelligent match" to achieve the best possible arrangements, using a very specific matching parameters and computerized approach. E-mail was the preferred mode of communication to foster ongoing relationship, with mentors further appreciating the importance of additional face-to-face contact.

We learned that the design and maintenance of a mentoring program should be dynamic with frequent assessments for improvement. An efficient monitoring vehicle was brief periodic online surveys. The frequency of these surveys could depend on the program, but annual surveys were adequate in this experience. In our program response to survey was close to 50% of participants. Results of the surveys were reviewed and discussed both, at meetings and conference calls with the Research Committee to identify process improvements. As a testament to the success of this program, 50% of the mentees surveyed in 2018 had already scheduled another meeting with their mentors, and all mentors were willing to continue sharing their expertise. We also believe that this program helped to enrich a national network of pediatrician scientists with similar research interests. It is possible that people with positive experiences were more likely to complete the survey. As such, some results may be biased toward positive experiences.

Challenges for a mentoring program in academic pediatric nephrology are great but not insurmountable. Limited funding opportunities across a small number of funding agencies in the field can make it difficult for junior investigators to identify and successfully compete for grant funding.Within small programs, mentees may have difficulty finding a mentor with the same or similar research interest or appropriate expertise. Mentors may have topical expertise but lack experience in pediatric research or grant review. Faculty from smaller programs may not have access to robust grant writing or career development curricula. Harnessing the strengths of pediatric academic societies addresses these challenges by bringing together mentees and outside mentors from different institutions.

We were proud of the fact that a significant number of early and midcareer women scientists benefited from the program. Per national NIH data, women are still under-represented at every stage of academic advancement. In 2015, for example, women were 44% of assistant professors, and 35% of professors (25). Our program successfully overcame that disparity.

Our program has some limitations. Due to the relatively small number of participants, we cannot provide detailed characteristics on age, race/ethnicity, and size/resources of their home institution. Although we chose mentors with strong track records of NIH-funded research, we did not collect information about their mentoring activities within their own institutions. In the future we are planning to collect more data about mentee's institutional environment (e.g., size of practice, characteristics of medical centers, local mentoring) as well as data about number of proposals funded by the NIH, industry and foundations.

Suggestions for the Design of Mentoring Programs

It is fundamental that mentors and mentees who entered a mentoring program have a clear understanding of the goals, objectives, and desirable outcomes of the collaboration. Mentors should be willing to commit to working one-on-one with mentees and ideally should have a successful track record of grant awards and research publications. Mentors should help young clinical scientists to plan, develop, grow, and manage their careers. Mentors also have an important role in helping junior faculty members become resilient in times of change, more self-reliant

in their careers, and more responsible as self-directed learners. Mentees should clearly understand expectations and should be willing to provide feedback to the program.

Based on our ASPN experience, we identified that the 10 key elements (in no specific order) for successful mentorship programs are to:

1. Provide concrete advice on specific research pursuits and promote interaction between mentors and mentees as well as other content experts with successful research careers;
2. Provide infrastructure that enables pairing of mentors and mentees, completion of goals statements, assurance of confidentiality, and successful program completion;
3. Facilitate planning and management of program assessments, track scheduling, and report documentation;
4. Inform program participants about available specialty focused NIH and non-NIH funding opportunities;
5. Complete thorough and consistent evaluations of the participants' progress;
6. Perform continuous review and evaluation of program assignments and activities throughout the program cycles and make appropriate changes;
7. Employ continuous QI techniques (Plan-Do-Study-Act) to improve programmatic effectiveness;
8. Provide enticements for senior investigators and experienced mentors to stay involved, such as Maintenance of Certification (MOC) Part 4 credits from the American Board of Pediatrics (ABP) for participants;
9. Train a future generation of mentors through role modeling and feedback.
10. Motivate others with dedication, enthusiasm and good will.

In summary, a structured and well-organized research mentorship program with strong medical society leadership, brings professional fulfillment to academic physicians. In addition, providing increased research mentorship helps to increase and sustain the research workforce, which results in more robust research and improved child health.

CONCLUSION

The ASPN Research Mentorship Program, initiated and supported within a relatively small medical society; showed early success in training a future generation of clinician-scientists who will be able to undertake valuable research that we hope will 1 day lead to better patient outcomes.

AUTHOR CONTRIBUTIONS

TV participated in the program and led the writing group, correspondent author. MD-G substantial contribution in Program improvement and correction of manuscript. DH led a mentorship program, participated in writing. JH led mentorship program, participated in writing. LH actively participated in mentorship program and manuscript writing. KR actively participated in mentorship program evaluation and manuscript writing. TB led the program, writing. DO led the program, writing. DS manuscript revision, suggestions, writing. SW led the program, participated in survey, data analysis and writing. EH led the program, survey, data analysis, graphs for paper, writing and revisions.

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REVIEW
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Overview of Monogenic or Mendelian Forms of Hypertension

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Monogenic or Mendelian forms of hypertension are described as a group of conditions characterized by insults to the normal regulation of blood pressure by the kidney and adrenal gland. These alterations stem from single mutations that lead to maladaptive overabsorption of electrolytes with fluid shift into the vasculature, and consequent hypertension. Knowledge of these various conditions is essential in diagnosing pediatric or early-onset adult hypertension as they directly affect treatment strategies. Precise diagnosis with specific treatment regimens aimed at the underlying physiologic derangement can restore normotension and prevent the severe sequelae of chronic hypertension.

Keywords: monogenic hypertension, Liddle syndrome, congenital adrenal hyperplasia, apparent mineralocorticoid excess, Gordon syndrome, familial hyperaldosteronism

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INTRODUCTION

Hypertension is one of the most prevalent diseases in the world, affecting an estimated 34% of individuals at or above the age of 20 within the United States alone (1). In contrast, the prevalence of hypertension among individuals aged 12–19 in the United States is 4.11% (2). Treatment of high blood pressure is paramount for improving cardiovascular health and preventing long term morbidity and mortality, per the World Heart Federation (3). The significance of untreated hypertension is emphasized by the severity of end organ damage and extreme systemic sequelae in patients with longstanding disease. The majority of hypertension results from a combination of behavioral and environmental factors; however, various genetic mutations have been identified as distinct causes as well. These genetic forms of hypertension stem from gain- or loss-of-function mutations within the mineralocorticoid, glucocorticoid, or sympathetic pathways (4). The term, monogenic hypertension, is used to describe specific genetic hypertensive disorders which inhibit normal renal and/or adrenal blood pressure regulation. It is especially important to keep these rare conditions in mind when diagnosing hypertension in children, as the younger the child, the more likely that their hypertension is due to a secondary cause. Recent advancements in genetic sequencing methodology have provided further insight into these conditions.

Monogenic hypertension drives volume expansion by three distinct mechanisms: (1) excessive sodium ion reabsorption by hyperactive channels, (2) hyperstimulation of mineralocorticoid receptors due to alterations in steroid synthesis, and (3) excess mineralocorticoid synthesis causing volume expansion (5, 6). However, one could conceptualize the latter two mechanisms as indirect

means of increasing salt reabsorption, which is the basis of the first mechanism. Monogenic hypertension is further classified based on serum renin and aldosterone levels. Low renin monogenic hypertension conditions can be further categorized by aldosterone level: low, normal, or high (**Table 1**). Another category is monogenic hypertension due to andrenergic/sympathetic excess. Treatment must be specific to each syndrome's unique derangements within the mineralocorticoid, glucocorticoid, or sympathetic pathways. However, as with all types of hypertension, treatment is aimed at the amelioration of cardiovascular morbidity and mortality associated with long standing high blood pressure. The various types of monogenic hypertension with specific evaluation and treatment considerations will be reviewed in this article. **Table 2** provides an overview of the causes of monogenic hypertension and their Online Mendelian Inheritance in Man (OMIM) numbers (7). **Figure 1** depicts the diagnostic evaluation for monogenic hypertension.

TABLE 1 | Basic classification scheme for causes of monogenic hypertension.

Low renin level	Low aldosterone levels	Liddle syndrome Congenital adrenal hyperplasia Apparent mineralocorticoid excess Gellers syndrome
	Normal aldosterone levels	Gordon syndrome (pseudohypoaldosteronism type II)
	High aldosterone levels	Familial hyperaldosteronism type I (glucocorticoid-remediable aldosteronism) Familial hyperaldosteronism type II Familial hyperaldosteronism type III Familial hyperaldosteronism type IV
Adrenergic/sympathetic excess	High metanephrine and normetanephrine levels	Familial pheochromocytoma
Vascular smooth muscle proliferation		Hypertension and brachydactyly syndrome

LOW ALDOSTERONE MONOGENIC HYPERTENSION

Case 1: A 10-year-old boy presented to the clinic with skeletal muscle weakness, paresthesia, nausea, and vomiting, all starting ∼5–6 months ago. He had no significant past medical history and was previously healthy. His mother had been diagnosed with hypertension at the age of 20 years, but no other significant family history was noted. The patient was not currently on any medications and had no known drug allergies. On examination, the child was mildly lethargic. Blood pressure measured 164/92 mmHg in the arms and 183/112 mmHg in the legs. The rest of the general physical examination was unremarkable. The patient's height and weight were at the 50th and 62nd percentile, respectively, based on the WHO growth chart. Laboratory work up included complete blood count, electrolytes, thyroid profile, liver profile, urinalysis (UA), and abdominal imaging was obtained. The patient's electrolytes were significant for hypokalemia with serum potassium levels of 2.1 mEq/L (normal 3.5–5.0 mEq/L) as well as a mild metabolic alkalosis with serum bicarbonate of 31 mmol/L (normal 22–29 mmol/L). The remaining labs and imaging studies were unremarkable. The patient was started on spironolactone for the elevated blood pressure and potassium supplements for hypokalemia and was asked to return for a follow up visit in 2 weeks.

After 2 weeks, the patient returned to the clinic with no change in symptoms. Lab values remained the same despite appropriate medication adherence. Plasma renin, serum aldosterone, 11-beta-hydroxylase, and 17-alpha-hydroxylase were ordered. Lab values revealed suppressed plasma renin <1.0 ng/mL/h (normal 2.8–39.9 ng/mL/h) as well as low aldosterone level 8.2 ng/mL/h (normal >20 mg/mL/h). The plasma aldosterone to plasma renin ratio was greater than 30 ng/dL. The child was diagnosed with Liddle syndrome and was started on amiloride. At the next follow up visit, the patient's prior symptoms resolved, blood pressure had returned to the normal range based on age, sex, and height; and the hypokalemia had resolved.

LIDDLE SYNDROME

Genetics and pathogenesis: Liddle Syndrome is caused by an autosomal dominant (AD), gain-of-function mutation of the epithelial sodium channel (ENaC) present in the collecting duct. The ENaC beta and gamma subunits, SCNN1B and SCNN1G, respectively, are most commonly affected (4, 8). Mutation of these subunits disrupts expression of proline-rich regions of the cytoplasmic carboxyl terminus and results in loss of regulatory binding sites for Nedd4-2, a ubiquitin ligase necessary for the breakdown of ENaC (8–10). Thus, ENaC remains constitutively active with increased Na^+ reabsorption and subsequent intravascular volume expansion culminating in hypertension (**Figure 2**) (8, 11, 12). Liddle Syndrome-associated Na^+ reabsorption is independent of aldosterone, and a hallmark of the disease is the suppression of renin and aldosterone. Hypokalemia ensues as excess Na^+ is continually absorbed with extrusion of K^+ via the Na/K-ATPase pump (8, 13). Mild metabolic alkalosis is also seen, as the increased Na^+ reabsorption increases the net negativity of the lumen, causing an increase in H^+ extrusion via the renal outer medullary potassium (ROMK) channel and H^+-ATPase pump located on alpha-intercalated cells (10).

Epidemiology: Liddle's is rare with only 30 patients having been formally diagnosed since 2008; however, many nephrologists perceive that it is an under recognized cause of hypertension (14). Liddle's has an estimated prevalence of 1.5% with genetic testing in a Chinese population and was found to have a 6% prevalence in hypertensive patients among a study of US veterans in Louisiana (15, 16). Age of onset for hypertension is typically young, between late childhood and adolescence (17). Patients lacking a family history have also been noted, indicating that LS should always remain in the differential for early-onset hypertension irrespective of the family history (8).

TABLE 2 | Overview of the causes of monogenic hypertension and their OMIM genotype and phenotype numbers.

Condition	Mode of inheritance	OMIM phenotype number(s)	OMIM genotype numbers(s)	Cytogenetic loci	Pathophysiology	Management
Liddle syndrome	Autosomal dominant	177200	600760 (SCNN1B) 600761 (SCNN1G)	16p12.2	Hyperactive ENaC reabsorbs sodium at elevated levels, resulting in volume expansion and hypertension	Patients present with early onset HTN with hypokalemia non-responsive to conventional therapy. Genetic testing confirms the diagnosis. Use ENaC inhibitory agents: amiloride, triamterene.
Congenital adrenal hyperplasia	Autosomal recessive	202010 (type IV)	610613 (CYP11B1)	8q24.3	Defects in steroid synthesis cause buildup of intermediate metabolites with MR activity	Patients present with HTN at very young ages along with atypical sexual development. Glucocorticoid supplementation to suppress ACTH expression treats HTN; potentially add MR antagonists for better control. Therapy should also be individualized to address aspects of sexual dysfunction.
	Autosomal recessive	202110 (type V)	609300 (CYP17A1)	10q24.32		
Syndrome of apparent mineralocorticoid excess	Autosomal recessive	218030	614232 (HSD11B2)	16q22.1	HSD11B2 deficiency allows excess cortisol stimulation at the MR	Therapy uses MR antagonists to alleviate overactivity and may call for ACTH suppression with excess cortisol
Geller syndrome	Autosomal dominant	605115	600983 (NR3C2)	4q31.23	Genetic mutations in the MR alter its structure and binding affinities, allowing atypical stimulation by other steroids, especially progesterone	Presents by early adult life; most critical in pregnant women. Management would be with delivery of the child and subsequent monitoring. Spironolactone is to be avoided.
Gordon syndrome (pseudohypoaldosteronism type II)	Autosomal dominant	145260 (type IIA)	Unspecified	1q31-1q42	Mutations in regulatory proteins for the NCC channel allow for unchecked activity, causing subsequent electrolyte and fluid overabsorption	Thiazide diuretic therapy directly treats NCC hyperactivity.
	Autosomal dominant	614491 (type IIB)	601844 (WNK4)	17q21.2		
	Autosomal dominant	614492 (type IIC)	605232 (WNK1)	12p13.33		
	Autosomal recessive or dominant	614495 (type IID)	605775 (KLHL3)	5q31.2		
	Autosomal dominant	614496 (type IIE)	603136 (CUL3)	2q36.2		
Familial hyperaldosteronism type I (glucocorticoid-remediable aldosteronism)	Autosomal dominant	103900	610613 (CYP11B1)	8q24.3	Unequal crossing over between the CYP11B1 and CYP11B2 genes generates a chimeric product that is ACTH-sensitive and produces aldosterone	Treatment with glucocorticoids to reduce ACTH secretion, supplemented with MR antagonists if necessary. Patients should be screened regularly for HTN-induced cerebrovascular sequelae
Familial hyperaldosteronism type II	Autosomal dominant	605635	600570 (CLCN2)	3q27.1	Hyperplasia or benign neoplasia within the adrenal cortex results in excess aldosterone production	Medical management with MR antagonists with potential surgical resection
Familial hyperaldosteronism type III	Autosomal dominant	613677	600735 (KCNJ5)	11q24.3	Gain-of-function mutations in potassium channels allow adrenal cortical cells to depolarize and subsequently activate aldosterone synthase	Medical management with MR antagonists with potential surgical resection
Familial hyperaldosteronism type IV	Autosomal dominant	617027	607904 (CACNA1H)	16p13.3	Gain-of-function mutations in calcium channels delay inactivation of cells, allowing enhancing aldosterone synthase activity	Medical management with MR antagonists with potential surgical resection

(Continued)

TABLE 2 | Continued

Condition	Mode of inheritance	OMIM phenotype number(s)	OMIM genotype numbers(s)	Cytogenetic loci	Pathophysiology	Management
Familial pheochromocytoma	Autosomal dominant	171300	605995 (KIF1B)	1p36.22	Neoplasia of the adrenal medulla generates heightened levels of norepinephrine and epinephrine	Medical management with catecholamine antagonists and other antihypertensives prior to surgical resection. Continuous monitoring and genetic testing may prove helpful with syndromic causes
			185470 (SDHB)	1p36.13		
			613403 (TMEM127)	2q11.2		
			608537 (VHL)	3p25.3		
			600837 (GDNF)	5p13.2		
			164761 (RET)	10q11.21		
			602690 (SDHD)	11q23.1		
			154950 (MAX)	14q23.3		
Hypertension and Brachydactyly Syndrome	Autosomal dominant	112410	123805 (PDE3A)	12p12.2	Gain-of-function mutations generate increased cAMP levels causing enhanced vascular smooth muscle proliferation, accompanied by brachydactyly due to dysfunctional chondrogenesis	High concentration milrinone therapy with possible benefits from phosphodiesterase inhibitors to increase cGMP levels

Gene names are in parentheses next to the genotype number, where applicable. HTN, hypertension; ENaC, epithelial sodium channel; ACTH, adrenocorticotropic hormone; MR, mineralocorticoid receptor; NCC, sodium chloride cotransporter.

Work Up: A strong family history of hypertension, suppressed renin/aldosterone levels and response to ENaC antagonism is highly suggestive, but a definitive diagnosis requires genetic testing (16). Random aldosterone/renin ratio can be used as a screening test, and a ratio >30 excludes the diagnosis (when expressed in ng/dL for aldosterone and ng/dL/h for renin) (15). Renin is suppressed by both elevated sodium levels and volume expansion; aldosterone is often less suppressed, resulting in the increased aldosterone/renin ratio. Liddle syndrome is alternatively known as pseudohyperaldosteronism due to its similarity to hyperaldosteronism with hypertension, hypokalemia, and metabolic alkalosis (18).

Treatment: Treatment consists of direct ENaC inhibition with potassium-sparing diuretics such as amiloride or triamterene. Spironolactone is not useful as the genetically altered ENaC is independent of mineralocorticoid regulation (8). A low sodium diet is also important in managing hypertension and counteracting the altered ENaC physiology (19).

Case 2: A 10-year-old female presented with hirsutism, particularly on her face as well as the pubic area. She first noticed the hair development 2 months ago. Her past medical history is significant for menarche at the age of 8 with irregular menstrual periods accompanied by breast development. Her maternal aunt also went through puberty around the age of 7–8. The patient was not on any medications at the time of office visit. On examination, the patient was alert with no signs of distress. Her blood pressure measured 168 /104 mmHg on manual auscultation. She is currently Tanner stage II, though her clitoral folds were noted to be enlarged. She is at the 80 and 90th percentiles for height and weight, respectively. Pertinent labs including a complete metabolic panel, adrenal profile, and thyroid profile were obtained. Lab results showed hypokalemia as well as elevated levels of multiple hormone precursors and androgens [serum potassium 2.1 mEq/L (normal 3.5–5.0 mEq/L), serum deoxycorticosterone 21 ng/dL (normal 2–13 ng/dL), serum dehydroepiandrostenedione sulfate (DHEAS) 392 μg/dL (normal <160 μg/dL), serum testosterone, 82 ng/dL (normal 0–30 ng/dL)]. Based on presentation and laboratory findings, the patient was diagnosed with congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. She was started on hydrocortisone with subsequent resolution of her hypertension and hypokalemia.

CONGENITAL ADRENAL HYPERPLASIA

Though 21α-hydroxylase deficiency is more common, 11β-hydroxylase deficiency (11OHD or CAH type IV) and 17α-hydroxylase deficiency (17OHD or CAH type V), are two subtypes of CAH known to cause monogenic hypertension (13). The respective enzymes regulate different steps in steroid synthesis, but 11OHD and 17OHD deficiency both cause elevated deoxycortisol and deoxycorticosterone levels. These intermediates have activity at the mineralocorticoid receptors. Unchecked mineralocorticoid activity leads to hypertension and hypokalemia. Both types are inherited in an autosomal recessive

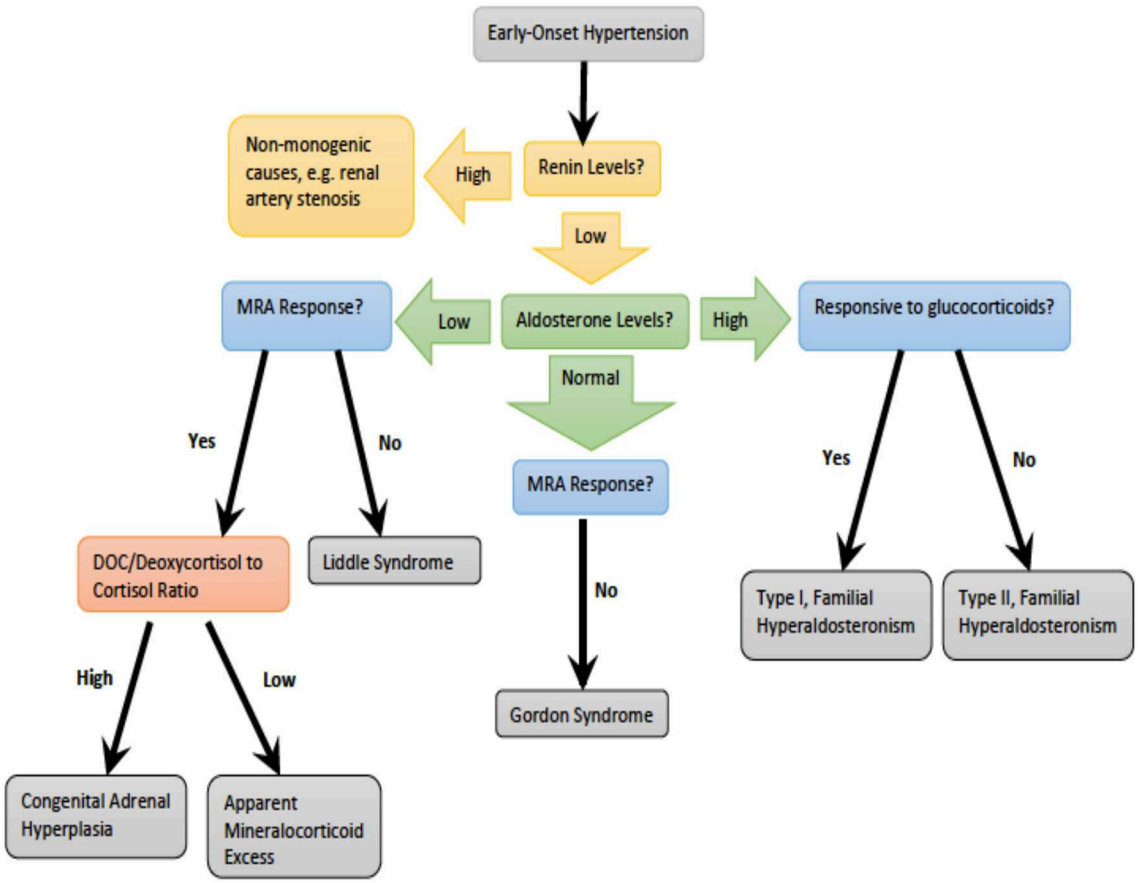

FIGURE 1 | Diagnostic evaluation for monogenic hypertension. DOC, deoxycorticosterone.

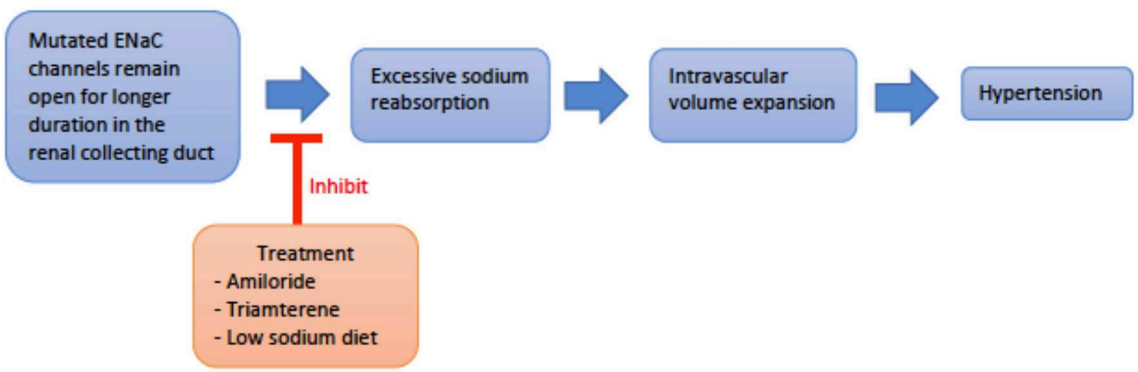

FIGURE 2 | An overview schematic of Liddle syndrome pathophysiology and treatment. ENaC, epithelial sodium channel.

(AR) fashion, stemming from inactivating mutations that prevent expression of the respective enzymes (20).

11β-Hydroxylase Deficiency

Genetics and Pathophysiology: CAH type IV, or loss of 11β-hydroxylase, prevents conversion of deoxycortisone and deoxycortisol into corticosterone and cortisol, respectively. Both deoxycortisone and deoxycortisol have weak activity at the mineralocorticoid receptor, but their accumulation in CAH type IV leads to significant mineralocorticoid activity and subsequent hypertension. As the conversion to glucocorticoids, androgens and estrogens are preserved, patients present with high levels of deoxycorticosterone, deoxycoritsol and androgens, mainly androstenedione and dehydroepiandrosterone (DHEA)

(**Figure 3**). This is a key factor in laboratory diagnosis after ACTH (adrenocorticotropic hormone) stimuation, as high levels of the androgens coupled with HTN prove helpful in differentiating 11OHD from other causes of CAH and monogenic hypertension (21).

Epidemiology: 11OHD is considered to be a rare condition that accounts for 5–8% of CAH cases (22). However, it has been seen with relatively high frequency among certain populations and accounts for 15% of CAH cases in Muslim and Jewish Middle Eastern populations (23). Detection of this disorder can be done by testing for 11β-hydroxylase activity or measuring tetrahydro-11-deoxycortisol in amniotic fluid or urine.

Workup: Like other CAH subtypes, 11OHD manifests with disorders of sexual development. Patients can potentially be identified at birth based on physical findings. Due to androgen accumulation, female infants undergo virilization, presenting with ambiguous genitalia at birth with enlarged clitoral folds (4). Androgen excess can also cause precocious puberty in both males and females. Regardless of variations in sexual development, patients present with hypertension at very young ages. Diagnosis relies on measuring levels of the adrenal steroid hormones to identify enzyme activity and defects. Further genetic testing for the CYP11B1 gene (cytogenetic locus 8q24.3) offers a definitive understanding of disease pathophysiology, guiding treatment (21).

Treatment: Treatment consists of glucocorticoid doses sufficient to decrease ACTH secretion, inhibiting stimulation of steroid synthesis and accumulation of mineralocorticoid receptor agonists. Spironolactone, amiloride, and calcium channel blockers may be further used to treat hypertension. Further considerations include individualized therapy to accommodate sexual development in patients and genital malformations in females may need surgical correction (24).

17α-Hydroxylase Deficiency

CAH type V, also known as P450C17α deficiency, manifests clinically as hypogonadism, hypokalemia, and hypertension. The gene, CYP17A1, located at cytogenetic locus 10q24.32 encodes 17α-hydroxylase with two key steroidogenic functionalities: 17α-hydroxylase and 17,20-lyase activity (21). Its 17α-hydroxylase function generates 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, which can be converted to cortisol. These products of the 17α-hydroxylase are also substrates for the 17,20-lyase, which generates DHEA and androstenedione to serve as precursors for androgen and estrogen synthesis. This rare disorder thus blocks the production of cortisol as its direct precursors are not synthesized, shunting steroid production toward the mineralocorticoids (**Figure 3**). Since the blockage is so early in the pathway, there is very little production of sex hormones (23). Male patients have ambiguous external genitalia and may even exhibit a female phenotype, while females present with primary amenorrhea and delayed sexual development (6).

Workup: Steroid analysis upon ACTH-stimulation would lead to an appropriate diagnosis, showing atypically elevated levels of pregnenolone and progesterone relative to 17α-pregnenolone and 17α-progesterone, respectively.

Treatment: Treatment for these patients would be the same as type IV regarding hypertension with the addition of sex hormone therapy (6).

Case 3: A 10-month-old infant came to the clinic with severe hypertension. The patient had no significant past medical history. Lab results as well as imaging reveal severe hypokalemia, mild left ventricular hypertrophy (LVH), and nephrocalcinosis on renal ultrasound. Further metabolic lab studies showed low aldosterone and renin levels. A 24-h urine tetrahydrocortisol (THF) + allo-tetrahydrocortisol (5α-THF) to tetrahydrocortisone (THE) was collected when prior labs detected normal deoxycorticosterone, corticosterone, 18-hydroxydeoxycorticosterone, and 18-hydroxycortisol levels. The 24-h urine sample of THF + 5αTHF confirmed the diagnosis of syndrome of apparent mineralocorticoid excess (AME). The patient was started on amiloride with potassium supplements. A follow up visit in 2 weeks showed normalization of blood pressure as well as normalized potassium.

APPARENT MINERALOCORTICOID EXCESS

Genetics and pathophysiology: The syndrome of apparent mineralocorticoid excess (AME) is an autosomal recessive disease caused by an inactivating mutation of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2). The cytogenetic location is on chromosome 16q22.1 (4). The mineralocorticoid receptor's prototypical ligand is aldosterone, but cortisol is also able to bind and activate the receptor. Thus, the primary function of HSD11B2 is to prevent cortisol from binding to the mineralocorticoid receptor by catalyzing its metabolism to cortisone which does not have mineralocorticoid activity (6, 23). As cortisol is expressed at much higher physiologic levels than aldosterone, HSD11B2 function is critical to maintaining proper control over mineralicorticoid receptor activation. With this mutation, cortisol is not metabolized and is able to bind to the mineralocorticoid receptor, causing clinical features similar to pseudohyperaldosteronism (**Figure 4**). However, in this disorder the defect is at the level of the receptor itself rather than down stream at the ENaC channel. However, due to this similarlity both groups of patients typically present with hypokalemia, hypertension, and metabolic alkalosis (25).

Epidemiology: AME has been observed in many ethnic groups, including Caucasians, African Americans, Asians, and American Indians. This disease presents in early childhood. Severe phenotypes can present with low birth weight and failure to thrive, and if left untreated, these patients have high mortality rates (13).

Workup: The diagnosis is based on laboratory findings of hypokalemia, metabolic alkalosis, hyporeninemia, hypoaldosteronemia, and an elevated urinary cortisol to cortisone ratio by THF + 5αTHF to THE in a 24-h urine collection (26). It is important to rule out chronic excess licorice or carbenoxolone ingestion; as these cause similar symptoms due to inhibition of the same enzyme as in AME, but typically resolve once the offending agent is removed (24).

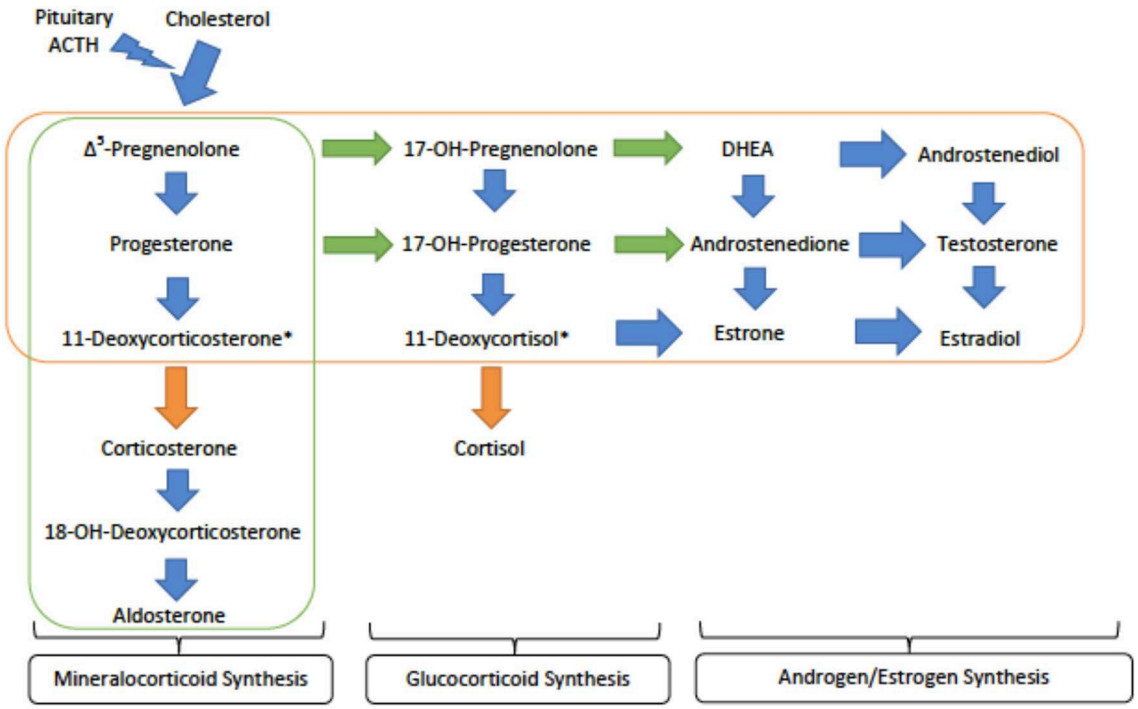

FIGURE 3 | An overview of the steroid synthesis pathways and the enzyme deficiences that lead to congenital adrenal hyperplasia (CAH) types IV and V. The orange arrows show the activity of 11β-hydroxylase (deficient 1 in type IV) while the orange rectangle surrounds the steroid hormones still produced in CAH type IV. The green arrows denote the activity of 17α-hydroxylase (deficient in type V) while the green rectangle encircles those hormones produced in CAH type V. ACTH - adrenocorticotropic hormone; DHEA - dihydroepiandrosterone. *These metabolites are active at the mineralocorticoid receptor and are important in generating hypertension.

Treatment: Treatment consists of mineralocorticoid receptor antagonists, such as spironolactone or eplerenone, along with potential potassium supplements and dietary sodium restriction (6, 24). Elevated cortisol levels may further warrant glucorticoid therapy to reduce ACTH-stimulated cortisol production and subsequent mineralocorticoid receptor stimulation (19, 27). Renal transplantation may be curative in some cases (28).

Case 4: A 17-year-old with a past medical history of hypertension came in for her routine checkup for her 34-week-old fetus. She had a family history of elevated blood pressure on her father's side. She complained of mild headaches for the past couple of weeks. On exam, her blood pressure was severely elevated. Laboratory testing including basic metabolic panel, thyroid function tests, and serum cortisol came back normal. Brain CT was normal. Her hypertension was attributed to pre-eclampsia and the patient was admitted for emergent C-section. After delivery, the patient's blood pressure was still mildly elevated and she received a dose of spironolactone. Two hours after taking the medication, her blood pressure worsened instead of improving. The patient was given other antihypertensives and her blood pressures improved. Genetic testing revealed a mutation of the mineralocorticoid receptor gene to confirm the diagnosis of Geller Syndrome.

GELLER SYNDROME

Geller syndrome, also referred to as constitutive activation of the mineralocorticoid receptor, results from a gain-of-function mutation on chromosome 4q31. Geller syndrome has an autosomal dominant inheritance pattern (17). This mutation within the mineralocorticoid receptor causes it to remain constitutively active due to changes to receptor sites that result in altered specificities to the steroid hormones. Steroid hormones, including progesterone, subsequently act as agonists of the mineralocorticoid receptor, as opposed to their normal antagonism (5).

The onset of hypertension starts before the age of 20 (17). It is essential to recognize this disease, as pregnancy can severely exacerbate the hypertension due to elevated progesterone levels. This causes activation of the mineralocorticoid receptors, though both aldosterone and renin are suppressed. Potassium levels are surprisingly normal in these patients. A clear diagnosis can be done by genetic testing for gene mutations in the mineralocorticoid receptor (17). Treatment is unlike many other causes of monogenic hypertension, as spironolactone is contraindicated and will worsen the disease. The mainstay for treatment during pregnancy is to deliver the fetus. Optimal management of non-pregnant females and males is not well-defined.

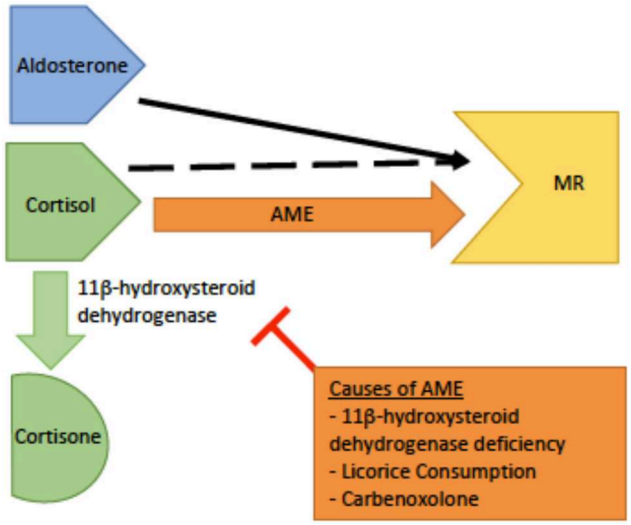

FIGURE 4 | A schematic of the pathophysiology of apparent mineralocorticoid excess (AME). The dashed black arrow indicates normal binding of cortisol to the mineralocorticoid receptor (MR) with conversion to cortisone by 11β-dehydrogenase.The orange arrow indicates AME. In AME, deficiency or inhibition of 11β -dehydrogenase leads to increased levels of cortisol increased cortisol-MR binding, and subsequent hyperactivity of the MR.

NORMAL ALDOSTERONE MONOGENIC HYPERTENSION

Case 5: A 9-year-old female was brought into the clinic by her mother for new onset dizziness and muscle weakness. The patient's height and weight were normal based on the WHO growth chart. Laboratory testing including chemistries were notable for severe hyperkalemia and hypocalcemia. All other lab values including PTH were normal. On exam her blood pressure was very elevated at 145/90 prompting renin and aldosterone testing. Renin levels were below normal and aldosterone was at the upper limits of normal. The patient was diagnosed with Gordon syndrome and she was started on a low dose thiazide.

Pseudohypoaldosteronism Type II (Gordon Syndrome)

Genetics and pathophysiology: Pseudohypoaldosteronism type II (PHAII), also referred to as Gordon syndrome, is autosomal dominant and affects the WNK serine-threonine kinase family (WNK1 and WNK4) (29). PHAII type B is due to a loss-of-function mutation of the WNK4 gene on chromosome 17q21.2, while PHAII type C results from gain-of-function mutation of the WNK1 gene on chromosome 12p12.3 (30). The most common clinical features seen are hyperkalemia, hyperchloremic metabolic acidosis, and normal-to-elevated levels of aldosterone. Mutation of the WNK genes leads to failure of endocytosis of the Na^+-Cl^- co-transporter (NCC). This loss of inhibitory regulation of NCC in the distal convoluted tubule causes hyperchloremic metabolic acidosis. More specifically, WNK4 activity directly inhibits NCC activity by reducing its expression on the extracellular membrane. In contrast, WNK1 inhibits WNK4 to reduce inhibition of NCC expression on the apical membrane. Thus, a loss-of-function in WNK4 or gain-of-function in WNK1 both generate a PHAII phenotype. The resulting increase in sodium reabsorption in the distal convoluted tubule inhibits normal sodium reabsorption downstream by ENaC (**Figure 5**). Without ENaC sodium reabsorption, ROMK potassium excretion as well as H-K exchange are both suppressed, driving the hyperkalemia and acidosis associated with Gordon's (13). Hypercalciuria is also found in these patients, and specifically hypercalciuria in the absence of elevated parathyroid hormone or serum calcium. The mechanism of urine calcium wasting is not completely understood, though multiple mechanisms have been postulated. WNK4 is known to modulate the activity of the transient receptor potential V5 channel (TRPV5), also located in the distal convoluted tubule. Decreased WNK4 function may thus decrease the normal inflow of calcium ions through TRPV5 in the DCT. Increased plasma sodium levels in PHAII may also inhibit the function of the Na^+/Ca^{2+} exchanger in the PCT. Ultimately, reduced calcium reabsorption places these patients at risk for urolithiasis and osteoporosis (31, 32).

Two additional genes, KLHL3 and CUL3, located at chromosome 5q31.2 and 2q36.2, respectively, have been identified as aiding WNK4 function; KLHL 3 mutations are implicated in PHAII type D, while CUL3 is implicated in PHAII type E (4, 33). Activation of WNK1 stimulates SGK1 (serum- and glucocorticoid-inducible protein kinase 1), which activates the NCC, increasing sodium reabsorption, and leading to hypertension (34).

Epidemiology: Hypertension generally manifests in adolescence to adulthood, but cases involving younger patients have been reported (31). It is important to note that Spitzer-Weinstein syndrome in children has similar clinical features, such as metabolic acidosis and hyperkalemia, and is thought to be an early presentation of Gordon syndrome (13). The prevalence of this disorder is unknown; only about 180 cases have been reported (31).

Workup: No criteria for the diagnosis of PHAII has been officially published. A clinical history and family history of a first-degree relative would aid in diagnosis, but a first degree-relative is not necessary. Molecular genetic testing is ideal for diagnosis, due to variation in severity and presentation.

Treatment: Electrolyte and blood pressure abnormalities can be treated with a low dose thiazide, which directly inhibits NCC hyperactivity. Patients should have routine follow up for assessment of electrolyte levels and blood pressure (31). Counseling patients to adhere to a low-sodium diet is an important consideration in long-term management of hypertension.

HIGH ALDOSTERONE MONOGENIC HYPERTENSION

Case 6: A 15-year-old boy presented to the emergency room with projectile vomiting, migraine and hypertension. The patient had no significant past medical or surgical history. A CT scan of the head showed that the patient had a minor bleed. A cerebral

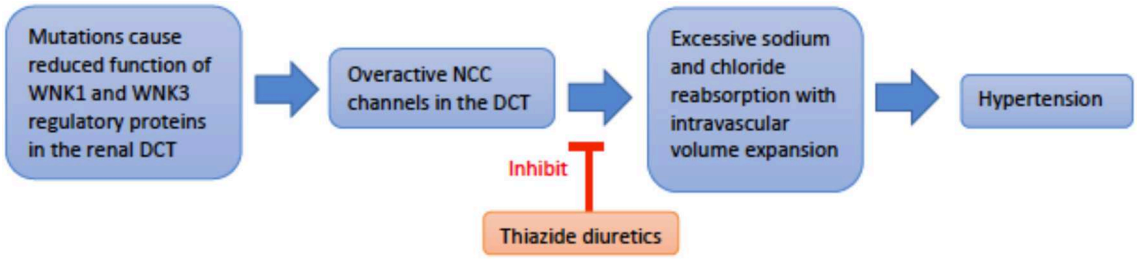

FIGURE 5 | An overview schematic of Gordon Syndrome pathophysiology and treatment. NCC is the sodium chloride co-transporter; DCT is the distal convoluted tubule.

angiogram showed rupture of a small aneurysm. Appropriate management, including acute blood pressure reduction, was performed and the patient survived. He presented to the clinic for a follow up visit. He is asymptomatic but still has elevated blood pressure, and laboratory testing demonstrates hypokalemia. All other labs are normal, except for low renin and elevated aldosterone levels. Adrenal vein sampling shows elevated levels of ACTH, and the patient is diagnosed with Familial Hyperaldosteronism type 1. The patient was started on dexamethasone and potassium supplements. Follow-up shows resolution of the hypertension.

Familial Hyperaldosteronism Type 1 (Glucocorticoid-Remediable Aldosteronism)

Genetics and pathophysiology: Inherited in an autosomal dominant fashion, familial hyperaldosteronism type 1 (FHT-I) is also also known as glucocorticoid-remediable aldosteronism (GRA) (20). This condition is due to alterations in the CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) genes that lie adjacent to each other on chromosome 8q24.3 (13, 35). Unequal crossover between these genes results in a chimeric variant that incorporates regulatory elements of CYP11B1 with the coding sequence of CYP11B2 (**Figure 6**). As a result the gene is regulated by ACTH rather than angiotensin II, however, the gene still controls aldosterone secretion (36). Normally, aldosterone is secreted by the zona glomerulosa of the adrenal gland, but due to this chimeric gene, aldosterone is ectopically secreted from the adrenal zona fasciculata instead (37). Increased production of aldosterone drives the key clinical features of hypertension and hypokalemia in the setting of suppressed renin levels (20).

Epidemiology: Most patients with this disease present with severe hypertension in childhood or even infancy. These patients also suffer from a high incidence of intracerebral hemorrhage (11–18%) with a high associated mortality rate (61%) at an early age (31.7 ± 11.3 years) (38).

Workup: Brain imaging with computed tomography angiogram (CTA) or magnetic resonance angiography (MRA) may be warranted due to clinical presentation and a strong family history of cerebrovascular incidents and early onset of hypertension (39, 40). Due to high rates of cerebrovascular events in GRA patients, screening is recommended every 5 years after puberty (41). Hypokalemia is usually mild and few go on to develop metabolic alkalosis. The plasma aldosterone (ng/dL) to plasma renin activity (ng/mL/h) ratio usually > 30 (normal<20). Many other diagnostic modalities such as the dexamethasone suppression test, adrenal imaging, and adrenal vein sampling may help in distinguishing FHT-I from other forms of hyperaldosteronism (13).

Treatment: Treatment for FHT-I includes glucocorticoids which suppress ACTH, and thus prevent the secretion of aldosterone (36). If glucocorticoids do not reduce the blood pressure, then mineralocorticoid receptor antagonists, such as spironolactone, or sodium epithelium receptor antagonists, such as triamterene or amiloride, can be used as second line therapy (39).

Case 7: A 14-year-old female presented with back pain and muscle spasms. The patient has been complaining of muscle spasms for the past 2 months but she believed it was due to her menstrual cycle. She was initially sent to obstetrics/gynecology for exploratory laparoscopy to rule out endometriosis. The exploratory laparoscopy was negative for endometriosis but the patient was noted to be hypertensive. She has a family history of hypertension. Laboratory work up was benign. A CT scan was completed and an adrenal tumor was identified. Biopsy revealed that it was a benign adenoma. Plasma aldosterone (ng/dL) to plasma renin (ng/mL/h) ratio was well above normal (normal<20). The patient was diagnosed with familial hyperaldosteronism type 1. The patient was given glucocorticoids and asked to come back the following week. The following week, the patient still had the same complaints and elevated blood pressures. The patient was then given mineralocorticoid receptor antagonists which lead to resolution of her symptoms and hypertension. The patient's diagnosis was changed from familial hyperaldosteronism Type 1 to Type 2.

Familial Hyperaldosteronism Type 2

Familial Hyperaldosteronism type 2 (FHT-II) is similar to FHT-I in that ectopic aldosterone synthesis occurs due to the loss of negative feedback seen with physiologic aldosterone secretion (42). However, the aldosterone synthase gene is not controlled by ACTH as in FHT-I, causing a non-dexamethasone-suppressible form of hyperaldosteronism. FHT-II had previously been mapped to chromosome 7p22, but no specific gene at this locus has been identified as the cause of the disease (43, 44).

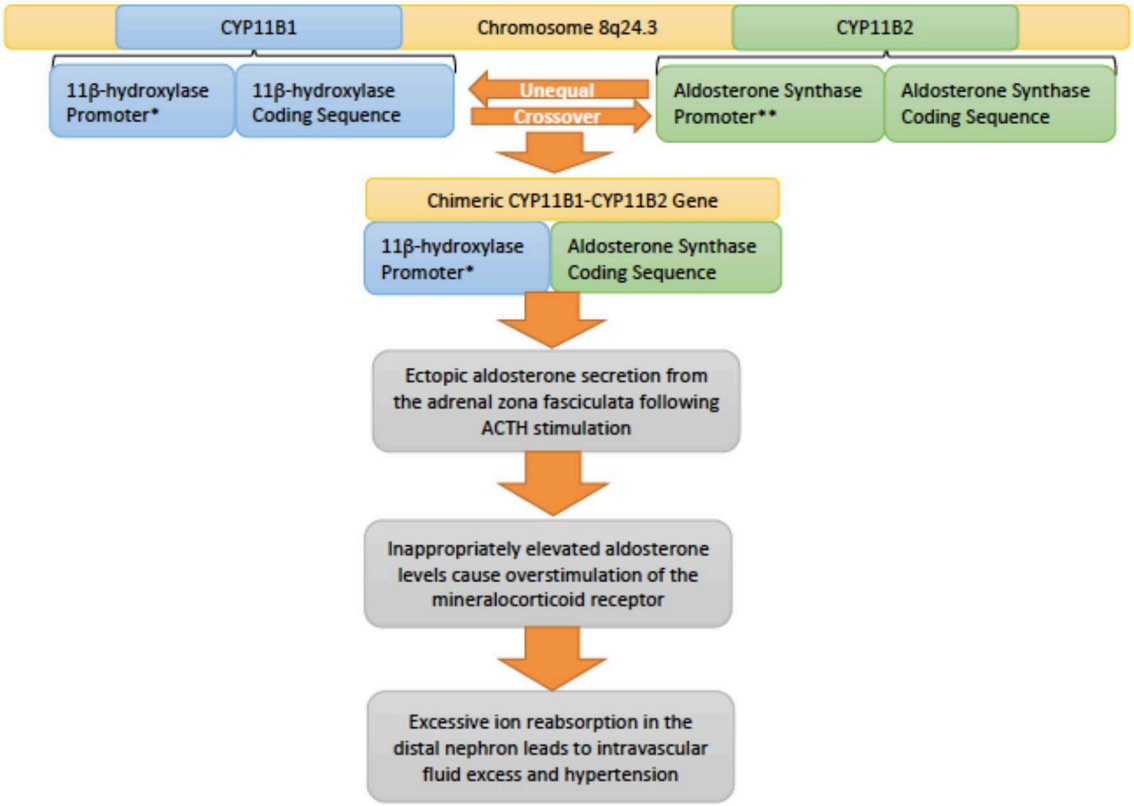

FIGURE 6 | An overview of the genetic pathophysiology of familial hyperaldosteronism type I. * Promoter responsive to adrenocotropic hormone (ACTH). **Promoter responsive to angiotensin II.

A recent study of patients diagnosed with FHT-II implicates gain-of-function mutations in the CLCN2 gene, encoding a chloride channel, ClC-2, located at chromosome 3q27.1 (45). The variant channels were shown *in vitro* to cause depolarization of adrenocortical cancer cell lines, leading to increased expression of aldosterone synthase and aldosterone production (45). FHT-II is characterized by bilateral adrenocortical disease and adenomas (42). The diagnosis of FHT-II should be confirmed by positive family history because FHT-II is clinically and biochemically indistinguishable from non-inherited primary aldosteronism (13). Genetic testing for CLCN2 mutations may prove helpful, though further work is needed to identify other possible causative genes (45). The mainstay of treatment for this disease is mineralocorticoid receptor antagonists or unilateral adrenalectomy (4).

Familial Hyperaldosteronism Type 3

Familial hyperaldosteronism type 3 (FHT-III) is an autosomal dominant condition caused by gain-of-function mutations in the KCNJ5 gene located at locus 11q24.3. KCNJ5 encodes a potassium channel, which loses its ionic selectivity in disease-causing variants to allow other cations, particularly sodium, to pass through. This allows for depolarization of the adrenal cortical cell, enhancing the expression of aldosterone synthase and subsequently raising aldosterone levels in patients (44). Treatment is similar to FHT-II and primary aldosteronism, ranging from mineralocorticoid antagonists to possible adrenalectomy depending on the severity of the hypertension (44, 46).

Familial Hyperaldosteronism Type 4

Familial hyperaldosteronism type 4 (FHT-IV) is an autosomal dominant condition caused by gain-of-function mutations in the CACNA1H gene located at chromosome 16p13.3 (44). CACNA1H encodes a transient opening calcium channel localized to the zona glomerulosa. The mutations implicated in FHT-IV cause the channel to be more likely to open at the baseline electrochemical gradient and remain open longer upon activation (47). These changes allow for greater influx of calcium ions, stimulating adrenal cortical cells and subsequently activating aldosterone synthase (47). As with other FHT types, treatment depends on the severity of the condition, possibly varying on the specific mutation, and ranges from medical management with mineralocorticoid antagonists to potential surgical adrenalectomy (46).

MONOGENIC HYPERTENSION DUE TO ADRENERGIC/SYMPATHETIC EXCESS

Case 8: A 7-year-old boy was brought to the clinic with a pounding headache. The child had also complained of palpitations for the past year. The patient had no other

complaints and had been healthy since birth. The patient was noted to have a severely elevated blood pressure of 210 / 130 mmHg. Physical exam was otherwise normal and routine laboratory values showed no significant findings. Urine metanepharines were collected and found to be elevated. Abdominal CT scan demonstrated a tumor adjacent to the adrenal glands. The diagnosis of pheochromocytoma was made and the tumor was surgically removed after appropriate perioperative blockade.

Familial Pheochromocytoma

Genetics and pathophysiology: Familial pheochromocytoma describes increased susceptibility to pheochromocytoma due to a variety of well-documented genetic mutations. Pheochromocytoma typically presents with paroxysmal symptoms, such as episodes of severe hypertension. Release of norepinephrine and epinephrine, parituclarly the former, is above and beyond physiologic levels. Pheochromocytoma susceptibility can be inherited alone or as a part of several syndromes. Von Hippel-Lindau disease (VHL) is associated with bilateral pheochromocytomas, retinal and cerebellar angiomas, renal and pancreatic cysts, and renal cell carcinoma. The mutation is on chromosome 3p25.3 and is a tumor-suppressor gene defect that leads to disease (48). RET, a proto-oncogene, is associated with type 2 multiple endocrine neoplasia syndrome (MEN 2) as well as non-syndromic pheochromocytoma (49). MEN 2 is inherited in autosomal dominant fashion due to a mutation on chromosome 10q11.21. MEN 2 has two subtypes MEN 2A and 2B. MEN 2A is associated with pheochromocytoma, medullary carcinoma of the thyroid, and hyperparathyroidism while MEN 2B is associated with pheochromocytoma, medullary carcinoma of the thyroid, and mucosal neuromas (50). Pheochromocytoma has also been associated with neurofibromatosis type I, caused by mutations in the NF1 gene, located at chromosome 17q11.2 (51). Solitary pheochromocytomas have also been shown to contain mutations in the aforementioned genes. One study of solitary tumors reported that 86% contained copy number alterations in genes associated with familial pheochromocytoma; changes in NF1 were found to be the most frequent at 26% of the tumors studied (52).

Epidemiology: Initially pheochromocytomas were considered to have 10% familial and 90% sporadic development. New technology in genetic testing has demonstrated that pheochromocytomas are 50% sporadic with 15–25% due to germ line mutations (53).

Workup: Catecholamine metabolism studies such as plasma free metanephrines and urinary fractionated metanephrines allow for successful screening of the condition and can be monitored for treatment response (54).

Treatment: Per The Endocrine Society clinical practice guideline, the treatment of functional pheochromocytomas begins with initiation of antihypertensive therapy followed by tumor resection. Medical management prior to surgery, known as pre-operative blockade, utilizes alpha-adrenergic antagonists (e.g., phenoxybenzamine or doxazosin) as firstline therapeutics. Other antihypertensives, particularly dihydropyridines and beta-adrenergic antagonists, may be used supplementarily. Diligent monitoring of blood pressure and catecholamine metabolism should take place throughout the perioperative course. Due to pheochromocytoma's association with the various aforementioned neoplastic syndromes, genetic testing may be recommended as a prognostic and preventative indicator (54).

MONOGENIC HYPERTENSION DUE TO VASCULAR SMOOTH MUSCLE PROLIFERATION

Hypertension and Brachydactyly Syndrome

Hypertension and brachydactyly (HTNB) describes an autosomal dominant syndrome caused by a mutation in the PDE3A gene located at chromosome 12p12.2; this gene encodes a phosphodiesterase that hydrolyzes cAMP. Variants of PDE3A that cause HTNB exhibit a gain-of-function due to altered enzyme phosphorylation (21). The mutant PDE3A enzymes consequently decrease cellular cAMP levels in vascular smooth muscle cells, allowing for proliferation. Uncontrolled proliferation of the smooth muscle eventually narrows the lumen of vessels, raising blood pressure. The associated brachydactly also stems from decreased cAMP levels, which lower the levels of PTHrP (parathyroid hormone related protein), a key moderator of chondrogenesis. Treatment aims to restore normal cAMP levels with high concentration milrinone, and there is possible benefit to increasing cGMP levels with phosphodiesterase inhibitors (21).

CONCLUSION

Hypertension is known as the silent killer; if not managed properly, it can lead to progressive end organ damage and death. Essential hypertension is often the result of multiple patient factors including polygenic inheritance and environmental factors, such as diet and physical activity. Monogenic hypertension refers to specific genetic mutations that interfere with normal renal and adrenal regulation of blood pressure. All forms of monogenic hypertension have low renin and can be further classified by the levels of aldosterone. Accurate diagnosis may require further hormonal studies or genetic testing. It important to have a high level of clinical suspicion and precisely diagnose these patients, as treatment is often quite different from traditional patients with essential hypertension. Appropriate treatment can decrease the morbidity and mortality associated with uncontrolled hypertension. With future advancements in genetic testing modalities and treatment options, there is hope for earlier recognition and improved management of the various forms of monogenic hypertension.

AUTHOR CONTRIBUTIONS

All the authors are responsible for the literature review, drafting and revision of the manuscript, and approved the final version of the manuscript.

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Rituximab for Children With Difficult-to-Treat Nephrotic Syndrome: Its Effects on Disease Progression and Growth

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Background: Since the early 2000s rituximab (RTX) has been thought of as an alternative treatment for steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS).

Objective: This study aimed to determine the effects of RTX treatment on disease outcome and growth in pediatric SSNS and SRNS patients.

Materials and Methods: The medical records of pediatric SSNS and SRNS patients that began RTX treatment at the mean age of 10.8 ± 5.1 years between 2009 and 2017 were retrospectively reviewed. Additionally, the effect of RTX on growth was evaluated based on patient height, weight, and BMI z scores.

Results: The study included 41 children, of which 21 had SSNS and 20 had SRNS. Mean age at diagnosis of NS was 5.8 ± 4.7 years. Mean duration of post-RTX treatment follow-up was 2.3 ± 1.6 years. Among the SSNS patients, 6 and 11 patients were steroid free and calcineurin inhibitor free at the last follow-up visit, respectively. The 1-year cumulative steroid and calcineurin inhibitor doses both decreased after RTX treatment, as compared to before RTX ($P = 0.001$ and $P = 0.015$, respectively). The median height *z*-score at the time of RTX initiation was −1.2 and the median height *z*-score at the last follow-up visit was −0.6 ($P = 0.044$). The median BMI *z*-score decreased from 1.6 (IQR; 0.9–3.0) at the time RTX was initiated to 1.1 IQR; [(−0.7)−2.5] at the last follow-up visit ($P = 0.007$). At the last follow-up visit 4 SRNS patients had complete remission and 4 had partial remission. The 1-year cumulative steroid dosage in the SRNS patients decreased significantly after RTX, as compared to before RTX ($P = 0.001$). The median height *z*-score at the time of RTX initiation was −0.8 and the median height *z*-score at the last follow-up visit was −0.7 ($P = 0.81$). The median BMI *z*-score decreased from 0.3 at the time RTX was initiated to −0.1 at the last follow-up visit ($P = 0.11$).

Conclusion: RTX has a more positive effect on disease outcome and growth in SSNS patients than in those with SRNS.

Keywords: rituximab, nephrotic syndrome, steroid, cyclosporine, growth

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INTRODUCTION

Idiopathic nephrotic syndrome (NS) is a major challenge in pediatric nephrology. The mainstay of NS treatment is steroid therapy. Nearly 80–90% of NS patients respond to steroid therapy and are considered to have steroid-sensitive NS (SSNS) (1). Approximately 60% of SSNS patients develop frequently relapsing NS (FRNS) or steroid-dependent NS (SDNS). In contrast, 10–20% of NS patients have steroid-resistant NS (SRNS) and do not respond to steroid therapy (2). Some SRNS patients can achieve partial or complete remission with calcineurin inhibitor (CNI) treatment (3). How best to treat NS patients that do not respond to steroids or CNIs remains a challenge. Moreover, patients with both FRNS/SDNS and SRNS usually require long-term steroid therapy, which is associated with the risk of serious side effects, including obesity, growth retardation, osteoporosis, glaucoma, cataracts, and hypertension (2).

Due to the problems associated with treating NS with steroids and CNIs, rituximab (RTX) has recently become an important alternative treatment option. RTX is an immunoglobulin IgG1-kappa type, mouse-human chimeric monoclonal antibody, with murine anti-CD20 variable sequence regions and human constant sequence regions. RTX binds specifically to the CD20 antigen, which is expressed on pre B-cells, and immature, mature, and memory B-cells, but not on plasma cells (4). Benz et al. (5) were the first to report the use of RTX in NS patients. Subsequently, additional case reports provided additional observational evidence of the positive effect of RTX on NS (6). Large series on the use of RTX reported that other immunosuppressive drugs could be withdrawn. However, the relapse rate is high when the CD19 and CD20 counts returned to normal (7). Reports of the efficacy of RTX for treating both SSNS and SRNS have been encouraging (8, 9).

The first RTX protocols for NS were based on lymphoma, with a once-weekly dose of 375 mg/m^2 for 4 weeks. This protocol was modified in many institutions as 2 weekly doses of 750 mg/m^2 plus maintenance dosing at regular intervals (4). In addition, some RTX researchers focused on RTX's effect on height and weight in children (2). Accordingly, the present study aimed to determine the long-term effects of RTX treatment on disease progression in children with SSNS and SRNS, and to analyze its effect on height and weight.

MATERIALS AND METHODS

Patients and Definitions

Medical records of Caucasian SSNS and SRNS patients aged 1–20 years that were treated with RTX between 2009 and 2017 were retrospectively reviewed. Patients that were included in the study met the following criteria: 1. Onset of idiopathic NS at age >1 year; 2. Initiation of RTX treatment at age <19 years. Standard definitions were used for NS, remission, relapse, and steroid resistance (10, 11). Frequently relapsing NS was defined as ≥2 relapses within 6 months of achieving initial remission or ≥4 relapses in any 12-month period. SDNS was defined as 2 consecutive relapses while tapering the prednisolone dose or within 15 d of prednisolone discontinuation. SRNS was defined as lack of remission despite prednisolone 2 mg/kg/d (maximum: 60 mg/d) for 4 weeks, followed by 3 methylprednisolone pulses (12). Complete remission was defined as a urinary protein/creatinine ratio (Up/Uc) <0.2 mg/mg and partial remission was defined as a Up/Uc of 0.2–2 mg/mg and serum albumin >2.5 g/dL. All SRNS patients were screened for genetic mutations that cause steroid resistance.

The study protocol was approved by the Hacettepe University Ethics Committee (GO18/1136) and written informed consent was provided by the patients' parents and patients who were >10 years of age. The study was conducted in accordance with the World Medical Association Declaration of Helsinki.

Immunosuppressive Therapies

Steroid therapy was initiated as 2 mg/kg/d (maximum: 60 mg/d) and continued for 4 weeks, followed by tapering as 1.5 mg/kg/d (maximum: 60 mg/d) q.o.d. for 4 weeks, 1 mg/kg/d q.o.d. for 4 weeks, 0.5 mg/kg/d q.o.d. for 4 weeks, and then 0.25 mg/kg/d on q.o.d. for 6–12 months (13). Relapse was treated with prednisolone 2 mg/kg/d (maximum: 60 mg/d) until remission (zero or trace proteinuria for 3 consecutive d), followed by prednisolone 2 mg/kg/d q.o.d. for 2 weeks, and then tapering (1 mg/kg/d q.o.d. for 2 weeks, 0.5 mg/kg/d q.o.d. for 2 weeks, and 0.25 mg/kg/d q.o.d. for 2 weeks and continued for 12 months). Cyclosporine 1–4 mg/kg/d b.i.d. was administered to maintain a through level of 50–100 ng/mL. Cumulative steroid and CNI dosages before and after RTX were also calculated. After obtaining parental/patient consent, patients were administered 2–4 weekly doses of RTX (375 mg/m^2/dose).

Each patient's attending physician decided on the number of RTX doses for induction and maintenance. Premedication consisted of diphenhydramine, acetaminophen, and methylprednisolone (1 mg/kg, max. 60 mg) 30 min before RTX infusion. The standard methylprednisolone dose was also included in the calculation of cumulative steroid doses. Maintenance RTX treatment was given to some patients every 6–12 months, according to the clinical assessment made by their physician. Following RTX treatment, prophylactic trimethoprim-sulfamethoxazole t.i.w. was routinely administered. In order to prevent relapse, maintenance immunosuppressive therapy was continued, which could be stopped in some of the patients. CD19 and CD20 counts were not performed in all the patients on a regular basis.

Growth Parameters

Height, weight, and BMI z-scores were assessed at the time RTX was initiated and at the last follow-up visit. Standard height, weight, and BMI scores were based on Turkish children growth curves. BMI was calculated as kg/height (m)^2.

Statistical Analysis

Descriptive statistical analysis was used to evaluate demographic and clinical data. Mean ± SD, median, and IQR (interquartile range) were calculated for numeric variables. Frequency tables were used to describe categorical data. The Mann-Whitney U-test or the independent t-test was used to compare 2 independent samples. Survival analysis was performed using Kaplan-Meier

analysis with overall log-rank testing. All data were analyzed using IBM SPSS Statistics for Windows v.21 (IBM Corp., Armonk, NY).

RESULTS

Among the 41 pediatric NS patients included in the study, 21 (51.2%) had SSNS (20 SDNS and 1 FRNS) and 20 (48.8%) had SRNS. Mean duration of follow-up after RTX treatment was 2.3 ± 1.6 years. Additional patient clinical characteristics are given in the **Table 1**.

SSNS Patients

In all, 21 (51.2%) of the NS patients had SSNS. All of these patients used steroid and CNI before RTX. Additional clinical features are given in the **Table 1**. The mean time from diagnosis of NS to the first RTX infusion was 6.4 ± 3.8 years. As induction treatment, RTX was given as 2, 3, and 4 doses in 4, 10, and 7 patients, respectively. Regular maintenance RTX treatment (every 6–12 months) was given to 10 patients (47.6%). Median number of cumulative RTX infusions was 4 (IQR; 3–6). Following RTX treatment, as maintenance treatment cyclosporine A was given to 10 of the patients and mycophenolate mofetil (MMF) was given to 2.

The median number of relapses during the first 2 years after RTX was 0 (IQR; 0–2), as compared to 4 (IQR; 2.5–6) during the 2 years before RTX ($P = 0.001$). In total, 8 (38%) of the SSNS patients relapsed after RTX. Among the relapsed patients, the mean time from RTX discontinuation to first relapse was 14.6 ± 11.7 months. In addition, among the relapsed patients RTX induction treatment was administered as 2 doses in 2 patients, 3 doses in 2 patients, and 4 doses in 4 patients. Kaplan-Meier analysis showed that age at diagnosis of NS, time from diagnosis of NS to RTX, age at initiation of RTX, renal histological diagnosis before RTX, and the number of RTX doses for both induction and maintenance were not associated with the first relapse after RTX treatment. The post-RTX relapse-free period did not differ significantly between the patients that received RTX induction treatment as 2, 3, and 4 doses ($p = 0.48$).

At the last follow-up visit 2 (9.5%) of the SSNS patients were in relapse, 3 (14.3%) in partial remission, and 16 (76.2%) in complete remission. At the last follow-up visit 6 of the patients were steroid free, 11 were still receiving steroid treatment of ≤0.5 mg/kg/d, and 4 were receiving steroid treatment at 0.5–1.1 mg/kg/d. At last follow-up visit, 11 of the SSNS patients were CNI free, 10 were still receiving CNI 2.4 ± 1.0 mg/kg/d, and 4 were both CNI free and steroid free.

Median cumulative steroid dose was 117.8 mg/kg (IQR; 75.6–152 mg/kg) for 0–12 months before RTX and 69.6 mg/kg (IQR; 51.4–133 mg/kg) for 12–24 months before RTX. At 0–12 months post RTX treatment the median cumulative steroid dose decreased to 34 mg/kg (IQR; 20–48 mg/kg; $P = 0.001$) and 24.5 mg/kg (7.5–74.8 mg/kg) 12–24 months post RTX ($P = 0.013$). The median cumulative cyclosporine dose was 811.5 mg/kg (IQR; 594.8–937.5 mg/kg) 0–12 months before RTX and 782.5 mg/kg (IQR; 519–1057.3 mg/kg) 12–24 months before RTX. At 0–12 months post RTX the median cumulative cyclosporine dose decreased to 583.5 mg/kg (IQR; 305.8–903 mg/kg) mg/kg ($P = 0.015$) and 725 mg/kg (IQR; 81.5–798 mg/kg) 12–24 months post RTX ($P = 0.028$; **Figures 1A,B**). Mean GFR at initiation of RTX and at the last follow-up visit was 202.1 ± 55.8 mL/min/1.73 m^2 and 176.9 ± 50.1 mL/min/1.73 m^2, respectively ($P = 0.14$).

At the initiation of RTX 18 (85.7%) of the SSNS patients had a height z-score <0 and their mean time from NS diagnosis to initiation of RTX was 6.6 ± 4.1 years. Among the SSNS patients, 3 (14.3%) had a height z-score >0 at initiation of RTX and their median time from NS diagnosis to initiation of RTX was 5.5 ± 1.4 years ($P = 0.60$). Steroid treatment duration of the patients with a height z-score <0 (6.8 ± 4.0 years) and >0 (4.0 ± 1.4 years) at the time of RTX was not statistically different ($P = 0.35$). Similarly, mean duration of cyclosporine treatment did not differ significantly according to height z-score (z-score <0: 3.7 ± 2.8 years; z-score >0: 2.0 ± 2.0 years; $P = 0.51$). In total, the height z-score in 11 (52.3%) SSNS patients improved after RTX treatment; the median height z-score at initiation of RTX was −1.2 [IQR; (−2.5)–(−0.3)], vs. −0.6 [IQR; (−1.9)–(−0.1)] at the last follow-up visit ($P = 0.044$). At the last follow-up visit the BMI z-score in 15 (71.4%) SSNS patients was lower than at initiation of RTX; the median BMI z-score decreased from 1.6 (IQR; 0.9–3.0) at the time of initiation of RTX treatment to 1.1 (IQR; [(−0.7)−2.5] at the last follow-up visit ($P = 0.007$).

SRNS Patients

In total, 20 of the NS patients (48.8%) had SRNS, all of which were also resistant to CNIs. Mean duration of follow-up after RTX was 2.0 ± 1.4 years. Additional clinical features are given in the **Table 1**. RTX induction treatment was administered as 2 doses in 2 patients, 3 doses in 4 patients, and 4 doses in 14 patients. Among the SRNS patients, 14 (70%) received maintenance RTX every 6–12 months. Median number of cumulative RTX infusions was 6 (IQR; 4–7). Rituximab treatment was discontinued in 5 SRNS patients that progressed to chronic renal failure (<90 ml/min/1.73 m^2).

At the last follow-up visit 4 (20%) of the SRNS patients had complete remission and 4 (20%) had partial remission, whereas 6 patients still had active disease with normal GFR (≥90 ml/min/1.73 m^2) and 6 others had a GFR <90 mL/min/1.73 m^2. Steroids were discontinued in 2 SRNS patients with complete remission and partial remission, and cyclosporine was withdrawn in 4 patients with partial remission. The 1-year cumulative steroid dose decreased significantly after RTX, as compared to before RTX (0–12 months). In contrast, the 1-year cumulative cyclosporine dose before and after RTX did not differ significantly (**Figures 1C,D**).

The median cumulative steroid dose 0–12 months before RTX was 77 mg/kg (IQR; 46.7–240.5 mg/kg), and 166 mg/kg (IQR: 56.3–572.3 mg/kg) for 12–24 months before RTX. The median cumulative steroid dose 0–12 months post RTX decreased to 56.2 mg/kg (IQR; 21.8–95.5 mg/kg; $P = 0.01$) and 56.5 mg/kg (IQR: 9.4–83.5 mg/kg) 12–24 months post RTX ($P = 0.063$). The median cumulative cyclosporine dose was 927 mg/kg (IQR; 496–1187.5 mg/kg) 0–12 months before

TABLE 1 | Demographic and clinical characteristics of the SSNS and SRNS patients.

Parameters	Total	SSNS	SRNS	P**
Boys (*n*) (%)/Girls (*n*) (%)	22(53.7)/19 (46.3)	10 (47.6)/11 (52.4)	8 (40)/12 (60)	0.43
Age of diagnosis of NS* (mean ± SD) (years)	5.8 ± 4.7	4.4 ± 3.6	7.3 ± 5.2	0.17
Age of first RTX* treatment (mean ± SD) (years)	10.8 ± 5.1	10.8 ± 4.3	10.9 ± 5.6	0.62
Duration of follow-up after RTX* treatment (mean ± SD) (years)	2.3 ± 1.6	2.5 ± 1.8	2.0 ± 1.4	0.44
Age at last visit (mean ± SD) (years)	13.0 ± 5.5	13.1 ± 5.6	12.9 ± 5.5	0.95
Number of the patients with renal biopsy before RTX* (FSGS/MCD/MGN/C1qN*) ($n = 36$)	19/14/2/1	5/10/1/0	14/4/1/1	0.64
Duration of steroid treatment before RTX* treatment (for SSNS; mean ± SD, for total and SRNS; median, IQR) (years)	4.3 (2.0–8.4)	6.4 ± 3.8	2.2 (1–5.8)	**0.028**
Duration of cyclosporine treatment before RTX* treatment (for SSNS; mean ± SD, for total and SRNS; median, IQR) (years)	1.5 (0.8–6)	3.5 ± 2.7	0.8 (0.5–2.8)	**0.018**
Serum albumin level at the time of RTX* (mean ± SD) (g/dl)	3.1 ± 0.9	3.7 ± 0.7	2.5 ± 0.8	**<0.001**
Urinary protein/creatinine ratio at the time of RTX* (median) (IQR) (mg/mg)	5.6 (0.14–7.9)	0.14 (0.1–1.3)	6.8 (1.4–15.4)	**<0.001**
Steroid dosage before RTX* (0–12 months) (median) (IQR) (mg/kg)	112.5 (57.6–176)	117.8 (75.6–152)	77 (46.7–240.5)	0.98
Steroid dosage after RTX* (0–12 months) (median) (IQR) (mg/kg)	36.3 (21–62)	34 (20–48)	56.2 (21.8–95.5)	0.15
Steroid dosage before RTX* (12–24 months) (median) (IQR) (mg/kg)	87.5 (53.5–220.5)	69.6 (51.4–133)	166 (56.3–572.3)	0.19
Steroid dosage after RTX* (12–24 months) (median) (IQR) (mg/kg)	28.7 (7.5–74.8)	24.5 (7.5–74.8)	56.5 (9.4–83.5)	0.68
Cyclosporine dosage before RTX* (0–12 months) (median) (IQR) (mg/kg)	831 (596.5–1057)	811.5 (594.8–937.5)	927 (496–1187.5)	0.34
Cyclosporine dosage after RTX* (0–12 months) (median) (IQR) (mg/kg)	743 (316.8–909)	583.5 (305.8–903)	770.5 (336.8–925.4)	0.63
Cyclosporine dosage before RTX* (12–24 months) (median) (IQR) (mg/kg)	750 (446–934.5)	782.5 (519–1057.3)	630 (397–929.5)	0.49
Cyclosporine dosage after RTX* (12–24 months) (median) (IQR) (mg/kg)	436 (0–735)	725 (81.5–798)	405.5 (0–555.5)	0.32

FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IQR, inter-quartile range; MCD, minimal change disease; MGN, membranous glomerulonephritis; NS, nephrotic syndrome; RTX, rituximab; SD, standard deviation. P is for comparison of related values of SSNS and SRNS.*
Bold values indicate the statistically significant (<0.05) parameters.

RTX, and 630 mg/kg (IQR; 397–929.5 mg/kg) 12–24 months before RTX. The median cumulative cyclosporine dose 0–12 months post RTX decreased to 770.5 mg/kg (IQR; 336.8–925.4 mg/kg; $P = 0.51$), as compared to 405.5 mg/kg (IQR; 0–555.5 mg/kg) 12–24 months post RTX ($P = 0.80$; **Table 1**). Mean GFR at initiation of RTX and at the last follow-up visit was 214.6±181.6 mL/min/1.73 m^2 and 128.5±63.2 mL/min/1.73 m^2, respectively ($P = 0.28$).

At the initiation of RTX 15 of the SRNS patients had a height *z*-score <0 and their median time from NS diagnosis to RTX initiation was 3.9 ± 3.9 years. Five patients had a height *z*-score >0 and their median time from NS diagnosis to RTX initiation was 2.8 ± 2.5 years ($P = 0.80$). The duration of steroid treatment did not differ significantly between the SRNS patients with a height *z*-score <0 (7.2 ± 10.4 years) and >0 zero (2.0 ± 1.5 years) at RTX initiation ($P = 0.23$). Similarly, the duration of cyclosporine treatment in the patients with a height *z*-score <0 (2.4 ± 3.7 years) and >0 (1.7 ± 1.3 years) at initiation of RTX did not differ significantly ($P = 0.74$). The height *z*-score improved in 8 of the SRNS patients after RTX treatment; the median height *z*-score at initiation of RTX was −0.8 [IQR; (−2.2)–(0.03)], vs. −0.7 [IQR; (−2.1)−0.4] at the last follow-up visit ($p = 0.81$). At the last follow-up visit the BMI *z*-score in 13 (65%) SRNS patients was lower than at initiation of RTX; the median BMI *z*-score decreased from 0.3 [IQR; (−0.8)−1.0] at initiation of RTX to −0.1 [IQR;(−1.0)−0.8] at the last follow-up visit ($p = 0.11$).

Adverse Effects

Only 1 SSNS patient and 2 SRNS patients had mild RTX infusion-related adverse reactions (tingling of the throat). In all patients, the rate of RTX infusion was decreased and these adverse effects did not persist. Additionally, they did not re-occur in following RTX doses. All patients continued RTX doses. None of the patients had serious infections, such as peritonitis, cellulitis, and thrombosis, or other adverse events.

DISCUSSION

The present study aimed to determine the effects of RTX on disease outcome and growth parameters in pediatric SSNS and SRNS patients. Fornoni et al. (14) observed that RTX can bind directly to the sphingomyelin phosphodiesterase

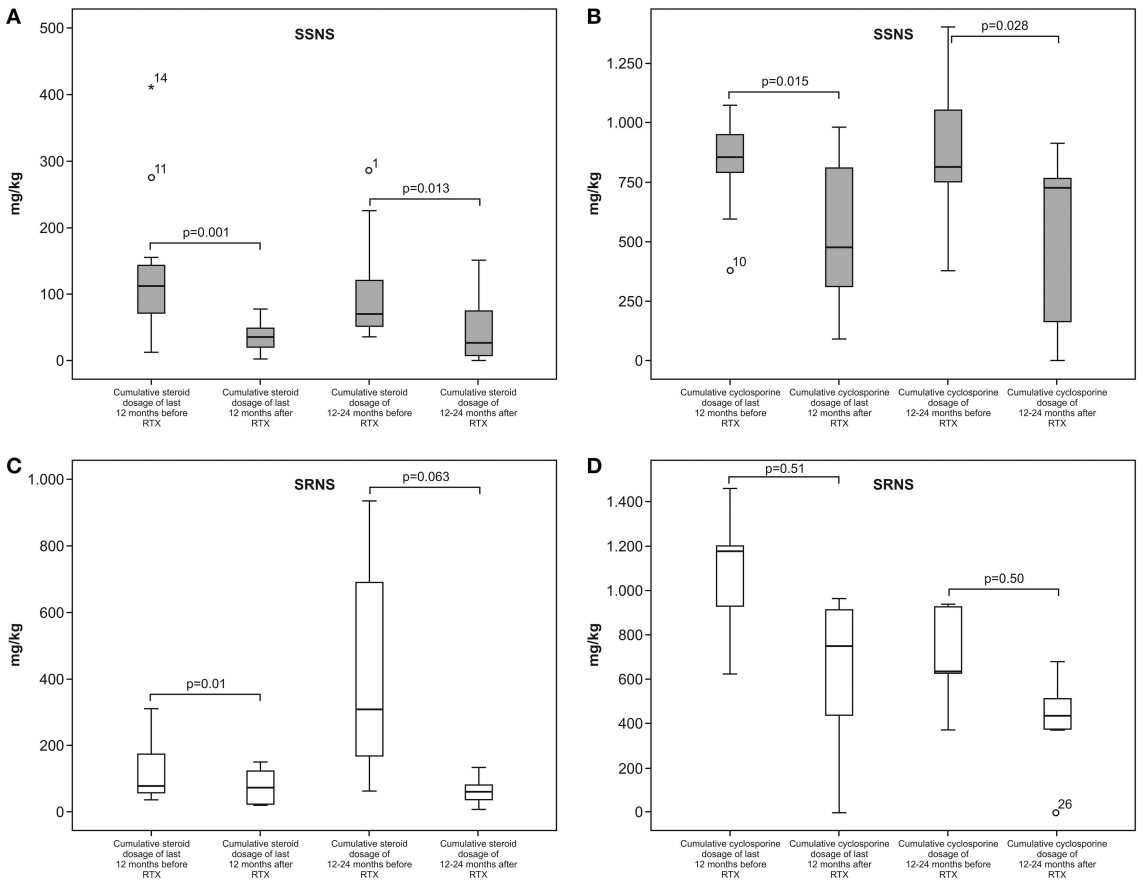

FIGURE 1 | (A,B) cumulative steroid and cyclosporine doses in the SSNS patients 12 months before and 12 months after RTX treatment. **(C,D)** cumulative steroid and cyclosporine doses in the SRNS patients 12 months before and 12 months after RTX treatment.

acid-like 3b protein (SMPDL3b), which is a component of phospholipid membranes that prevents SMPDL3b down-regulation in podocytes. RTX's relevance to NS was first reported in 2004 (5); subsequently, numerous studies on RTX and NS were performed: however, there remains a lack of consensus regarding the use of RTX for treating NS, including the optimal regimen for initial treatment (dose and number of doses). Kamei et al. (15) reported that Japanese patients with a single dose of RTX had a higher rate (75%) of relapse than those given multiple doses. The researchers concluded that the ability of a single RTX dose to prevent relapses was transient and that most relapses occur in association with recovery of B-cells. Kemper et al. (7) studied 37 SDNS patients and reported that among the 21 that received 1 dose of RTX, 64.8% relapsed. The researchers also noted that the time to first relapse was shorter in the patients that received 1 or 2 doses of RTX, as compared to those that received 3 or 4 doses. Lastly, they reported that the number of RTX induction doses did not affect long-term prognosis.

Fujinaga et al. (16) studied the predictors of relapse and long-term outcome in 43 SDNS patients given a single dose of RTX as induction treatment. In all, 91% of their patients relapsed. In addition, the relapse-free survival rate in the patients aged <12.5 years at the time of initial RTX treatment was shorter than in those aged ≥12.5 years. Furthermore, age at RTX initiation and the duration of relapse-free periods was positively correlated. The researchers also noted that the early relapse rate after RTX was significantly higher in the patients with shorter CD19 depletion. Tellier et al. (17) studied the long-term outcome in 18 SDNS patients with a median duration of follow-up of 3.2 years. They observed a relapse rate of 56% during a 2-year period following RTX treatment. In addition, 44.5% of the patients were free of therapy at the last follow-up visit. In the present study 38% of the SSNS patients relapsed following RTX treatment, but age and the number of RTX doses administered for both induction and maintenance were not associated with the first post-RTX relapse. Furthermore, in the present study 20% of SSNS patients were free of oral drug therapy.

Findings regarding the efficacy of maintenance immunosuppressive drug treatment following RTX are inconsistent. Ito et al. (18) reported that maintenance therapy with MMF is effective in SDNS patients, whereas Fujinaga et al. (19) compared MMF and cyclosporine after 1 dose of RTX in SDNS patients, reporting that treatment failure occurred more frequently in those given MMF and that the sustained remission

rate was higher in the cyclosporine group. In the present study most of the NS patients received cyclosporine as maintenance therapy and the type of maintenance therapy did not affect the time to first relapse after RTX treatment.

Sinha et al. (8) published one of the largest studies on RTX in NS (SSNS and SRNS) patients. They included 101 SDNS patients, of which 82 received 2 doses of RTX initially. The authors found that both cumulative steroid dosage and number of relapses were lower at in time periods 6 and 12 months after RTX compared to 6 and 12 months prior RTX. Similarly, in the present study the cumulative steroid dose 12 months after RTX was lower than 12 months before RTX. Moreover, the present study also calculated the cyclosporine dose before and after RTX, observing that the cumulative cyclosporine dose was lower after RTX than before RTX. More recently, Ravani et al. (20) performed a multicenter, open label, non-inferiority trial with pediatric SDNS patients that had developed SDNS during the previous the 6–12 months and were maintained in remission with high-dose prednisone. Their patients were divided into 2 groups: prednisone (control) and single-dose RTX (intervention). They concluded that RTX was not inferior to steroids for the treatment of SDNS.

The rate of response to RTX in SRNS patients is lower than in SSNS patients, even when SSNS patients receive RTX during periods of relapse. The first report of RTX treatment in SRNS patients included 5 Indian children treated with RTX, of which 3 achieved complete remission and 2 achieved partial remission (21). Prytula et al. (22) studied 27 SRNS patients with different RTX regimes. In this cohort, 17 patients received two doses of 750 mg/m^2 every 14 days and 6 patients received single dose of RTX (375 mg/m^2). The rest of the patients received other treatment regimes of RTX. After these RTX doses, 6 patients (22%) achieved full remission and 6 patients (22%) had proteinuria with serum albumin >30 g/L. Sinha et al. (8) reported a series of 58 NS patients that were steroid and calcineurin resistant. Most of the patients ($n = 39$) received 4 doses of RTX, and complete remission and partial remission were observed in 12.1 and 17.2% of the patients, respectively. Fujinaga et al. (23) studied the long-term outcome of early RTX treatment in 6 NS patients that were both cyclosporine and steroid resistant. Following RTX treatment, and then intravenous pulse methylprednisolone and/or high-dose prednisolone, all patients achieved complete remission.

The findings above, in whole, show that the response to RTX varies geographically. The present study's findings fall between those of the 2 geographic regions described above. Complete or partial remission was observed in 40% of the present study's patients, respectively. Some of the SSNS and SRNS patients in the present study received periodic RTX dose, as reported earlier (24). Based on the present findings, we think that such dosing regimens might have a positive effect on remission.

The effect of RTX on growth in children is another contentious issue. Kamei et al. (25) measured the height of 21 SSNS patients aged ≤10 years at initiation of RTX and at the last follow-up visit, reporting that the height *z*-score did not differ significantly between the 2 time points. Sato et al. (2) investigated the effect of RTX on height and weight in 13 SDNS patients, and observed that the height *z*-score for all 13 patients was higher at the last follow-up visit and the height *z*-score of 10 of the 13 patients increased significantly following RTX treatment. Lastly, they noted that the obesity index in 12 of the 13 patients improved significantly following RTX treatment. More recently, Basu et al. (26) compared the efficacy of RTX and tacrolimus in SDNS patients in a randomized clinical trial. They reported a higher 12-month absolute change in the height *z*-score in the RTX arm, as compared to the tacrolimus arm. In the present study the height *z*-score in 50% of the patients improved following RTX treatment and the median height *z*-score at the last follow-up visit was higher than at the time of initiation of RTX treatment. RTX had a similarly positive effect on BMI in the present study's SSNS and SRNS patients. These positive effects of RTX on growth were less prominent in SRNS patients than in the SSNS patients; only 40% of the SRNS had an improved height *z*-score after RTX treatment, and the median height *z*-score among the SRNS patients at the last follow-up visit and at the time of initiation of RTX did not differ significantly. These findings might have been due to the shorter duration of steroid treatment before RTX in SRNS patients than in the SSNS patients.

In conclusion, the positive effects of RTX on disease progression and growth (height and BMI) are more prominent in SSNS patients than in SRNS patients. RTX causes decreased number of relapses, cumulative steroid, and calcineurin dosages.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RT, BG, FO, and AD: research formulation and study design. KÇ, Mİ, and BG: data acquisition. RT, BG, and MH: data analysis/interpretation. MH and BG: statistical analysis. RT: supervision/mentorship. Each of the authors contributed important intellectual content during manuscript drafting and/or revision, and approved the final version. Furthermore, they all accept responsibility for the overall work, including the accuracy and integrity of all portions of the work.

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Altered B-Lymphocyte Homeostasis in Idiopathic Nephrotic Syndrome

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Background: B-cell-deleted therapy has been successfully used for children with idiopathic nephrotic syndrome (INS), suggesting that B cells may be involved in the pathogenesis of INS. B cells are a heterogenous population comprised of subpopulations distinguished by their phenotypes. However, few studies have focused on the alteration of B-cell homeostasis in INS.

Methods: We measured the levels of B-cell subsets in the blood of 87 INS children via flow cytometry, prior to treatment with steroids. INS patients were divided into steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS) groups based on their sensitivities to steroids after a one-month follow-up. Subsequently, we compared these INS patients with age- and sex-matched patients with relapse ($n = 35$) and remissions ($n = 32$), as well as healthy controls ($n = 75$).

Results: We found that 65 SSNS patients exhibited an altered peripheral-blood B-cell-subset distribution, with increased levels of total, transitional, memory, IgM (immunoglobulin M) memory and switched-memory B cells compared to 22 SRNS patients. The proportion of total B cells was significantly higher in the SSNS group (22.1 ± 6.7% L, $p < 0.001$) than in the SRNS, remission, and control groups. In contrast, the levels of B-cell subsets in SRNS patients were generally the same as those in remission patients and healthy controls. Patients in relapse presented elevated memory B cells compared to those in other groups. The area under the ROC (receiver operating characteristic) curve of transition B cells at initial onset for the prediction of SSNS was 0.907 (95% confidence interval, 0.835–0.979). The analysis rendered an optimal cut-off value of 2.05 (% Lymphocyte) corresponding to 79.1% sensitivity and 90.9% specificity.

Conclusions: We observed and verified that B-cell subsets are significantly altered in children with SSNS. We propose that elevated transitional B cells may be a promising marker for predicting successful immunosuppressive therapy during the initial onset of INS. Further research is needed to determine the function of memory B cells in INS.

Keywords: idiopathic nephrotic syndrome, B cells, children, transitional B cell, flow cytometry

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most frequent glomerular disease during childhood. It is a clinical syndrome characterized by massive proteinuria, hypoalbuminemia, hyperlipidemia, and edema due to increased glomerular permeability. Although the role of B cells in several renal diseases is well-established (1), their contribution to the pathogenesis of INS is still being debated (2). The best supporting evidence for a role of B cells in INS comes from rituximab, a B-cell-specific antibody, which has been successfully used to treat patients with steroid-dependent or frequent relapse, and most of these patients have maintained remission in the absence of B cells (3–5). These findings imply that B cells are involved in the pathogenesis of INS.

B cells are a heterogeneous population that include different subsets characterized by their membrane phenotypes and/or cytokine-production profiles (6). B cells are capable of antibody production, antigen presentation, and immunomodulation. It has previously been reported—albeit debated—that CD19+ B cells increase during the acute phase of INS, but decrease to normal levels in the remission phase (7). It has also been reported that after treatment with rituximab, switched-memory B-cell recovery is associated with INS recurrence (8). In a recent study, elevated levels of memory B-cell subsets were considered to be closely related to the onset of INS (9). These studies suggest that different subpopulations of B cells play different roles in INS. However, few studies have focused on the alteration of B-cell homeostasis in children with INS.

Most INS is sensitive to prednisone treatment, and only a small number of patients exhibit steroid resistance (10). Steroid-resistant nephrotic syndrome (SRNS) is different from steroid-sensitive nephrotic syndrome (SSNS), and SRNS is currently recognized as a genetic disease or a disease due to other pathogenic factors. Therefore, we believe that SRNS and SSNS are also significantly different in terms of their corresponding subpopulations of B cells, and some B-cell subsets may serve as biomarkers for predicting one's sensitivity to steroid-therapy at the initial onset.

In the present study, we compared the distribution of B-cell subsets in the peripheral blood of SSNS and SRNS patients, as well as in healthy controls and in INS patients in relapse and remission. We hope that this investigation of altered B-cell subsets in INS may lead to the discovery of novel biomarkers for predicting INS-treatment responses.

MATERIALS AND METHODS

Patients

This study was conducted between March 2018 and March 2019 at the National Center for Children's Health in China. Children with active nephrotic syndrome during the initial onset were recruited for the collection of blood samples before the treatment with prednisone. These children were followed up for at least 4 weeks. Patients who achieved remission with prednisone (60 mg/m^2/day) within 4 weeks of treatment were classified as having SSNS, whereas those who did not respond were classified as having SRNS. Additionally, we included children with INS relapse and children with INS remission. Finally, 75 age- and sex-matched healthy children receiving routine medical examinations were included as healthy controls.

The diagnosis of INS was based on the presence of generalized edema, proteinuria (urine protein + + + or more by the heat-precipitation method and/or spot-urine protein/creatinine > 2 mg/mg), hypoalbuminemia (serum albumin < 2.5 g/dl) and hypercholesterolemia (serum cholesterol > 5.72 mmol/L). Patients were considered in relapse if they had edema, a serum albumin level of < 2.5 g/dl, and a urinary protein/creatinine (mg/mg) ratio of > 2.0, and they were considered to be in remission if they had no edema and their urinary protein/creatinine ratio was < 0.40 in a random urine sample.

Exclusion criteria were as follows: (1) age < 1 year old or > 10 years old; (2) positive family history of kidney disease; (3) systemic diseases, such as lupus and purpura; or (4) active infections, such as Epstein-Barr virus and cytomegalovirus.

Our treatment criterion followed the Improving Global Outcomes (KDIGO) guidelines of 2012 for kidney disease (11), which recommends the induction treatment of the initial episode of nephrotic syndrome in children with daily oral prednisone at 2 mg/kg/day (maximum 60 mg/day) for 4 weeks. No patients received or were ready to receive other forms of immunosuppression.

The study protocol was approved by the Capital Medical University Institutional Review Board, and informed consent was obtained from the parents or the authorized representative of each child.

Cell Isolation

The blood of each child was collected in the morning after 1–2 days of hospitalization, and 3 mL of blood was collected in a heparin-anticoagulation tube. Blood samples were placed in a 4–6°C refrigerator and were analyzed within 6 h. Peripheral-blood mononuclear cells (PBMCs) were isolated from heparinized blood using the Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density-gradient centrifugation method. Freshly isolated heparin-anticoagulated blood diluted in phosphate-buffered saline (PBS, 1:1) was layered on the surface of Ficoll at a ratio of 2:1 and was centrifuged at 1,600 g for 20 min at 20°C, with no breaks. The cells were harvested from the Ficoll interface.

Flow Cytometry

To identify different B-cell subpopulations, PBMCs were stained with the following fluorochrome-conjugated antibodies: CD19-FITC (fluorescein isothiocyanate), CD24-PE (phycoerythrin), CD27-PE (phycoerythrin), CD38-PerCP (peridinin chlorophyll protein), IgM-APC (allophycocyanin), and IgD-PerCP (peridinin chlorophyll protein) (BD Biosciences, San Jose, CA, USA). The antibodies were then analyzed by a multicolor flow cytometer (FACSCalibur; BD

Biosciences). Gated events (30,000) on living lymphocytes were analyzed for each sample. Four-color data acquisition was performed by FACS Calibur, and data were analyzed with CellQuest analysis software (BD Biosciences, San Diego, CA, USA).

Subsets of gated $CD19^+$ (total) B cells were identified on the basis of the expression of surface markers as follows (**Figure 1**): transitional ($CD24^{high}CD38^{high}$), mature/naïve ($CD24^{low}CD38^{intermediate}$), and memory ($CD24^{high}$ $CD38^-$) or ($CD27^+$) B cells. Memory B-cell subclasses ($CD19^+CD27^+$) were also defined as IgM memory ($IgM^+IgD^{intermediate}$) or switched memory (IgM^-IgD^-) and they were expressed as the percentage of total circulating lymphocytes (8).

Statistical Analyses

Data were analyzed using SPSS v.19 (SPSS Inc., Chicago, IL, USA). Data are summarized as the mean ± SEM and median (interquartile range) for continuous variables, as well as the percentage and frequency for categorical variables. Statistically significant differences were analyzed using independent *t*-tests or Mann-Whitney *U* tests for continuous variables and chi-squared tests for categorical variables. A receiver operating characteristic (ROC) curve was constructed and the area under the ROC curve (AUC) was calculated to assess the predictive strength. Optimal cutoff points to maximize both sensitivity and specificity were also determined. Statistical tests were two-tailed, and a *P*-value of < 0.05 was considered statistically significant. Additionally, a *p*-value < 0.005 (i.e., 0.05/10) was used to indicate statistical significance after Bonferroni correction.

RESULTS

Baseline Characteristics of the Study Population

The demographic characteristics of the subjects enrolled are presented in **Table 1**. A total of 156 patients with nephrotic syndrome were included in the study and were divided according to the stages of their diseases. Eighty-seven patients at disease onset (before any immunosuppressive treatment) were further divided according to their responses to prednisone (SSNS = 65, SRNS = 22). Additionally, there were 35 patients in relapse and 32 patients in stable (at least 1 month) remission. There was no difference in age or gender among the groups. Compared with that of the remission group and the control group, the albumin level in the disease-onset group and the relapse group was lower and the 24-h urine-protein level was higher. Serum-creatinine levels were normal for all patients and healthy controls.

In the relapse group and the remission group, all patients were treated with PDN. No children were treated with other immunosuppressants at the time of enrollment. For the IgA and IgM levels, there were no statistically significant differences among the groups. Additionally, the IgG level at the remission period was significantly lower than that during the active and relapse periods. The IgE level was significantly higher in SSNS patients than in other groups. The total T-cell ratio did not differ significantly among groups, and the CD4/CD8 ratio was slightly higher in the SSNS group than in the other populations. The proportion of NK cells was significantly lower in SSNS and relapsed groups than in other groups, and children in the SSNS group had a lower proportion of NK cells than those in the relapsed group.

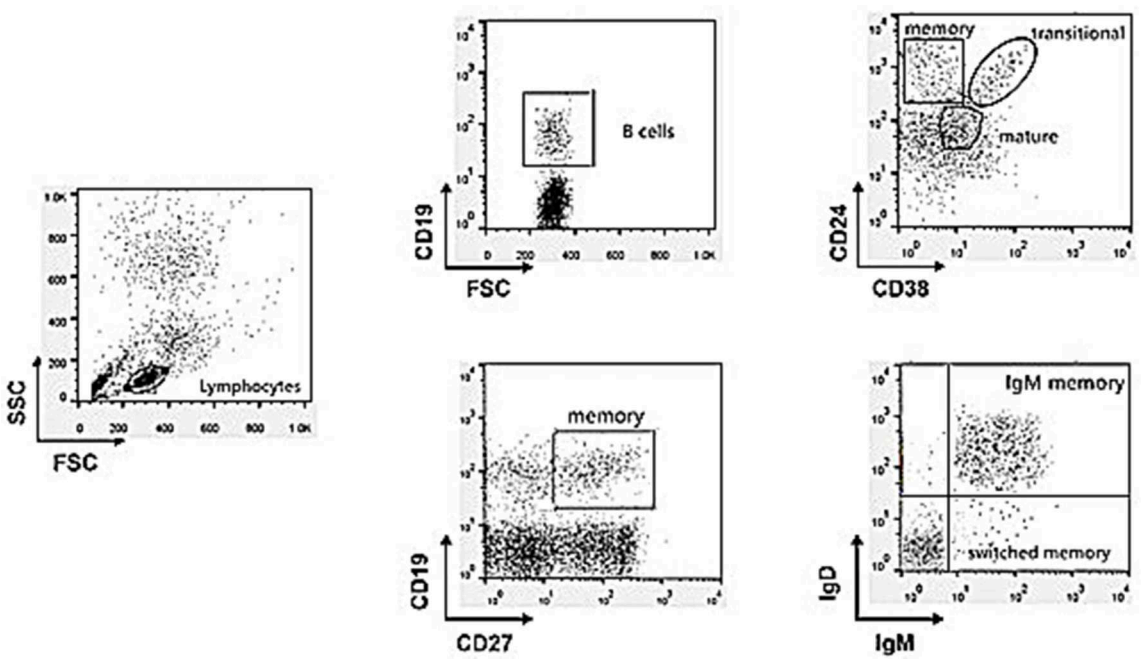

FIGURE 1 | Gating strategy to discriminate the different B cell subpopulations by multicolor flow cytometry analysis.

TABLE 1 | Characteristics of patients with nephrotic syndrome and controls.

Parameter	Onset		Relapse ($n = 35$)	Remission ($n = 3$ 2)	Health controls ($n = 75$)
	SS ($n = 65$)	SR ($n = 22$)			
Age, years	5.2 ± 2.9	5.5 ± 4.3	5.6 ± 4.2	5.5 ± 2.8	5.2 ± 2.7
Sex, male	44 (67.7%)	16 (72.7%)	23 (65.7%)	22 (68.7%)	48 (64.0)%
Serum albumin, g/L	18.1 ± 5.8‡‡	20.9 ± 6.0‡‡	23.2 ± 8.3‡‡	35.4 ± 7.6$^{\#\#,††}$	–
Serum creatinine, g/L	32.8 ± 11.8	37.5 ± 15.7	37.2 ± 20.3	35.1 ± 7.0	–
24-h urine protein, mg/kg	132.1 ± 78.9‡‡	148.1 ± 103.5‡‡	135.3 ± 102.1‡‡	13.2 ± 9.5$^{\#\#,††}$	–
IgA, g/l	1.19 ± 0.78	1.10 ± 0.71	1.17 ± 0.62	1.31 ± 1.06	–
IgG, g/l	3.07 ± 2.9$^{\#,†,‡‡}$	3.98 ± 2.11‡‡	4.24 ± 2.41‡‡	6.98. ± 2.74$^{\#\#,††}$	–
IgM, g/l	1.57 ± 0.92	1.59 ± 0.94	1.50 ± 0.45	1.41 ± 0.57	–
IgE, g/l	216.2 (59.2, 537.8)$^{\#\#,†,‡‡}$	90.6 (42.4, 284.0)	45.0 (20.1, 346.5)	76.2 (55.7, 104.09)	–
T cell, %L	70.9 ± 8.9	71.6 ± 7.2	71.5 ± 8.2	71.4 ± 5.3	–
CD4+ T, %L	40.5 ± 8.2	36.9 ± 8.4	36.8 ± 8.2	39.1 ± 4.2	–
CD8+ T, %L	24.3 ± 6.0$^{\#,†,‡}$	29.9 ± 7.6	29.7 ± 7.0	27.9 ± 5.0	–
CD4/CD8,	1.8 ± 0.6$^{\#,†,‡}$	1.3 ± 0.5	1.2 ± 0.5	1.4 ± 0.3	–
Nature kill, %L	5.7 ± 3.0$^{\#\#,††,‡‡}$	8.0 ± 4.1‡‡,†	6.5 ± 3.8$^{‡‡,\#}$	13.5 ± 3.3	–
B cell, %L	22.1 ± 6.7$^{\#\#,†,‡‡,**}$	12.7 ± 6.1†	18.5 ± 7.4$^{\#,‡‡,*}$	13.7 ± 3.3††	14.1 ± 3.3†
Transitional B, %L	5.3 ± 3.8$^{\#\#,††,‡‡,**}$	2.0 ± 1.5‡	2.0 ± 1.8	2.5 ± 2.0$^{\#}$	2.0 ± 1.4
Mature B, %L	22.8 ± 9.6*	22.4 ± 8.9‡,*	23.7 ± 7.5‡,*	27.6 ± 8.0$^{\#,†}$	30.0 ± 11.0$^{\#,†}$
Memory B, %L	4.5 ± 2.4††,*	3.5 ± 2.0††,*	7.7 ± 5.5$^{\#\#,**,‡‡}$	4.6 ± 3.0††,*	2.8 ± 1.5$^{††,\#,‡}$
IgM memory B, %L	1.5 ± 0.8††,‡,*	1.0 ± 0.8††	1.9 ± 0.9$^{\#\#,‡‡,**}$	0.9 ± 0.5††	1.0 ± 0.5††
Switched memory B, %L	1.3 ± 0.8	1.0 ± 0.4††	1.4 ± 0.5$^{\#\#,‡‡,**}$	1.0 ± 0.3††	1.1 ± 0.4††

SS, steroid sensitive; SR, steroid resistant; Ig, Immunoglobulin; %L, Percentage of lymphocytes.
$^{\#}p < 0.005$; $^{\#\#}p < 0.001$, compared to SRNS patients at onset.
$^{†}p < 0.005$; $^{††}p < 0.001$, compared to patients in relapse.
$^{‡}p < 0.005$; $^{‡‡}p < 0.001$, compared to patients in remission.
$^{*}p < 0.005$; $^{**}p < 0.001$, compared to controls.

B-Cell Subsets Are Significantly Altered in Children With SSNS

The proportion of total B cells was significantly higher in the SSNS group (22.1 ± 6.7% L, $p < 0.001$) than in the SRNS, remission, and control groups (**Figure 2**). Although prednisone was being applied, the children in the relapse group also had an increased proportion of total B cells compared with that in the SRNS, remission, and control groups (18.3 ± 5.5% L, $p < 0.005$). There were no statistically significant differences among the SRNS, remission and control groups in terms of the proportion of total B cells ($p > 0.005$).

The proportion of mature B cells did not differ significantly in SSNS, SRNS, and relapse groups ($p > 0.005$), but was slightly higher in the remission patients and healthy controls ($p < 0.005$).

The proportion of transition B cells was significantly higher in the SSNS group (5.3 ± 3.8% L, $p < 0.001$) than in the other groups, and was significantly lower in the relapse group and the remission group after treatment with PDN. These proportions were not different between the relapse, remission, and healthy control groups. There was no significant increase in the proportion of SRNS at the time of onset ($p > 0.005$).

Memory B cells did not increase significantly at the time of initial onset, but increased significantly in the relapse phase compared with those of other groups (7.7 ± 5.5% L, $p < 0.001$). This phenomenon was due to an increase in the proportion of IgM-memory B cells (1.93 ± 0.86% L) and switched-memory B cells (1.39 ± 0.51% L).

Transition B Cells as a Biomarker for SSNS

Transition B cells differed greatly in patients with newly diagnosed SSNS and SRNS. Hence, we attempted to use transition B cells as biomarkers to predict the outcome of PDN therapy. ROC curves were used to assess the potential utility of transition B-cell detection in patients with SSNS at the time of initial onset. The area under the ROC curve of transition B cells for the prediction of SSNS was 0.907 (95% confidence interval, 0.835–0.979). The analysis rendered an optimal cutoff value of 2.05% L corresponding to a 79.1% sensitivity and 90.9% specificity (**Figure 3**).

DISCUSSION

This cohort study from China confirmed that B-cell subsets are altered in an SSNS population in children. We tested 87 newly diagnosed INS children before any immunosuppressive treatment, and all patients were followed up for 1 month.

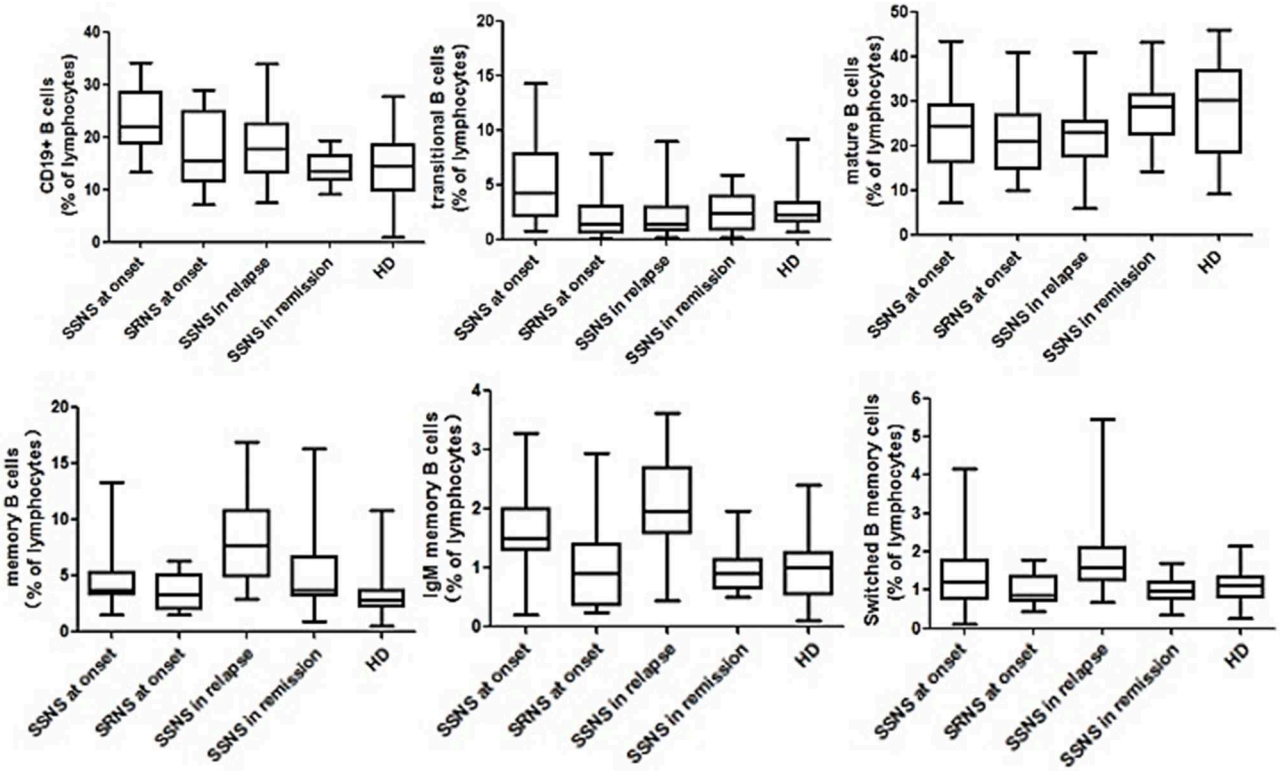

FIGURE 2 | Multicolor flow cytometry analysis is circulating B cell subsets in NS patients and controls. B cells cell subsets from patients at disease onset ($n = 87$, in which SSNS = 65, SRNS = 22), in relapse ($n = 35$), in remission ($n = 32$), and healthy controls ($n = 75$) were compared with each other. All of them were expressed as percentages of total lymphocytes. Each box plot represents the median and the 25th and 75th centiles.

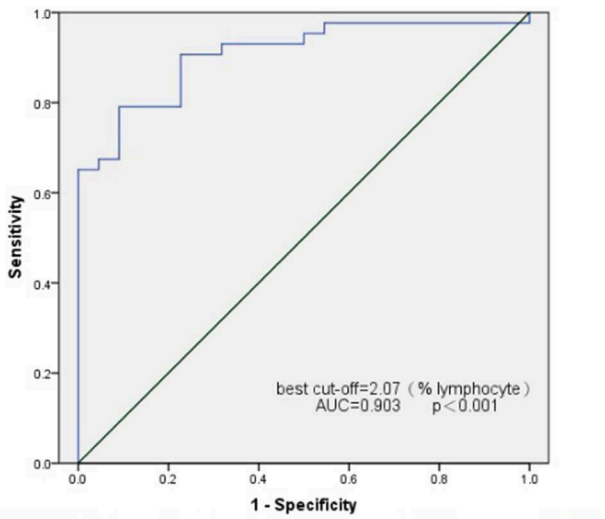

FIGURE 3 | Receiver operating characteristics (ROC) curve analysis for transitional B cell as a marker for the identification of SSNS.

Other groups were age- and sex-matched. According to the efficacy of prednisone, patients were divided into SSNS and SRNS groups.

An increased level of B cells has already been reported in SSNS, but conflicting results also exist (12). Colucci et al. suggested that prednisone treatment is the cause of these controversial conclusions (9). We confirmed the theory that $CD19^{+}$ B cells were significantly elevated in initial-onset NS children before steroid treatment, which is consistent with reports by Colucci et al. (9). We also observed an increase in B cells in the relapse group compared with that in the remission group, but this increase was not as high as that of the SSNS group during first onset, which may be related to the effects of immunosuppressive therapy.

We further compared the changes in B-cell subsets in SSNS and SRNS groups during the first onset. We found that transitional B cells ($CD19+CD24^{hi}CD38^{hi}$) were significantly elevated in the SSNS population compared to those in the SRNS population. This is consistent with the results reported in a previous study (9). When using a cut-off value of 2.05 (% of lymphocytes), transitional B cells allowed observers to distinguish SSNS from SRNS with an AUC of 0.907, an optimized sensitivity of 79.1%, and a specificity of 90.9%. This suggests that transitional B cells may be a biomarker for early screening for steroid-sensitive nephrotic syndrome.

It is worth mentioning that in recent years, some transitional B cells have been found to secrete the anti-inflammatory factors, interleukin-10 (IL-10) and transforming growth factor-β

(TGF-β), and to express inhibitory surface molecules, which are considered to have immunomodulatory effects (13). The importance of regulatory B cells has been shown in several autoimmune conditions. For example, a prolonged presence of transitional B cells has been associated with long-term non-relapsing of systemic lupus erythematosus (14). Moreover, transitional B lymphocytes are associated with protection from kidney-allograft rejection (15). The opposite result showed a positive correlation between CD38+CD19+ B cells and IL-10+CD19+ B cells and hepatitis B virus-associated membranous nephropathy (16). Our present study showed that transitional B cells exhibited a markedly abnormal increase in early disease, and that transitional B cells showed a markedly rapid and sustained decrease after steroid administration. Although our present study demonstrates that transitional B cells may be used as steroid-sensitive biomarkers in children with NS, it is necessary to further study the mechanisms of transition B-cell elevation and their role in the pathogenesis of SSNS.

Memory B cells are one of the focuses of current research on children with primary nephrotic syndrome. The first report by Colucci et al. showed that delayed reconstitution of memory B cells was protective against relapse after rituximab (8). This finding was partly confirmed by a study from India (17). However, Bhatia et al. observed that total B cells and memory B cells recovered earlier in relapsers, but Colucci et al. considered that this is related to the prolonged depletion of memory B cells rather than of total B cells. In our present study, memory B cells were significantly elevated in the relapse group and also in the SSNS group during onset. Overall, these phenomena may imply that memory B cells are involved in the pathogenesis of SSNS. One hypothesis of memory B cells in NS is that the Epstein-Barr Virus is able to establish latent benign infection in memory B cells that display phenotypes similar to those of antigen-selected memory B cells. A specific anti-EBNA1 antibody internalized in podocytes via the neonatal FC receptor might cross-react with a major podocyte protein (18). These possibilities have not been explored and should be investigated further.

The class conversion of immunoglobulins is the basis for the body to produce different classes of antibodies and serve different functions. We observed that SSNS had low IgG levels and high IgE levels. Following statistical analyses, we did not find any correlation between IgG and IgE with serum albumin and B-cell subsets. The role of antibodies in INS has been proposed by a number of clinical observations and experimental studies. Dantal et al. showed that a permeability factor inducing albuminuria may bind to an immunoglobulin and induce decreases of IgG (19). IgE is often associated with allergic diseases, and elevated IgE may be due to a response to elevated plasma IL-13 in allergic disease (20).

The current study also has limitations. First, although we showed that the percentage of transitional B cells appeared to be a marker of steroid sensitivity with reasonable sensitivity and specificity, this finding requires further verification in a separate group of recently diagnosed nephrotics. Second, although children in the relapsing and remission groups never received other immunosuppressive agents, their concurrent use of steroids may have had an effect on the levels of B cells and their subpopulations.

In conclusion, we validated that there is an alteration of B-cell subsets in children with SSNS, and that transition B cells may be a biomarker for SSNS during the initial onset before steroid treatment. Future studies should investigate the function of B-cell subsets at different periods of NS, which will help to further elucidate the pathogenesis of NS.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

The study protocol was approved by Beijing Children's Hospital at Capital Medical University. All procedures performed in our studies were in accordance with these ethical standards. All parents of the individual participants included in the study provided written informed consent.

AUTHOR CONTRIBUTIONS

CL and XL designed the research. CL wrote the paper. ZC, QS, JF, QM, and NZ were responsible for collecting clinical pathology. LH was responsible for statistical processing. XW and JG completed the laboratory work.

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Dextran-Sulfate Plasma Adsorption Lipoprotein Apheresis in Drug Resistant Primary Focal Segmental Glomerulosclerosis Patients: Results From a Prospective, Multicenter, Single-Arm Intervention Study

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Background: Focal segmental glomerulosclerosis (FSGS) causes end stage renal disease (ESRD) in significant proportion of patients worldwide. Primary FSGS carries poor prognosis and management of FSGS patients, refractory to standard treatments or resistant to steroids, remains a major challenge. Lipoprotein apheresis is a therapeutic approach for drug resistant primary FSGS and post-renal transplant primary FSGS recurrence.

Objectives: To examine the safety and probable benefit at 1, 3, 6, 12, and 24-months following completion of apheresis treatment using Liposorber® LA-15 system in patients with nephrotic syndrome (NS), due to refractory primary FSGS or primary FSGS associated NS, in post renal transplant children.

Material and Methods: Prospective, multicenter, single-arm intervention study using Liposorber® LA-15 system. Patients ≤21 years old with drug resistant or drug intolerant NS secondary to primary FSGS with glomerular filtration rate (GFR) ≥60 ml/min/1.73 m^2 or post renal transplant patients ≤21 years old with primary FSGS associated NS were included in the study. Each patient had 12 dextran-sulfate plasma adsorption lipoprotein apheresis sessions over a period of 9 weeks. All patients were followed up at 1, 3, 6, 12, and 24-months following completion of treatment.

Results: Of 17 patients enrolled, six were excluded from the outcome analysis (protocol deviations). Of the remaining 11 patients, all but one have completed apheresis

treatments. Three patients were lost to follow-up immediately after completion of apheresis and excluded from outcome analysis. At one-month follow-up, 1 of 7 patients (14.3%) attained partial remission of NS while 2 of 4 subjects (50%) and 2 of 3 subjects (66.7%) had partial/complete remission at 3- and 6-months follow-up, respectively. One of two patients followed up for 12 months had complete remission and one patient had partial remission of NS after 24 months. Improved or stable eGFR was noted in all patients over the follow-up period.

Conclusion: The results of our multicenter study showed improvement in the response rates to steroid or immunosuppressive therapy and induced complete or partial remission of proteinuria in some of the patients with drug resistant primary FSGS. The main limitation of our study is the small number of subjects and high dropout rate.

Keywords: focal segmental glomerulosclerosis, lipoprotein apheresis, nephrotic syndrome, liposorber, proteinuria

BACKGROUND

Focal segmental glomerulosclerosis (FSGS) causes end stage renal disease (ESRD) in significant proportion of patients in the United States and worldwide (1, 2). FSGS is characterized by nephrotic or subnephrotic range proteinuria due to sclerosis and scarring of the glomerulus, which often progresses to ESRD (1, 3). Primary FSGS is idiopathic and commonly occurs in children and young adults, while secondary FSGS is frequently seen in older adults due to cytomegalovirus, human immunodeficiency virus (HIV) infection, systemic lupus erythematosus (SLE), sickle cell disease, hepatitis, reflux nephropathy, illicit drug use, and certain malignancies (1, 3). Pathology underlying FSGS is podocyte injury resulting in protein leak, capillary expansion, synechiae formation, and proliferation of mesangial matrix (3, 4). Proteinuria is the main presentation in FSGS and other symptoms are secondary to urinary protein loss, which include hypoalbuminemia, edema, hypertension, and hyperlipidemia (5, 6). Primary FSGS carries a poor prognosis as spontaneous remission is rare and it tends to rapidly progress to ESRD within 2–8 years in patients with persistent nephrotic range proteinuria (2, 7, 8).

The mainstay of treatment for primary FSGS is to control while preventing and decreasing the rate of progression to ESRD. Corticosteroids are most effective and commonly used treatment to induce remission of proteinuria in FSGS patients. However, remission rate is only 20–50% with steroid therapy for a mean duration of 3.7 $\pm$ 2 months (8, 9). Most steroid resistant FSGS patients develop ESRD (8). Furthermore, successful steroid therapy may come at the cost of severe adverse effects, such as growth impairment, hypertension, and immune suppression (6, 8). Steroid resistant or steroid intolerant FSGS patients can be treated with cyclophosphamide or a calcineurin inhibitor, but the response rates are very low (<25%) with significant complications (1, 8, 10). Moreover, long-term remission is uncertain in cyclophosphamide or calcineurin inhibitor responsive patients (1, 8).

FSGS recurs in 30–40% of renal transplant patients, and causes allograft injury in 20–30%, and graft loss in 40–50% of these patients (11). Furthermore, FSGS recurrence is the leading cause of graft failure in children and has the lowest 5-year graft survival rate for living donor renal transplant recipients compared to other renal disorders (7). Despite all the treatment options, management of FSGS patients, refractory to standard treatments or resistant to steroids, remains a major challenge. Lipoprotein apheresis therapy is used for drug resistant (corticosteroids and/or calcineurin inhibitors) primary FSGS and post-renal transplant primary FSGS reoccurrence. Lipoprotein apheresis selectively removes VLDL, LDL, and triglycerides without affecting serum HDL levels (12–14). Although underlying mechanism by which lipoprotein apheresis reduces proteinuria is unknown, several studies have shown that lipoprotein apheresis induces remission of drug resistant NS in FSGS patients (12, 14–19).

OBJECTIVES

The primary objective was to examine the safety and potential benefit of lipoprotein apheresis at 1 month following a 9-week

TABLE 1 | Demographics of patients.

	n (%)
Age (years)	
6–8	5 (29.4)
9–11	4 (23.5)
12–14	6 (35.3)
15–17	0
18–20	2 (11.8)
Sex	
Male	8 (47)
Female	9 (52.9)
Race/ethnicity	
Caucasian	9 (52.9)
African American	4 (23.5)
Hispanic/Latino	2 (11.8)
Unknown	2 (11.8)

course of dextran-sulfate plasma adsorption lipoprotein apheresis for the treatment of patients with primary FSGS associated NS, who are refractory or intolerant to standard therapy, or primary FSGS associated NS, in post renal transplant children. The secondary objective was to examine the safety and potential benefit of dextran-sulfate plasma adsorption lipoprotein apheresis at 3, 6, 12, and 24-months following apheresis treatment in the same patient population.

MATERIALS AND METHODS

Definitions Used in the Study

Nephrotic syndrome is defined as having a first morning void urine protein to creatinine ratio (UPCR) of >2 (g/g).

Drug resistant NS due to FSGS is defined as failure to attain partial or complete remission of NS with corticosteroids and/or calcineurin inhibitors (standard therapy) after at least 8 weeks of treatment in FSGS patients.

Drug intolerant NS due to FSGS is defined as patient not tolerating standard therapy due to severe adverse effects (such as growth impairment, hypertension, obesity, immune suppression, diabetes mellitus, etc.) with or without adequate clinical response.

Partial remission of NS is defined as first morning void sample UPCR of 0.2-2 (g/g) or decrease in UPCR ≥50% of initial screening value.

Complete remission of NS is defined as first morning void sample UPCR <0.2 (g/g).

Study Design

Prospective, multicenter, single-arm intervention study of dextran-sulfate plasma adsorption lipoprotein apheresis using Liposorber® LA-15 system. Liposorber® LA-15 system has been

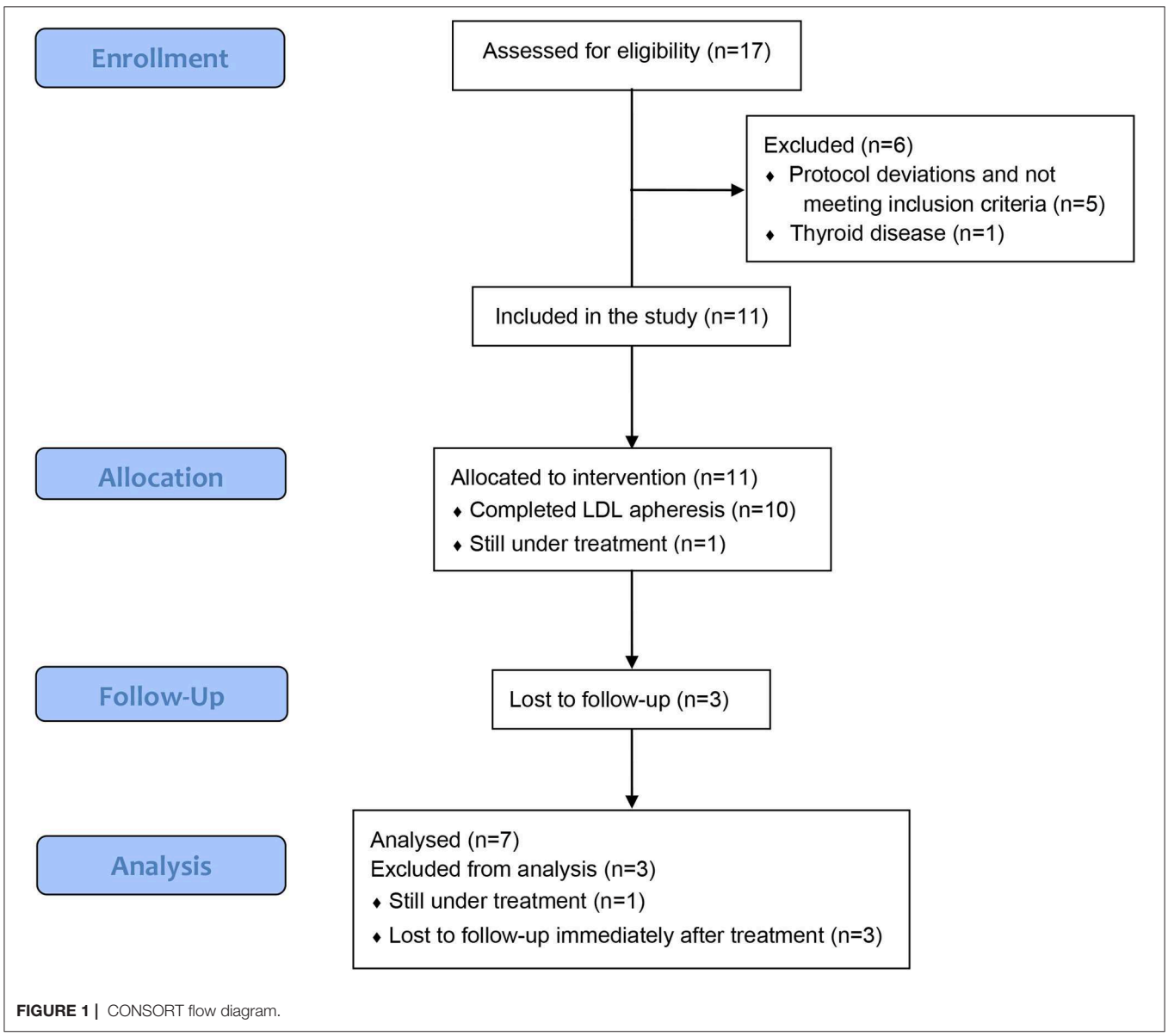

FIGURE 1 | CONSORT flow diagram.

TABLE 2 | Follow-up visit data and nephrotic syndrome status for study subjects.

Patient ID		Indication	Parameter	Baseline	After final treatment	1M F/U	3M F/U	6M F/U	12M F/U	24M F/U	Notes
NCH001	Patient 1	Post-transplant	NS	NS	Partial	Partial	Partial	Partial	Withdrawal		Withdrawal after 6-month F/U
			UPCR (g/g)	44.33	13.02	17.43	12.81	17.51			
			SCR (mg/dl)	0.8	0.4	0.6	0.6	0.6			
			eGFR (ml/min/1.73 m^2)	62.2	125.4	83.6	83.0	83.9			
			LDL (mg/dl)	60	71	269	344	498			
NDE001	Patient 2	Primary FSGS	NS	NS	NS	NA	NS		Withdrawal		Withdrawal after 3-month F/U
			UPCR (g/g)	8.11	3.84	Not performed	6.27				
			SCR (mg/dl)	0.7	0.7	0.7	0.8				
			eGFR (ml/min/1.73 m^2)	89.4	91.0	89.7	78.7				
			LDL (mg/dl)	212	30	181	189				
NDE002	Patient 3	Primary FSGS	NS	NS	NS	NS	Partial	Complete	Complete	Par	24-month F/U completed
			UPCR (g/g)	6.33	<5.0	3.33	0.90	0.18	0.08	0.36	
			SCR (mg/dl)	0.8	0.4	0.6	0.6	0.7	0.7	0.7	
			eGFR (ml/min/1.73 m^2)	84.9	172.2	112.9	114.3	98.3	100.3	100.4	
			LDL (mg/dl)	345	23	96	70	78	45	62	
NDE003	Patient 4	Post-Transplant Recurrence	NS		Not performed			Withdrawal			Withdrawal after final treatment (w/o F/U)
			UPCR (g/g)	5.05							
			SCR (mg/dl)	0.6							
			eGFR (ml/min/1.73 m^2)	95.8							
			LDL (mg/dl)	73							
NDE004	Patient 5	Primary FSGS	NS	NS			Excluded				Excluded from the study
			UPCR (g/g)	8.16							
			SCR (mg/dl)	0.7							
			eGFR (ml/min/1.73 m^2)	72.9							
			LDL (mg/dl)	N/A							
NDE005	Patient 6	Post-Transplant Recurrence	NS	NS	NS			Withdrawal			Withdrawal after final treatment (w/o F/U)
			UPCR (g/g)	19.52	25.58						
			SCR (mg/dl)	0.8	1.3						
			eGFR (ml/min/1.73 m^2)	76.6	47.1						
			LDL (mg/dl)	64	47						
ACH001	Patient 7	Primary FSGS	NS	NS							Excluded from the study
			UPCR (g/g)	1.05			Excluded				
			SCR (mg/dl)	1.9							
			eGFR (ml/min/1.73 m^2)	39.8							
			LDL (mg/dl)	165							

(Continued)

TABLE 2 | Continued

Patient ID		Indication	Parameter	Baseline	After final treatment	1M F/U	3M F/U	6M F/U	12M F/U	24M F/U	Notes
ACH002	Patient 8	Primary FSGS	NS	NS	NS	Partial	Complete	Partial	Complete	Complete	24-month F/U completed
			UPCR (g/g)	1.98	0.71	0.39	0.10	0.42	0.17	0.16	
			SCR (mg/dl)	0.3	0.3	0.4	0.4	0.4	0.4	0.5	
			eGFR (ml/min/1.73 m^2)	170.7	170.3	129.1	129.8	130.1	132.2	109.4	
			LDL (mg/dl)	126	26	98	91	115	179	95	
ACH003	Patient 9	Primary FSGS	NS	NS	NS	NS	NS	NS	NS		Withdrawal after 12-month F/U
			UPCR (g/g)	1.81	3.48	2.67	2.11	4.01	3.78	Withdrawal	
			SCR (mg/dl)	1.2	1.2	1.4	1.2	1.4	2.2		
			eGFR (ml/min/1.73 m^2)	60.0	60.0	51.9	60.9	52.5	33.9		
			LDL (mg/dl)	96	21	98	86	138	143		
ACH004	Patient 10	Primary FSGS	NS				Excluded				Excluded from the study
			UPCR (g/g)	0.08							
			SCR (mg/dl)	1.2							
			eGFR (ml/min/1.73 m^2)	158.5							
			LDL (mg/dl)	103							
ACH006	Patient 11	Post-Transplant Recurrence	NS	NA							
			UPCR (g/g)	Not performed							Under treatment
			SCR (mg/dl)	0.7							
			eGFR (ml/min/1.73 m^2)	72.7							
			LDL (mg/dl)	132							
LLU001	Patient 12	Post-Transplant Recurrence	NS	NS	NS	NS	Withdrawal				Withdrawal after 1-month F/U
			UPCR (g/g)	4.78	3.01	3.66					
			SCR (mg/dl)	2.1	0.5	0.4					
			eGFR (ml/min/1.73 m^2)	84.7	103.8	129.8					
			LDL (mg/dl)	N/A	4	81					
LLU002	Patient 13	Primary FSGS	NS	NS	NS	NS	Withdrawal				Withdrawal after 1-month F/U
			UPCR (g/g)	4.1	5.21	4.58					
			SCR (mg/dl)	0.3	0.3	0.3					
			eGFR (ml/min/1.73 m^2)	153.0	159.1	160.8					
			LDL (mg/dl)	N/A	7	110					
LLU003	Patient 14	Post-Transplant Recurrence	NS	NS	NS	Partial	Partial	Partial	Partial	Partial	24-month F/U completed
			UPCR (g/g)	1.09	1.66	1.49	0.70	0.67	0.37	0.34	
			SCR (mg/dl)	0.8	1.0	1.0	0.9	0.9	0.9	1.1	
			eGFR (ml/min/1.73 m^2)	78.0	13	65.3	69.3	69.3	69.3	71.7	
			LDL (mg/dl)	44	13	100	78	86	115	103	

(Continued)

TABLE 2 | Continued

Patient ID		Indication	Parameter	Baseline	After final treatment	1M F/U	3M F/U	6M F/U	12M F/U	24M F/U	Notes
HDV001	Patient 15	Primary FSGS	NS	NA	NA						Withdrawal after final treatment (w/o F/U)
			UPCR (g/g)	Not performed	Not performed			Withdrawal			
			SCR (mg/dl)	0.7	0.6						
			eGFR (ml/min/1.73 m^2)	83.2	98.8						
			LDL (mg/dl)	56	27						
CHS001	Patient 16	Primary FSGS	NS	NS	NA	NA	NS	NS	NS		12-month F/U completed
			UPCR (g/g)	5.42	Not performed	Not performed	12.51	8.83	2.77		
			SCR (mg/dl)	0.9	0.6	0.4	0.3	0.3	0.3		
			eGFR (ml/min/1.73 m^2)	60.3	89.5	134.2	179.0	183.0	191.4		
			LDL (mg/dl)	N/A	15	389	370	350	163		
CHS002	Patient 17	Primary FSGS	NS	NS	NS	NS		Withdrawal			Withdrawal after 1-month F/U
			UPCR (g/g)	28.04	8.33	38.4					
			SCR (mg/dl)	0.3	0.4	0.5					
			eGFR (ml/min/1.73 m^2)	216.1	163.5	130.8					
			LDL (mg/dl)	>DL*4	>DL*4	14					

NS, nephrotic syndrome; F/U, follow up; UPCR, urine protein to creatinine ratio; SCR, serum creatinine; eGFR, estimated glomerular filtration rate; LDL, low density lipoprotein.

described in the **Appendix A** (20). The study is registered on Clinical Trials.gov, which is a resource provided by the U.S. National Library of Medicine, and the number is as following: NCT02235857. The clinical sites (see **Appendix A**) have their Institutional Review Board (IRB) approval and they have been updated annually. Following FDA approval of the Liposorber® LA-15 system and the study plan in October 2013, the study was initiated in July 2014 with the enrollment completion in August 2018, expected follow up completion in October 2020, and expected final report submission in January 2021. Total duration of the study from initiation to completion of follow up will be 76 months. IRB approvals for the study from the respective clinical sites have been obtained. Informed consent from the patients or legal guardians of minor children (age <18 years) and assent from the children who can understand the language and procedure have been obtained. Sample size was calculated considering both primary safety and primary potential benefit objectives ($n = 30$). A maximum of 35 subjects from 3 to 10 sites will be enrolled in the study.

Study Population

Inclusion criteria: Patients ≤21 years old with drug resistant or drug intolerant NS secondary to primary FSGS with glomerular filtration rate (GFR) ≥60 ml/min/1.73 m^2 or post renal transplant patients ≤21 years old with primary FSGS associated NS. Patients with refractory or recurrent NS due to FSGS were also included if standard therapy was contraindicated. *Exclusion criteria:* Patients were excluded if they are >21 years old, pregnant, lactating or plan to conceive before completion of the study, unable to sign informed consent or adhere to follow up schedule, participating in another interventional study and weight <18 kgs. Patients were also excluded if they are on angiotensin converting enzyme (ACE) inhibitors that cannot be withheld for 24 h before each apheresis session, presently on other antihypertensives which cannot be withheld on the day of apheresis, life expectancy less than the study endpoint or medical condition that interferes with study schedule and outcome, known allergies to dextran sulfate or heparin or ethylene oxide, receiving vitamin K antagonists, severe hemophilia or hemorrhage diathesis, severe gastrointestinal ulcers, uncontrolled hypotension or hypertension, decompensated heart failure or valvular disease, unstable angina, acute myocardial infarction, uncontrolled cardiac arrhythmias, severe apoplexy, unresolved infection, hepatic or thyroid abnormalities.

Study Protocol

Eligible patients were started on lipoprotein apheresis using Liposorber® LA-15 system. Each patient had a total of 12 apheresis sessions (twice/week for 3 weeks then once/week for 6 weeks) over a period of 9 weeks. Patients received medications at the discretion of the treating physician or as per institution's standard of care. Few exceptions were: ACE inhibitors should not be given for at least 24 h before each apheresis session (to avoid bradykinin release), other antihypertensives should be withheld on the day of each apheresis treatment until completion of the session. No initiation, discontinuation or routine

TABLE 3 | Trends in eGFR and UPCR.

	Baseline eGFR (ml/min/1.73 m^2)	Last eGFR (ml/min/1.73 m^2)	Δ eGFR (ml/min/1.73 m^2)	Trend	Baseline UPCR	Last UPCR	Δ UPCR	Trend
Patient 1	62	84	+22	Increase	44.3	17.5	−26.8	Decrease
Patient 2	89	79	−10	Stable	8.1	6.3	−1.8	Stable
Patient 3	85	100	+15	Increase	6.3	0.4	−5.9	Decrease
Patient 12	85	130	+45	Increase	4.8	3.7	−1.1	Stable
Patient 13	153	161	+8	Stable	4.1	4.6	+0.5	Stable
Patient 16	60	191	+131	Increase	5.4	2.8	−2.6	Decrease
Patient 17	28	38	+10	Increase	216	131	−85	Decrease

eGFR, estimated glomerular filtration rate; UPCR, urine protein to creatinine ratio.

changes in dosages of immunosuppressives (e.g., corticosteroids, calcineurin inhibitors, etc.), ACE inhibitors or angiotensin receptor blockers (ARBs) for 2 weeks before first apheresis treatment until 1 month following last apheresis treatment unless deemed absolutely medically necessary. Initiation of low dose corticosteroids (<0.3 mg/kg or <15 mg/day whichever is lower) can be considered for edema during the study period and was not interpreted as treatment failure. All the patients were followed up at 1, 3, 6, 12, and 24 months following the last apheresis session, and data was being collected as per **Appendix A**.

Patient Participation Endpoints

All enrolled patients are expected to participate in the study for 24 months following last apheresis session. Participation will end if subject completes all the scheduled 24 month follow up visits, is lost to follow up, withdraws from the study, has reoccurrence of NS during 24 months follow up period, conceives during the study, requires plasmapheresis, develops ESRD, achieves complete remission at the time of baseline, death or study closure.

Study Endpoints

The primary potential benefit endpoint was the percentage of subjects with partial or complete remission at 1-month post treatment, and the primary safety endpoint s the rate of device or procedure related serious adverse events (SAEs) happening during apheresis treatments and until 1 month after last apheresis session. Secondary endpoints were complete or partial remission or persistent NS at 1, 3, 6, 12, and 24 months following last apheresis session, and percentage of subjects who attained partial or complete remission at the 3, 6, 12, and 24 months follow up visits. Secondary endpoints also include incidence of adverse events (AEs) during apheresis treatments, incidence of all AEs and SAEs within 3, 6, 12, and 24 month follow up, and percentage of subjects showing an increase or decrease in lab values including percent change from baseline and at 1, 3, 6, 12, and 24 months follow up.

Statistical Analysis

Clinically relevant baseline variables will be tabulated. Continuous variables will be reported as means and standard deviations and categorical variables will be reported as percents. Covariate analysis may be performed to identify predictors of SAEs and/or remission. Covariate analysis will also be performed with transplant status (i.e., pre-transplant, post-transplant) as a variable to identify additional predictors of SAEs and/or remission. Survival analysis techniques such as Kaplan-Meier or Cox Proportional Hazards will be incorporated if censoring of data occurs. Descriptive analysis of clinical parameters of NS (UPCR, serum total serum creatinine, eGFR, and LDL) stratified by the status of medication change (Yes vs. No) was provided.

RESULTS

A total of 17 patients from 6 sites have been enrolled into the study. The majority of the patients are Caucasians (52.9%) with the maximum number in the age group 12–14 years (6/17, 35.3%) followed by 6–8 years (5/7, 29.4%) (**Table 1**). Of 17 patients, six were excluded from the outcome analysis; one patients who did not start treatment due to thyroid disease and five who received treatment but did not meet inclusion/exclusion criteria (protocol deviations due to UPCR <2) (**Figure 1**). Of remaining 11 patients, 10 had completed 12 apheresis treatments over a period of 9 weeks and one is still receiving apheresis treatments (**Table 2**). Of the 10 patients who completed apheresis treatments, three patients were lost to follow-up immediately after completion of apheresis and were excluded from outcome analysis (**Table 2**).

One of the seven patients (14.3%) attained partial remission of NS at the 1-month follow-up visit and one patient whose UPCR data was missing during 1-month follow-up, had NS at the 3-month follow-up visit. Furthermore, 2 of 4 subjects (50%) and 2 of 3 subjects (66.7%) had partial/complete remission at 3- and 6-months following lipoprotein apheresis, respectively (**Table 2**). One out of two patients who were followed for 12 months had complete remission while one patient who was followed up for 24-months had partial remission of NS. Trends in eGFR and UPCR before (baseline) and after treatment are tabulated in **Table 3**. Improvement or stable eGFR was noted in all the patients (7/7, 100%) over the follow-up period. Details of steroids and immunosuppressive therapies are listed in **Table 4**. Reported side effects were nausea, vomiting, diarrhea, abdominal pain, fever/infection, pharyngitis, headache, lightheadedness,

TABLE 4 | Experimental subject's medication list.

	Medications at enrollment	During the course of LDL apheresis treatment
Patient 1	Prednisone Mycophenolate mofetil Tacrolimus Amlodipine Losartan	No medication
Patient 2	Prednisone Tacrolimus Pravastatin Lisinopril	Losartan (Before 2nd session)
Patient 3	Prednisone Tacrolimus Losartan	No medication
Patient 4	Prednisone Losartan Lisinopril Mycophenolate mofetil	No medication
Patient 5	Data not available as treatment not started due to thyroid disease	N/A
Patient 6	Prednisone Valganciclovir Ketoconazole Bactrim Tacrolimus Amlodipine	Prednisone (Before 2nd session) Cyclophosphamide (Before 3rd session) Tacrolimus (Before 4th session) Amlodipine (Before 7th session) Mycophenolate mofetil (Before 9th session) Metolazone (Before 11th session)
Patient 7	Prednisone Pravastatin Tacrolimus Valsartan Amlodipine	No medication
Patient 8	Prednisone Cyclosporine Mycophenolate mofetil Lisinopril	Prednisone (Before 2nd and 12th session) Mycophenolate mofetil (Before 11th session)
Patient 9	Cyclosporine Simvastatin Enalapril Chlorothiazide Amlodipine	Losartan Amlodipine (Before 9th session)
Patient 10	Data not available as treatment was not performed	N/A
Patient 11	Prednisone Pravastatin Mycophenolate mofetil Cyclosporine Valacyclovir Clonidine Cozaar Amlodipine Bactrim Aranesp	Aranesp (Before 2nd session) Methylprednisolone (Before 8th session)
Patient 12	No medication	No medication
Patient 13	No medication	Tacrolimus (Before 6th session)
Patient 14	No medication	Mycophenolate mofetil (Before 10th session)
Patient 15	Prednisone Mycophenolate mofetil Tacrolimus Amlodipine Isradipine Labetalol	Tacrolimus (Before 3rd, 7th, 9th, 10th session)
Patient 16	Prednisone Mycophenolate mofetil Simvastatin Amlodipine Isradipine	Simvastatin (Before 12th session)
Patient 17	Prednisone Amlodipine	Cyclosporine (Before 5th, 7th, and 9th session) Prednisone (Before 8th, 9th, and 10th session) Losartan Amlodipine (Before 8th session) Mycophenolate mofetil (Before 12th session)

malaise, hypotension, leg cramps, allergic reaction, pneumonia, bacteremia, and anemia.

DISCUSSION

Our study showed that partial/complete remission rates of NS at 1, 3, 6, 12, and 24-month follow-up after completion of lipoprotein apheresis treatment were 14.3, 50, 66.7, 50, and 100%, respectively with stable or improvement in eGFR in all the patients. Management of steroid resistant primary FSGS is a challenge to date and bear poor prognosis given <25% response rate with uncertain long-term outcomes in patients treated with cyclophosphamide and calcineurin inhibitor therapy (1, 8, 10). Lipoprotein apheresis has been shown to improve the response rates to steroid or immunosuppressive therapy and induce complete or partial remission of proteinuria with histological recovery from the disease (12–19, 21–23). The mechanism by which lipoprotein apheresis produces favorable effects on the outcomes of primary FSGS is poorly understood, however, several theories have been put forth (24); (1) Improvement in macrophage function due to decrease in lipotoxic effect on glomeruli/interstitium as a result of reduction in lipid levels, (2) Better response to steroids and calcineurin inhibitor therapy as a result of lowering lipid levels, (3) improvement in endothelial dysfunction due to decrease in vascular cell adhesion molecule-1 (VCAM-1), (4) Better blood flow due to the removal of fibrinogen and other anticoagulants, (5) Vasodilation due to fall in thromboxane A2 levels and rise in the levels of vascular endothelial growth factor (VEGF), nitric oxide, bradykinin, and endothelial derived growth factor, (6) Anti-inflammatory effect due to reduction in LDL oxidation, C-reactive protein, intercellular adhesion molecule-1 (ICAM-1) and P-selectin, and (7) Reduced levels of vascular permeability factor in the circulation (24).

In the US, Liposorber LA-15® system received Humanitarian Device Exemption approval from FDA for lipoprotein apheresis in the treatment of primary FSGS in both adults and children who are refractory to standard treatment, have a GFR ≥60 mL/min/1.73 m^2, or are post-renal transplantation (25). Our study showed reduced remission rate at 1-month follow-up (14.3 vs. 64%) compared to a study by Hattori et al. (12). However, the limitation of our study is the small number of subjects and high dropout rate. Hattori et al. studied effectiveness of lipoprotein apheresis monotherapy vs. combination of lipoprotein apheresis with prednisolone therapy in 11 children with NS secondary to primary FSGS unresponsive to cyclosporine (CsA) and steroids (12). Lipoprotein apheresis alone did not have significant impact on lowering LDL levels or reducing proteinuria, however, significant reductions were noted in total cholesterol and triglycerides levels. Combination therapy of lipoprotein apheresis with prednisolone induced partial/complete remission of NS in 7 of 11 (64%) children at 1 month following lipoprotein apheresis. Of these seven children, five achieved complete remission and had normal renal function at median of 4.4 years after lipoprotein apheresis, and remaining two had partial remission at 1 month after lipoprotein apheresis (stable renal function at 4.5 years follow up in one patient while the other one eventually developed ESRD after 7.8 years) (12).

The prospective observational survey on the long-term effects of lipoprotein apheresis on drug-resistant nephrotic syndrome (POLARIS) showed remission of NS (urine protein <1.0 g/day) in 21 of 44 (47.7%) patients followed for 2 years (26). Clinical parameters immediately after lipoprotein apheresis therapy that contributed significantly for favorable outcome were serum total protein (4.9 ± 0.7 g/dl), serum albumin (2.9 ± 0.8 g/dl), serum creatinine (1.2 ± 0.7 mg/dl), eGFR (61 ± 27.2 ml/min/m^2), urine protein (1.7 ± 1.8 g/day), triglycerides (240.2 ± 156.3 mg/dl), total cholesterol (194.3 ± 65.6 mg/dl), LDL (83.1 ± 60.4 mg/dl), HDL (66.5 ± 18.3 mg/dl), fibrinogen (271.1 ± 77.2 mg/dl), and thrombin-antithrombin III complex (14.7 ± 38.6 ng/ml) (26). In addition, published literature has revealed few case studies showing remission of NS following dextran-sulfate plasma adsorption lipoprotein apheresis along with steroid and immunosuppressive therapies in children with drug resistant FSGS (14, 18, 19).

Available evidence from the published literature shows that lipoprotein apheresis enhances response rates to steroids and CsA in both adult and pediatrics NS patients as the effectiveness of lipoprotein apheresis monotherapy in the treatment of NS is not known (12, 16, 17, 21, 26). This is because VLDL and LDL decreases glucocorticoid receptor binding sites and impede glucocorticoid actions (24). Due to this, combination of lipoprotein apheresis with CsA or steroids or both is recommended by the Japanese Society of Pediatric Nephrology (27). In our study, most of the patients were on steroids and immunosuppressive therapies (**Table 4**). Additionally, maximum benefit can be achieved by starting lipoprotein apheresis early in the course of the disease process as response rates are better in patients with highly selective proteinuria and presence of advanced interstitial fibrosis indicates poor prognosis (12). Besides these benefits, a recent retrospective analysis involving five adult patients showed that lipoprotein apheresis prevented recurrence of FSGS in renal transplant recipients (28). Two sessions of lipoprotein apheresis was administered to all patients before renal transplantation except one patient who had single session (28). Survival of renal graft was noted in all patients without FSGS recurrence during 2–22 months of observation period following renal transplantation (28).

Common side effects that are associated with extracorporeal circulation can be encountered during lipoprotein apheresis, however, there are no serious adverse effects reported (27). ACE inhibitors should be discontinued during lipoprotein apheresis to avoid hypotensive events and shock (27). Restricting the use of lipoprotein apheresis therapy to children who weigh >30 kg is also recommended due to consumption of a significant amount of blood by the extracorporeal circuit (27). However, the lower weight limit for lipoprotein apheresis treatments for our study was recently set to 18 kg.

CONCLUSION

FSGS is the main cause of ESRD among children with a high recurrence rate. Primary FSGS carries a poor prognosis as spontaneous remission is rare and it more rapidly progresses to ESRD. Despite all the available treatments, management of FSGS patients refractory to standard treatments remains a major challenge. Lipoprotein apheresis therapy may be beneficial for drug resistant (corticosteroids and/or calcineurin inhibitors) primary FSGS and post-renal transplant primary FSGS reoccurrence. Results of our multicenter study showed partial remission rate of NS was 14.3% at 1-month following lipoprotein apheresis. Furthermore, partial/complete remission rates at 3 and 6-month follow-ups were 50 and 66.7%, respectively. In addition, all patients showed stable or improved eGFR during the follow-up period. The main limitation of our study is small number of subjects and high dropout rate.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

RR, VK, CS-K, AQ, KT, RM, ML, SM, and JZ contributed to the conception and design of the study. RR, VK, CS-K, AQ, KT, RM, ML, RC, SM, JS, TB, and JZ helped organize the database and wrote sections of the manuscript. All authors contributed to manuscript revision and read 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.

APPENDIX

Appendix A: IRB approval has been given by the following institutions.

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Neonatal Acute Kidney Injury: A Survey of Perceptions and Management Strategies Amongst Pediatricians and Neonatologists

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Background: Neonatal Acute Kidney Injury (AKI) occurs in 40–70% of critically ill newborn infants and is independently associated with increased morbidity and mortality. Understanding the practice patterns of physicians (neonatologists and pediatricians), caring for neonates in India is important to optimize care and outcomes in neonatal AKI.

Aim: The aim of this study was to identify differences in physician's perception and practice variations of diagnosis, management, and follow-up of newborn infants with AKI in India.

Methods: An online survey of neonatologists and pediatricians in India caring for newborn infants with AKI.

Results: Out of 800 correspondents, 257 (135 neonatologists and 122 pediatricians) completed the survey, response rate being 32.1%. Resources available to the respondents included level III NICU (59%), neonatal surgery (60%), dialysis (11%), and extracorporeal membrane oxygenation (ECMO, 3%). Most respondents underestimated the risk of AKI due to various risk factors such as prematurity, asphyxia, sepsis, cardiac surgery, and medications. Less than half the respondents were aware of the AKIN or KDIGO criteria, which are the current standard criteria for defining neonatal AKI. Only half of the respondents were aware of the risk of CKD in preterm neonates and nearly half were unaware of the need to follow up with a pediatric nephrologist.

Conclusions: Similar to other regions worldwide, there exists a knowledge gap in early recognition, optimal management and follow up of newborn infants with AKI amongst Indian physicians.

Keywords: neonatal, acute kidney injury, AKI, dialysis, preterm, survey

INTRODUCTION

Neonatal Acute Kidney Injury (AKI) is common amongst the Neonatal Intensive Care Unit (NICU) infants and is a major contributor of neonatal mortality and morbidity (1, 2). Although precise prevalence is unknown, reported frequency of neonatal AKI ranges from 6 to 24% (3, 4). Since nephrogenesis is incomplete before 36 weeks' gestation, AKI has a significant short term and long-term impacts on renal health necessitating a long term follow up.

Progress in neonatal AKI has been slow, primarily due to under recognition of AKI by neonatologists and pediatricians and due to the lack of universally accepted diagnostic criteria. Several neonatal AKI definitions have been proposed in the past but more recently, the Kidney Diseases: Improving Global Outcomes (KDIGO) neonate modified classification is gaining more acceptance amongst neonatologists, pediatricians, and nephrologists. The neonate modified KDIGO is a tiered description of AKI based on changes in oliguria severity and serum creatinine (SCr) levels (5, 6). Moreover, the data on neonatal AKI from the Indian subcontinent is very small and regional risk profile predisposing newborn to develop AKI is not well-understood. Reported incidence of neonatal AKI in India ranges from 3.4 to 4.2% of all NICU admissions (7, 8). Understanding the practice patterns of neonatologists and pediatricians caring for neonates in India is important to optimize care and outcomes in neonatal AKI.

The objective of this study was to identify differences in physicians' (neonatologists and pediatricians) perception and practice variations of diagnosis, management, and follow-up of newborn infants with AKI in India after the publication of the AWAKEN study.

METHODS

Study Design

The study followed a cross-sectional survey design. The target population for the survey was neonatologists and pediatricians caring for newborn infants. The study was conceptualized and developed by the Indian Neonatal Kidney Collaborative Study Group, which includes seven pediatric nephrologists and two neonatologists.

Survey Instrument

The survey instrument was created on an online survey platform (Survey Monkey) by the Indian Neonatal Kidney Collaborative Study Group that included pediatric nephrologists and neonatologists. It was evaluated for both face and content validity by the investigators and approved by the Indian Society of Pediatric Nephrology. The survey was pilot tested on a group of neonatologists and pediatricians before distribution. Feedback from the pilot testing was used to modify and finalize questions. The survey instrument included questions referencing demographic data on providers, years of experience, level of neonatal care provided, type-severity-number of patients seen monthly (preterm infants, intubation with assisted ventilation, birth asphyxia/hypoxic ischemic encephalopathy), and availability of pediatric subspecialists including nephrologists. The survey instrument also included a common clinical scenario of AKI occurrence in preterm infants in the NICU (see below) and asked questions to elicit differences in physicians (neonatologists and pediatricians) perception and practice variations of diagnosis, management, and follow-up.

Survey Distribution

The survey was administered electronically via email along with instructions and a hyperlink to the survey. The participants were notified of the voluntary nature of participation, confidentiality and non-compensation for participation. Lists of email addresses of neonatologists and neonatal practitioners were assembled by liaising with the Neonatology Chapter of Indian Academy of Pediatrics (IAP Neochap). The invitation was sent to 800 neonatologists and pediatricians nationwide in India during the month of January 2018. To encourage response rates, an additional survey reminder was emailed 1 month later.

Statistical Analysis

Descriptive statistics was used to analyze data. A panel of one pediatric nephrologist (SKS) and two neonatologists (GA, SW) was formed to determine the optimal answers for the questionnaire of published literature or consensus.

RESULTS

Participant Characteristics

Out of 800 correspondents, 257 completed the survey with response rate being 32.1%: out of which 52% were neonatologists and 48% were pediatricians. Amongst all the respondents, 29.5% worked in a teaching hospital and 39% in private practice, while 59% were attached to a level III NICU. The majority (71%) of respondents were between ages 30–50 years, 15% $\leq$ 30 and remaining 14% over 50 years. Twenty five percent of respondents had clinical experience of $<$3 years, 54% had 3–15 years' experience and 21% had $>$15 years' experience.

Participant Exposure to Patient Variety and Resources

Figure 1 demonstrates the number of newborn infants at risk (preterm, ventilated, birth asphyxia) for AKI dealt by the respondents per month. Nearly two-third (60.3%) of the institutes of respondents had pediatric-neonatal surgery capabilities, whereas neonatal dialysis facilities were available for 11% and extracorporeal membrane oxygenation (ECMO) for only 3%. Only 31% of the respondents had the facility to access the services of a pediatric nephrologist.

Perception and Practice Variations Regarding Diagnostic Criteria (Definitions) for Neonatal AKI

Table 1 demonstrates the perception of respondents in the incidence of neonatal AKI in the presence of various risk factors

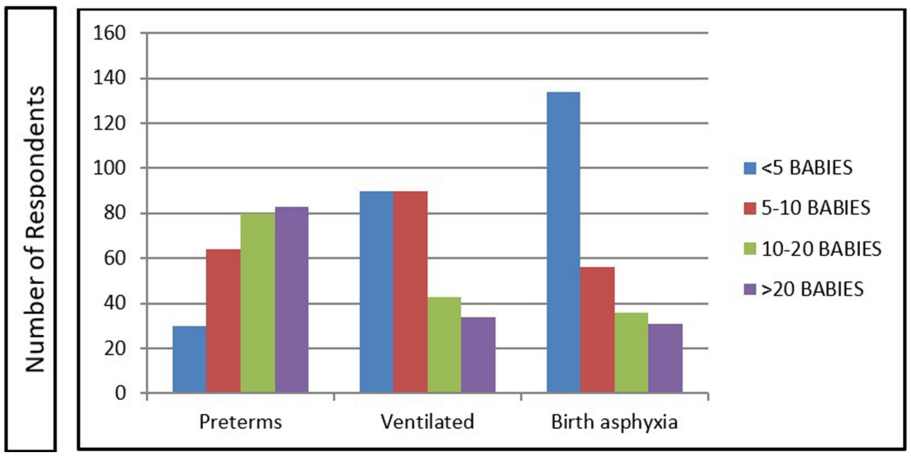

FIGURE 1 | Newborn infants at risk for AKI seen by the respondents per month.

TABLE 1 | Perception of respondents regarding the incidence of neonatal AKI in various clinical conditions.

	Incidence of neonates developing AKI				
Clinical conditions	**<20**	**21–40**	**41–60**	**>60**	**Not sure**
Preterm, *n* (%)	210 (81.7)	20 (7.8)	2 (0.8)	1 (0.4)	24 (9.3)
Asphyxia, *n* (%)	127 (49.4)	81 (31.5)	32 (12.5)	7 (2.7)	10 (3.9)
Cardiac surgery, *n* (%)	63 (24.5)	34 (13.2)	17 (6.7)	3 (1.1)	140 (54.5)
Sepsis, *n* (%)	133 (51.6)	71 (27.6)	34 (13.4)	7 (2.8)	12 (4.6)
Medications, *n* (%)	169 (65.8)	24 (9.4)	2 (0.8)	3 (1.1)	59 (22.9)

(prematurity, asphyxia, cardiac surgery, sepsis, medications). There was significant disagreement in the above perceptions and AKI decided by the expert panel. The panel notes, AKI in preterm infants was 41–60%, in asphyxia 21–40%, cardiac surgery 41–60%, sepsis 21–40%, and due to medications was 41–60%. Prematurity was considered to be a low risk factor for AKI by most of the respondents (81.7%) and only 2 (0.8%) respondents matched with the panel's opinion. More than 50% of respondents were unaware that cardiac surgery was a high-risk factor. The incidences of neonatal AKI due to the use of various medications was considered to be 41-60% by the expert panel but only 2 (0.8%) respondents had a similar response.

Familiarity with various diagnostic criteria of AKI was variable with most respondents being familiar (to least familiar) with pRIFLE (57.5%), AKIN (49.8%), and KDIGO (19.8%), whereas 16.3% were unaware of any criteria. However, in their own clinical practice to diagnose AKI, respondents most frequently (in decreasing order) used rise in serum creatinine (57%), urine output (51%), pRIFLE (39%), AKIN (28%), and KDIGO (11%). As per the expert panel, a pRIFLE diagnostic criterion was not useful in neonates whereas other criteria (AKIN, KDIGO, Urine output, rise in serum creatinine) could be used for diagnosing neonatal AKI.

Practice Variations in Diagnosis, Management, and Follow-Up of 27-Week Preterm Infants With AKI

Clinical Scenario

A 27 weeks' gestation extreme preterm infant on empiric IV ampicillin, IV amikacin and on continuous positive airway pressure (CPAP) since birth was noted to have a significant systolic murmur associated with tachypnea and feeding intolerance on day 7. Following an echocardiogram confirmation of patent ductus arteriosus (PDA), 3 doses of IV indomethacin were administered day 8 onwards. Baseline serum creatinine (SCr) before indomethacin administration rose from 0.6 mg/dl (Day 7) to 1.0 mg/dl (Day 10). Subsequent SCr values ranged from 0.8 to 0.7 mg/dl (Days 11–15). Enalapril and furosemide were added on Day 15 for ongoing clinical symptoms. SCr rose to 2.1 mg/dl on Day 17. Enalapril and furosemide were stopped.

Assessment of Risk Factors for AKI

Respondents believed that AKI developed most likely (to least likely) after the administration of indomethacin (49%), prior to the administration of indomethacin (40%), and 11% believed that AKI occurred after the administration of furosemide and enalapril. Respondents graded the relative importance of risk factors in the development of AKI (in decreasing order of importance) as the administration of amikacin (26%), prematurity (21%), PDA (19%), administration of enalapril (17%), or furosemide (14%), and antenatal antibiotics (3%). The expert panel opined that the preterm infant developed AKI after adding indomethacin and all the factors described in the case scenario (Use of amikacin, presence of PDA, use of enalapril, use of furosemide, prematurity, use of antenatal antibiotics) could be considered as risk factors for developing AKI.

Evaluation Strategies

Respondents would involve a pediatric nephrologist with variable frequency on day 7 (34%), day 10 (44%), day 17

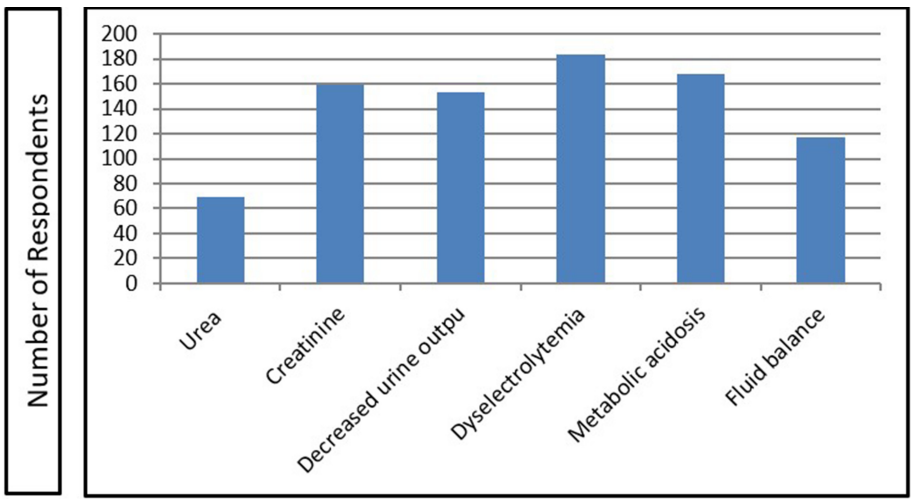

FIGURE 2 | Criteria to initiate dialysis in the case scenario if renal function deteriorated.

(10%), and the remaining 12%, only if the dialysis was deemed necessary. The majority of the responses agreed with the opinion of the panel who suggested involving pediatric nephrologist on day 10, when creatinine increased from 0.6 to 1.0 mg/dL. In reference to sending the first SCr in the scenario, 14% of respondents would send it within 12–24 h of age, 36% at 24–48 h, 36% at 48–72 h and remaining 14% would order SCr prior to initiating the Indomethacin. There, the respondents differed from the panel's view that the first serum creatinine should have been ordered before administering indomethacin.

Management Strategies

Figure 2 demonstrates the criteria, which the respondents would use to initiate dialysis if the renal function in the case scenario deteriorated. Most common (to least common) criteria that the respondents would use to initiate dialysis were dyselectrolytemia (80%), metabolic acidosis (73%) and rising SCr (69%). The panel members had a consensus that urine output, dyselectrolytemia, metabolic acidosis, and fluid imbalance could be used as the criteria but urea and creatinine values should not be the criteria for initiation of dialysis. In such a scenario, majority of respondents would use peritoneal dialysis (84%), followed by hemodialysis (7%), and the rest (9%) would prefer continuous renal replacement therapy (CRRT), which almost matched with the view of panel members.

Follow-Up

Only 43% of respondents corroborated with the panel's view: that in the case scenario, the preterm infant was at risk for developing chronic kidney disease (CKD). In addition, over half (52%) of the respondents would not arrange a follow-up of the infant with a pediatric nephrologist, even though the panel suggested that all such infants should be followed up with a pediatric nephrologist.

DISCUSSION

In this first of its kind national survey in India of neonatologists and pediatricians caring for newborn infants in regard to neonatal AKI. The data demonstrated significant differences among practices for recognizing and defining AKI, renal function surveillance, nephrology consultation, dialysis options and follow up practices. The respondents demonstrated a variable degree of knowledge gaps of current definitions, early diagnosis, optimal management strategies, and follow up of newborn infants with AKI. This data may help in formulating future strategies to close these gaps.

The most common medical conditions in neonates associated with AKI are prematurity (42.2%) and congenital heart disease (CHD, 11.7%), with major independent risk factors being mechanical ventilation, hypervolemia, CHD and metabolic acidosis (9). In this survey, most respondents underestimated the risk of AKI due to various risk factors such as prematurity, asphyxia, sepsis, cardiac surgery, etc. There was a disagreement between the respondents, the views of the expert panel, and the published literature. In the study by Mian et al., the incidence of AKI in premature infants was 26% with incidence showing an inverse relation with the gestational age (GA) of the neonate. In infants between 22 and 25-weeks, AKI was seen in 65% of patients, while in infants between 26 and 28 weeks, this incidence was seen at 25% and a mere a 9% in infants between 29 and 32 weeks (10). In birth asphyxia, the incidence reported by Kaur et al. was 47.1% with incidence varying from 9.1% in moderate asphyxia to 56% in severe asphyxia (11). As per the study by Blinder et al., AKI incidence in neonates after cardiac surgery was 52% (12).

In order for these studies to be compared, standardized definition are required for neonatal AKI. This standard definition allows for the outcomes associated with AKI along with their epidemiology to be studied. To increase the accuracy of the results, AKI definitions have been changed [from pRIFLE to AKIN to KDIGO; (5, 6)]. More than half the respondents in

our study were aware of pRIFLE criteria, which the expert panel considered least useful in neonates. Less than half the respondents were aware of AKIN or KDIGO criteria, which are the current standard criteria for defining neonatal AKI. Few respondents were aware about the new neonatal modified KDIGO classification. More than half used serum creatinine or urine output as the lone criteria. In a similar survey by Kent et al., nearly half of the neonatologists were unaware of the staged definitions of neonatal AKI (13). This study shows that there remains a gap in understanding regarding neonatal AKI and efforts at education are justified in the country.

A modifiable risk factor for AKI is the exposure to nephrotoxic medications. Rhone et al. have shown that during the stay of premature infants in the NICU, their exposure to nephrotoxic medications is 14 days on average (14). In our survey, most respondents felt that the risk of neonatal AKI due to various medications was <20%. This was in disagreement with the expert panel as they considered the incidence to be 41–60%. In the case scenario, only 49% respondents attributed AKI due to indomethacin use whereas only 26% felt that amikacin use can lead to AKI. This event brings up concerns as aminoglycosides and indomethacin have been shown to be frequently associated with AKI (15). Education in association with use of nephrotoxic medications usage in neonates can potentially minimize both the risk and incidence of AKI in neonates.

In recent years, renal replacement therapy has moved from being the last treatment option to a therapy designed to inhibit the effects of AKI in patients early (16). An interesting finding was that the good understanding between the respondents and the expert panel in response to the indications of dialysis and the mode of renal replacement therapy. Peritoneal dialysis was the most common mode while 9% considered CRRT as the first line of renal replacement therapy. The feasibility and technology available for doing CRRT in an extreme preterm infant needs to be considered.

There is strong evidence to convey that there is an increased risk of CKD with each episode of AKI. There is also evidence that extreme prematurity and exceedingly low birth weight are associated with CKD. However, there is currently no follow-up study reported that is large enough to evaluate the relationship of the risk of CKD and AKI in neonates. Small single-center studies have shown an increased risk of CKD in certain neonatal populations (17–19). In this survey, only half of the respondents were aware of the risk of CKD in preterm neonates and nearly the other half were unaware of the need to follow up with a pediatric nephrologist. A follow up of AKI at 3 months post discharge is recommended by the KDIGO guidelines. In this survey, only 31% had exposure to a pediatric nephrologist. This suggests that neonatologists/pediatricians may fail to diagnose AKI and lead to delayed consultations and treatments. Multiple studies have illustrated that lower mortality rates in adults is associated with early nephrology consultation (20, 21).

This study has several limitations including a small sample size, which may not be representative of the total cohort. Additionally, there was a very limited response rate which may have been due to the resource limitations, the lack of neonatology fellowship programs, and the small handful of practicing neonatologists in this country. Another limitation is that the results are subject to both survey bias and non-response error. Conceivably, physicians with interest in or previous experience in managing neonatal AKI were more likely to respond to this survey. As is the case with any survey, this one is also limited by its population set and may not be representative of the whole population.

In conclusion, this study demonstrates that there exists an important gap in knowledge about the effects of AKI in neonates. Increased efforts at education are crucial in order to improve clinical outcomes in the country. The development of guidelines and increase in education may potentially minimize the incidence and risk associated with neonatal AKI. Evaluating the long-term consequences of neonatal AKI and improving the management and its outcomes will require collaborations in both clinical care and research.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SS, GA, SR, RJ, SW, and RoC helped in study design, carried out the survey, analyzed the data, and drafted the manuscript. SS, GA, RaC, NN, DK, RS, AI, RR, and SW supervised the collection, analysis of data, did critical revision, and finalization of the manuscript.

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Circulating *de novo* Donor Specific Antibodies and Carotid Intima-media Thickness in Pediatric Kidney Transplant Recipients, A Pilot Study

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Background: The presence of circulating *de novo* donor specific anti-HLA antibodies (dnDSA) has been implicated in an immune-mediated form of accelerated systemic arteriosclerosis in adult heart and kidney transplant recipients, however this has not been previously investigated in pediatric kidney transplant recipients. Carotid intima-media thickness (CIMT) is a reliable method for detection of arteriosclerosis. We hypothesized that children who develop dnDSA after kidney transplant would have increased CIMT compared with those who remain dnDSA negative.

Methods: A prospective, controlled pilot cohort study of 38 transplant patients and 20 healthy controls was conducted to investigate the association between CIMT and development of dnDSA after kidney transplant. CIMT, anthropometrics, blood pressure and lipid panel were measured at 1, 18, and 30 months post-transplant. DSA was checked at 6, 12, 18, 24 and 30 months post-transplant. CIMT of DSA positive transplant recipients was compared to DSA negative and controls.

Results: Of the 38 transplant recipients, 7 patients developed dnDSA by 18–30 months post-transplant. Among 5 dnDSA positive patients who did not receive treatment for DSA prior to CIMT measurement (n=6 observations), the median CIMT was 0.505 mm (95% CI 0.454–0.560 mm) at 18–30 months post-transplant, compared to 0.455 mm (95% CI 0.440–0.470) in DSA negative transplant recipients (n = 54 observations of 30 patients) and 0.450 mm (95% CI 0.436–0.460) in the healthy controls (20 observations of 20 patients). Presence of dnDSA was independently associated with a 7.8% increase in CIMT compared to those without dnDSA (p=0.006), after adjusting for race, hypertension, dyslipidemia, and abdominal obesity.

Conclusions: Development of dnDSA was associated with increased CIMT, an indicator of arteriosclerosis, in a cohort of dnDSA positive pediatric kidney transplant recipients. The association between dnDSA and CIMT was independent of traditional CV risk factors, including hypertension, dyslipidemia, and abdominal obesity.

Keywords: donor specific antibodies, carotid intima-media thickness, cardiovascular, arteriosclerosis, pediatric, kidney transplant

INTRODUCTION

Pediatric kidney transplant recipients are at high risk for cardiovascular (CV) disease, the second leading cause of mortality in this population (1). Traditionally, factors such as hypertension, dyslipidemia, and obesity have been associated with increased CV risk in both the general population (2) and in kidney transplant recipients (3, 4). However, recently, the presence of circulating *de novo* donor specific anti-HLA antibodies (dnDSA) have been implicated in an immune-mediated form of accelerated systemic arteriosclerosis in adult heart and kidney transplant recipients (5, 6). In addition, one pediatric study has identified a link between dnDSA and antibody-associated arteriosclerosis in heart transplant recipients (7). This form of antibody-associated arteriosclerosis is a condition characterized by arterial inflammation and fibrointimal thickening of the arteries caused by endothelial activation, a pro-inflammatory and pro-coagulant state of the endothelial cells lining the lumen of the blood vessels.

The study by Loupy et al. demonstrated that the presence of circulating dnDSA was significantly associated with severe allograft arteriosclerosis, independent of traditional CV risk factors in adult kidney transplant recipients (5). In addition, patients with dnDSA-associated arteriosclerosis had decreased allograft survival, increased occurrence of major adverse cardiovascular events, and increased mortality.

Carotid-intima media thickness (CIMT) is one known reliable indicator of arteriosclerosis that can be utilized to assess CV risk in renal transplant recipients (1, 4). The association of dnDSA with arteriosclerosis and CV risk has not been previously investigated in pediatric kidney transplant recipients. Therefore, the aim of our pilot study was to investigate the effect of dnDSA on CIMT as a marker of arteriosclerosis in pediatric kidney transplant recipients, based on the hypothesis that the presence of circulating DSA promotes vascular proliferation resulting in increased CIMT.

PATIENTS AND METHODS

Patients and Controls

A prospective, controlled cohort study was conducted to investigate the association between CIMT and the development of dnDSA after kidney transplant. Children, 3–20 years of age, who received a kidney transplant between September 2010-January 2015 at Children's National Hospital in Washington DC were eligible to enroll in the study at the time of transplant. Patients with an estimated glomerular filtration rate (eGFR) $<$60 mL/min/1.73 m^2 at 1 month post-transplant, multi-organ transplant, or nephrotic-range proteinuria were excluded from the study.

Healthy children 3–20 years of age served as controls. The healthy control participants were recruited from the local pediatric community, reflecting similar environmental exposures to the transplant cohort, and the racial distribution of the control population was matched to that of the transplant group.

Approval was obtained from the IRB at Children's National Hospital (IRB protocol number 2024). Informed consent was obtained from all participants, and the study was conducted in accordance with the Helsinki Declaration of 1975.

Measurement and Classification of dnDSA

Transplant patients were monitored for development of dnDSA at 6, 12, 18, 24, and 30 months post-transplant, as well as for-cause. Donor-specific antibodies were tested using multiple solid phase assay platforms. In cases where flow panel reactive antibody (PRA) Screen was positive, both SAB (One Lambda, Inc., Canoga Park, CA, USA) and phenotype bead (LABScreen PRA assay by One Lamba, Inc.) assays were then performed. DSA was determined to be positive when mean fluorescence intensity (MFI) was $>$1000. DSA were further classified as weak if MFI was 1000–2000, strong if MFI was $>$2000–10,000, or very strong if MFI was $>$10,000.

For purposes of the study, transplant patients were classified as DSA positive (DSA+) if strong or very strong DSA was detected at 18 and/or 30 months post-transplant. Observations of CIMT at the time of DSA+ detection, prior to receiving any pharmacologic treatment for DSA or acute cellular-mediated rejection were included in the analysis. Any CIMT measurements performed after administration of treatment for DSA+ and/or acute cellular-mediated rejection, such as pulse methylprednisolone, alemtuzumab, rituximab, and bortezomib, were excluded from the analysis. CIMT measurements of patients with weak DSA (MFI $>$1000–2000) were also excluded from the analysis. Patients were classified as DSA negative (DSA-) if they never developed any dnDSA during the first 30 months post-transplant. In patients who were DSA+ at 18 months post-transplant but became DSA- by 30 months post-transplant, the 30 month data point was excluded from the analysis to minimize confounding effects of prior DSA positivity.

CIMT

CIMT was measured at baseline (0–1 months post-transplant), 18, and 30 months post-transplant in the transplant group, and at a single study visit in the healthy control group. CIMT was visualized using B-mode ultrasound imaging of the arterial far wall segments of the right and left common carotid arteries and carotid bulbs. Imaging was performed according to a standard protocol by a single pediatric sonographer using aiE33 xMatrix DS Ultrasound System (Philips North America Corporation, Andover, MA, USA). Intima-media thickness of the distal, mid-, and proximal segments of right and left common carotid arteries and carotid bulbs were analyzed by a pediatric cardiologist blinded to the clinical information. A composite of the eight measured segments was then created to represent the CIMT.

Abbreviations: CV, Cardiovascular; dnDSA, *De novo* donor specific antibody; DSA+, Donor specific antibody positive; DSA-, Donor specific antibody negative; CIMT, Carotid intima media thickness; eGFR, Estimated glomerular filtration rate; PRA, Panel reactive antibody; MFI, Mean fluorescence intensity; BMI, Body mass index; WHr, Waist to height ratio; CDC, Center for disease control; NHANES, National Health and Nutrition Examination Survey; HDL, High density lipoprotein; LDL, Low density lipoprotein; WC, Waist circumference; GEE, Generalized estimating equation; QIC, Quasi-likelihood under the independence model criterion; ESRD, End stage renal disease.

Traditional CV Risk Factors

Parameters traditionally used to evaluate CV risk, including BMI, waist-to-height ratio (WHr), fasting lipid panel, and blood pressure, were measured at 1, 18, and 30 months post-transplant in the transplant recipients. The children in the healthy control group had BMI, WHr, and blood pressure measured once, at the time of CIMT measurement. Presence of general obesity and abdominal obesity were assessed using BMI and WHr, respectively. Weight in kilograms and height in centimeters were measured and used to calculate BMI [weight (kg)/height(m^2)]. Participants were then classified as BMI-obese if they had a BMI $\geq$ 95th percentile-for-sex-and-age on the Center for Disease Control (CDC) growth charts (8). Waist circumference (WC) was measured using a Gulick II fiberglass tape measure (Country Technology, Gays Mills, WI) at the upper-most lateral border of the right ileum, according to standard National Health and Nutrition Examination Study (NHANES) procedures (9). WHr was calculated as a ratio of waist in centimeters divided by height in centimeters. Participants were then classified as WHr-obese if WHr $\geq$ 0.5 (10).

Cut-points for abnormal lipid parameters for high density lipoprotein (HDL), low density lipoprotein (LDL), and triglycerides were defined according to 2011 Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents for children age 3–19 (11), and by the Adult Treatment Panel III criteria for those >19 years of age (12). Dyslipidemia was defined as the presence of at least one abnormal lipid parameter (HDL, LDL, or triglyceride). Hypertension was defined by diagnosis of hypertension (systolic and/or diastolic blood pressure >95th percentile for age-sex-and -height on 3 different occasions) and requiring antihypertensive medication (13). Estimated glomerular filtration rate (eGFR) was calculated using the updated Schwartz equation for children under 18 years of age and the MDRD equation was used for those $\geq$ 18 years of age.

Induction and Maintenance Immunosuppression

The transplant recipients received Thymoglobulin for induction immunosuppression, followed by maintenance immunosuppression with either with a steroid-withdrawal or steroid-based protocol, depending on their immunological risk. The steroid-withdrawal protocol was used for "standard immunological risk" patients, defined as those receiving their first transplant, with cPRA <20%, and ESRD diagnosis not due to autoimmune disorder or FSGS. The steroid-based protocol was used for "high immunological risk" patients, defined as those with history of a prior transplant, diagnosis of FSGS or autoimmune disorder, highly sensitized patients, or those with delayed graft function (as defined by need for dialysis in the immediate post-transplant period). The details of the induction therapy protocols have been previously published (14). Maintenance immunosuppression for those on the steroid-based protocol consisted of tacrolimus, mycophenolate mofetil (MMF), and prednisone, while those on the steroid-withdrawal received tacrolimus and MMF.

Statistical Analysis

Statistical analyses were conducted using Stata 14.0 (StataCorp LP, College Station, TX, USA). Analyses of the demographic and clinical characteristics of the study and control populations were conducted using Fisher's exact test for categorical variables and Wilcoxon rank-sum for continuous variables.

The median (95% CI) of the CIMT observations of the DSA+ and DSA- transplant groups at 18–30 months post-transplant and of the control group (single time point) are presented as descriptive statistics in the Results section and in graphical form in **Figure 1**. In order to establish a temporal reference point, the median CIMT of the DSA+ and DSA- transplant groups are also depicted at baseline (0–1 months post-transplant). Due to the longitudinal nature of the data, bivariate statistical tests of significance were not appropriate, and therefore **Figure 1** represents a descriptive presentation of the data only.

Unadjusted and adjusted linear regression models using generalized estimating equations (GEE) to account for repeated measures were used to evaluate association between CIMT and DSA status at 18–30 months post-transplant. Observations at 18 and/or 30 months post-transplant time points were included in the GEE regression analysis if both a CIMT measurement and a DSA measurement were performed at that time point. In total, 60 observations of 38 patients were included in this analysis. GEE regression analysis was selected due to its ability to account for the within-patient correlation inherent to longitudinal serial data, as well as the fact that this statistical method is robust to missing data (15). In addition, multivariable linear regression was performed at baseline (0–1 months post-transplant) to determine whether there was a pre-existing difference in CIMT between the two transplant groups prior to the onset of DSA. Due to the non-normal distribution of CIMT in the transplant group, all continuous CIMT data were log-transformed, and therefore results of the regression models are presented as a % change in CIMT. Quasi-likelihood under the independence model criterion (QIC) was used to determine the best-fit statistical model. The most robust multivariable model is reported and was adjusted for race, hypertension, dyslipidemia, and abdominal obesity by WHr.

Statistical Power Analysis

Ad-hoc power analysis indicates that the sample size of 60 observations of 38 patients provided 87% power to detect a significant association ($p < 0.05$) between dnDSA and CIMT using a 5-parameter regression model. The power analysis was based on a full model $R^2 = 0.52$, reduced model $R^2 = 0.44$, and change in $R^2 = 0.08$. Therefore, the study was adequately powered to detect the primary outcome measure, a significant association between CIMT and DSA, by GEE multiple regression analysis.

RESULTS

Study Population

Of 70 patients who received a kidney transplant at Children's National between September 2010 and January 2015, 61 patients met the study inclusion criteria. Of these 61 eligible patients, 43

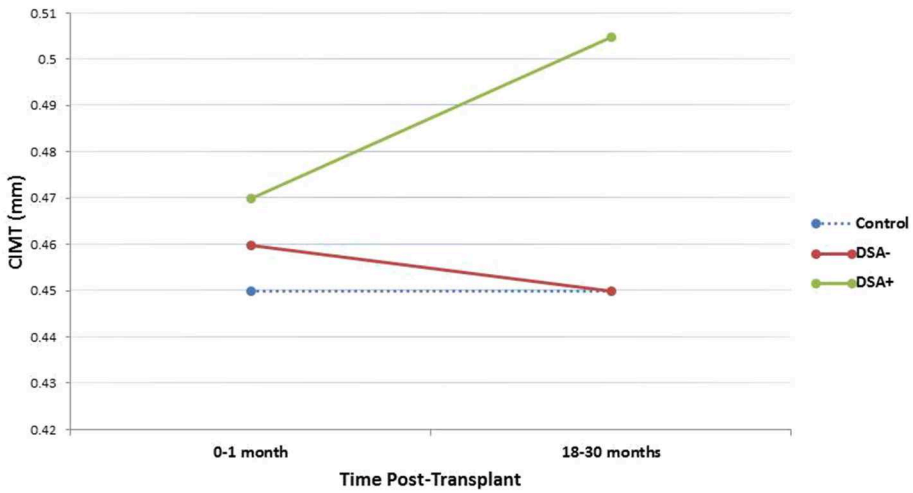

FIGURE 1 | Median CIMT observations of the group who remained DSA negative decreased from 0.460 to 0.455 mm between baseline (0–1 month) and 18–30 months, while CIMT increased from 0.470 to 0.505 mm in the group who developed dnDSA at 18–30 months post-transplant. Median CIMT of healthy controls was 0.450 mm (one measurement).

consented to enroll in the study, and 38 of these enrolled patients completed the required study procedures. In total, 38 transplant recipients contributed 60 observations of CIMT between 18 and 30 months post-transplant to the analysis.

Within the transplant group, 18.4% of patients (7/38) had developed strong or very strong dnDSA by 18–30 months post-transplant and were thus classified as DSA+ for purposes of the study. Of the 7 DSA+ patients, 2 patients received pharmacologic treatments for DSA+ acute cellular-mediated rejection prior to their 18 month CIMT imaging, and therefore were excluded from the analysis. One additional patient had weak dnDSA detected at 18 months and was therefore excluded from the analysis. Six observations (4 observations at 18 months and 2 at 30 months post-transplant) of the remaining 5 DSA+ patients who did not receive treatments prior to CIMT measurement were included in the analysis. Of a possible 60 CIMT observations of the 30 DSA- transplant patients at 18 and 30 months post-transplant, 54 observations were included in the analysis. The 6 observations of DSA- participants were missing for the following reasons: 2 patients moved out of the area after 18 months, 2 patients missed the 18 month study visit, and 2 patients missed the 30 month study visit.

In addition, 20 healthy children contributed 20 observations to the study (one CIMT observation per each control participant).

Characteristics of Patients and Controls

The demographics and clinical characteristics of the DSA+, DSA- and healthy control groups are summarized in **Table 1**. Among the transplant patients included in the dnDSA positive study group, antibody profiles included 3 patients with strong Class II, 1 with very strong Class II, and 1 with a combination of very strong Class I and II. There were no differences in the age or race distribution of the DSA+, DSA-, or control groups ($p > 0.05$). Male sex was more prevalent in the DSA- vs. the control group ($p = 0.02$), but did not differ between the DSA + and DSA – groups and also did not differ between the DSA + and control groups ($p > 0.05$). In addition, male gender was not significantly associated with CIMT in the multivariate analysis, as shown in **Table 2**. There were no differences in the proportion of patients with pre-emptive transplant or the mean duration of dialysis prior to transplant between DSA + and DSA- groups ($p > 0.05$). There were no statistical differences in prevalence of hypertension, dyslipidemia, BMI-obesity, abdominal obesity, steroid protocol or in eGFR between DSA+ and DSA- groups at 18 months post-transplant ($p > 0.05$).

CIMT

At 18–30 months post-transplant, DSA+ transplant recipients had median CIMT of 0.505 mm (95% CI 0.454–0.560 mm), compared to 0.455 mm (95% CI 0.440–0.470) in DSA- transplant recipients and 0.450 mm (95% CI 0.436–0.460) in the healthy controls. In order to visualize a temporal relationship between DSA and CIMT, we also examined the CIMT of the DSA+ and DSA- groups at baseline (0–1 months post-transplant), prior to development of DSA. As shown in **Figure 1**, the median CIMT of the group who remained DSA- during the first 30 months post-transplant decreased slightly from 0.460 mm (95% CI 0.430–0.490) to 0.455 mm (95% CI 0.440–0.470) between baseline and 18–30 months post-transplant, while the median of CIMT of the group who became DSA+ increased from 0.47 mm (95% CI 0.440–0.491) at baseline to 0.505 mm (95% CI 0.454–0.560 mm) over the same time period. Due to the longitudinal nature of the data, bivariate statistical comparison was not appropriate. As

these CIMT comparisons are descriptive only in nature and do not account for other potentially confounding factors, unadjusted and adjusted multivariable regression analyses were performed.

ASSOCIATION OF DSA WITH CIMT

18–30 Months Post-transplant, Unadjusted Analysis

Results of the unadjusted GEE regression analysis showed that the presence of DSA and African American race were associated with increased CIMT in transplant recipients. As shown in **Table 2**, DSA positive transplant recipients had 10.2% thicker CIMT compared with DSA negative transplant recipients ($p = 0.03$), and CIMT was 9.4% thicker in the African American transplant recipients compared with non-African Americans ($p = 0.0001$), prior to adjusting for any covariates. The remaining variables, which included BMI-obesity, WHr-obesity, hypertension, dyslipidemia, sex, age, steroid therapy, and eGFR at the time of CIMT measurement were not significantly associated with CIMT in the unadjusted analysis. Duration of dialysis prior to transplant was also not significantly associated with CIMT at 18–30 months post-transplant.

18–30 Months Post-transplant, Adjusted Analysis

After adjusting for potential confounding factors, multivariable GEE analysis showed that the presence of DSA was independently associated with a 7.8% increase in CIMT compared to those transplant recipients without DSA ($p = 0.006$). In addition, African American race was independently associated with a 9.3% increase in CIMT among transplant recipients ($p = 0.0001$). As presented in **Table 3**, the best-fit multivariable model was adjusted for race, abdominal obesity, hypertension, and dyslipidemia. Abdominal obesity was associated with a 3.7% increase in CIMT, with borderline statistical significance ($p = 0.059$).

TABLE 1 | Demographics and clinical characteristics.

	Transplant group		Control group
	DSA+	DSA-	
Age at transplant (mean ± SEM)	12.5 ± 2.9 years	11.8 ± 0.8 years	11.6 ± 0.9 years
African American Race (%)	60%	53.3%	50%
Male (%)	40%	66.6%*	30%*
Duration of dialysis prior to transplant (mean ± SEM)	13.7 ± 5.6 months	23.8±0.4 months	NA
Pre-emptive transplant	40%	30%	NA
Hypertension (%)**	60%	66.6%	0%
Dyslipidemia (%)**	20%	30%	NA
BMI-Obesity (%) * *	50%	36.7%	0%
Abdominal obesity (%)** (WHr≥0.5)	60.0%	53.3%	0%
Steroid protocol (%)‡	60.0%	53.3%	NA
eGFR at 18 months (mean ± SEM)	96.4± 14.8 ml/min/1.73m^2	96.6 ± 5.1 ml/min/1.73 m^2	NA
dnDSA	➢ 60% strong Class II ➢ 20% very strong Class II ➢ 20% very strong Class I and II	No DSA	NA

Categorical variables were compared by Fisher's exact test and continuous variables by Wilcoxon rank-sum. There were no differences in the age or race distribution of the DSA+, DSA-, or control groups ($p > 0.05$). Male sex was more prevalent in the DSA- vs. the control group ($p = 0.02^*$), but did not differ between the DSA + and DSA – groups and also did not differ between the DSA + and control groups ($p > 0.05$). There were no differences in any of the remaining variables between DSA + and DSA- groups ($p > 0.05$).
***Proportion of patients affected at any point during the 18–30 month post-transplant time period.*
‡Indicates proportion of patients who received maintenance steroid therapy.

TABLE 2 | Association of variables with CIMT (unadjusted analysis).

Variable	% change in CIMT[a] (95% CI)	*p*-value
dnDSA	10.2% (3.3 to 17.1%)	0.03*
African American race	9.4% (6.2 to 12.6%)	0.0001*
Hypertension	1.4% (−2.0 to 4.5%)	0.42
Dyslipidemia	−1.1% (−3.7 to 1.4%)	0.17
Obesity by BMI	−3.9% (−7.1 to 1.0%)	0.13
Abdominal Obesity (WHr)	0.23% (−3.1 to 3.5%)	0.89
Dialysis duration	0.08% (−0.03 to 0.20%)	0.16
eGFR	−0.02% (−0.07 to 0.03%)	0.43
Steroid therapy	−1.4% (−5.6 to 2.7%)	0.51
Male sex	−1.0% (−5.7 to 3.6%)	0.66
Age	−0.4% (−3.7 to 2.9%)	0.80

**Significant association with CIMT by GEE linear regression, without adjusting for any covariates.*
[a]Due to non-normal distribution, continuous CIMT data were log-transformed, and therefore results are presented as a % change in CIMT.

TABLE 3 | Association post-transplant dnDSA with CIMT (adjusted analysis).

Variable	% change in CIMT[a] (95% CI)	*p*-value
dnDSA	7.8% (2.2 to 13.4%)	0.006*
African American race	9.3% (5.5 to 13.1%)	0.0001*
Abdominal Obesity (WHr)	3.7% (0.0 to 7.5%)	0.059
Hypertension	0.70% (−2.8 to 4.2%)	0.69
Dyslipidemia	−1.6% (−5.2 to 2.0%)	0.38

**Significant association with CIMT by GEE linear regression, adjusted for race, hypertension, dyslipidemia, and abdominal obesity by WHr ($p < 0.05$).*
[a]Due to non-normal distribution, continuous CIMT data were log-transformed, and therefore results are presented as a % change in CIMT.

Temporal Validation of Relationship of DSA and CIMT, Adjusted Analysis

Multivariable linear regression was also performed to establish a timeline of the relationship between DSA status and CIMT among the transplant recipients. Results of this analysis showed that at baseline, prior to development of any DSA, neither group of transplant recipients had increased CIMT (coefficient −0.7%, 95% CI −6.3 – 4.0%, $p = 0.81$). As in the 18–30 month analysis, this linear regression model was adjusted for race, abdominal obesity, hypertension, and dyslipidemia. The results of this analysis demonstrate that association of increase in CIMT with DSA+ patients did in fact develop after the onset of dnDSA, and not before.

DISCUSSION

This pilot study is the first to demonstrate that development of dnDSA is associated with an indicator of arteriosclerosis, evidenced by increased CIMT, in a cohort of DSA positive pediatric kidney transplant recipients. Transplant patients with dnDSA exhibited thicker CIMT in comparison to transplant recipients without dnDSA at 18–30 months post-transplant, while CIMT of DSA- transplant recipients was similar to that of healthy controls. The association between dnDSA and CIMT was independent of traditional CV risk factors, including hypertension, dyslipidemia, and abdominal obesity. Our data also showed a temporal relationship between development of dnDSA and increased CIMT, as the association was not present at the time of transplant, prior to development of dnDSA.

Antibody-associated arteriosclerosis was previously described by Loupy et al. in a population of adult kidney transplant recipients (5). This study showed that presence of circulating dnDSA was associated with arterial inflammation and complement fraction C4d deposition resulting in intimal thickening of the arteries, and higher levels of DSA correlated with greater severity of arteriosclerosis (5).

Our findings are also supported by studies of dnDSA-associated vasculopathy in adult and pediatric heart transplant recipients (6, 7), which showed that circulating dnDSA were associated with increased risk of coronary artery vasculopathy, allograft failure, and mortality. The mechanism of action for accelerated antibody-associated arteriosclerosis has been previously demonstrated in animal models, as direct administration of anti-HLA antibodies to vascular endothelium induces release of inflammatory cytokines and complement activation, resulting in intimal hyperplasia and vasculopathy (16, 17).

The study by Loupy et al. also showed that adults with antibody-associated arteriosclerosis had 2.5 and 4.1 fold increased risk of major adverse CV events compared to those with non-antibody associated arteriosclerosis and those without significant arteriosclerosis, respectively, independent of other CV risk factors. The authors hypothesized that complement activation and release of inflammatory agents triggered by circulating dnDSA trigger not only injury localized to the allograft, but also ignite a generalized systemic process of arteriosclerosis, similar to that seen in other autoimmune diseases (5).

As major adverse CV events are not common in children, we used CIMT, a well-validated indicator for the early detection of CV disease, to identify arteriosclerosis in our study population. It was interesting to note that at the time of transplant, all transplant recipients had similar CIMT. In the group that went on to develop dnDSA, CIMT increased from 0.470 mm at baseline to 0.505 mm at 18–30 months post-transplant, while the DSA negative group had a slight decrease in CIMT from 0.460 to 0.455 mm (similar to the median CIMT of healthy controls, 0.450 mm) over the same time period (**Figure 1**). Previous longitudinal cohort studies of CIMT in children with ESRD suggest that CIMT would be expected to remain stable or slightly improve over time after kidney transplantation (18, 19). The results of our study suggest that the DSA negative transplant recipients follow the expected pattern, exhibiting stable or slightly improved CIMT, achieving a median CIMT matching that of race-matched healthy controls by 18–30 months post-transplant, while children who develop dnDSA exhibit an increasing trend in CIMT over time post-transplant.

Our results indicate that CIMT of DSA+ transplant recipients is approximately 0.05 mm higher than those of DSA- patients and healthy controls. Due to the rarity of major CV events in children, the exact magnitude of increase in CIMT that corresponds to increase in CV risk is not known. However, literature indicates that in adults, each absolute increase in cIMT by 0.1 mm corresponds to an increase in risk of stroke by 13–18% and an increase in risk of myocardial infarction by 10–15% (20). Therefore, based on this data combined with meta-analyses of pediatric studies of CIMT, an absolute increase in of 0.05 mm in CIMT a child is expected to be clinically significant in terms of portending future CV risk (21). The lack of significant association between CIMT with hypertension, dyslipidemia, and BMI that was observed in our study is in agreement with that of other pediatric solid organ transplant populations, as reported in a recent meta-analysis (21). Abdominal obesity as measured by WHr demonstrated a stronger magnitude of association with increased CIMT among our transplant cohort than did BMI, with borderline statistical significance ($p = 0.059$). Prior studies suggest that WHr is likely a more sensitive indicator of CV risk than BMI in children with kidney disease (22), but further research is needed to investigate its relationship to CIMT.

Strengths of our study included a prospective study design with a healthy control group that was matched by race to the transplant recipients. Matched race distribution between the study and control groups is important due to the known association of increased CIMT in African American children compared to other races (23). This was a pilot study, and as such was limited by small sample size, particularly in the DSA+ group. Our study was sufficiently powered to detect the association between DSA and CIMT, however larger multicenter studies would be needed to investigate more granular associations such as the type and strength of anti-HLA antibodies with severity

of arteriosclerosis. In addition, although we demonstrated a temporal relationship between DSA development and increased CIMT, we cannot prove causality in the absence of histologic evidence. Our study design did incorporate several measures to isolate the possible effects of dnDSA on CIMT from those of the treatments given to patients with dnDSA. We excluded CIMT measurements from DSA+ patients who had already received therapeutic interventions for DSA with concomitant acute cell mediated rejection. In addition, our statistical analysis controlled for possible side effects of maintenance steroid therapy, including obesity, dyslipidemia and hypertension during the first 30 months post-transplant. Larger studies of longer duration post-transplant would be needed to examine possible long-term effects of dnDSA on cardiovascular risk profile in patients with persistent DSA.

In summary, dnDSA was found to be associated with increased CIMT in a cohort of pediatric kidney transplant recipients. The novel finding identified in our pilot study should be followed up by larger studies to verify this association and to elucidate the mechanism, effects, and possible therapies to treat antibody-associated arteriosclerosis in kidney transplant recipients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors (KS, SC, AM) contributed to the conception, design, data collection and analysis, as well as writing and revision of the manuscript for this study. In addition, AM, pediatric nephrologist and Director of Kidney Transplantation, was the PI of the study and oversaw all of the research and regulatory aspects of the study. Statistical analysis was performed by KS, and analysis of CIMT was performed by pediatric cardiologist, SC.

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The Incidence of Paediatric Acute Kidney Injury Identified Using an AKI E-Alert Algorithm in Six English Hospitals

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Objective: Acute kidney injury (AKI) is a significant cause of morbidity and mortality among hospitalised patients. The objectives in this study were (i) to investigate the incidence of AKI using the National Health Services (NHS) AKI e-alert algorithm as a means of identifying AKI; and (ii) in a randomly selected sub-group of children with AKI identified using the algorithm, to evaluate the recognition and management of AKI.

Patients and Methods: Retrospective cross-sectional study with initial electronic retrieval of creatinine measurements at six hospitals in England over a six-month period. Results were evaluated using the NHS AKI e-alert algorithm with recognition and management of AKI stages 1, 2 and 3 reviewed in a sub-set of randomly selected patient case notes. Patients aged 29 to 17 years were included. AKI stage 1 was defined as a rise of 1.5 – ≤2x baseline creatinine level; AKI stage 2 a rise of ≤ 2.0 and < 3.0; AKI stage 3 a rise of ≥ 3.0. Urine output was not considered for AKI staging.

Results: 57,278 creatinine measurements were analysed. 5,325 (10.8%) AKI alerts were noted in 1,112 patients with AKI 1 (62%), AKI 2 (16%) and AKI 3 (22%). There were 222 (20%) <1y, 432 (39%) 1 ≤ 6y, 192 (17%) 6 ≤ 11y, 207 (19%) 11 ≤ 16y, and 59 (5%) 16–17y. Case notes of 123 of 1,112 [11.1%] children with AKI alerts were reviewed. Confirmed AKI was recognised with a documented management plan following its identification in $n = 32$ [26%] patients only.

Conclusions: In this first multicentre study of the incidence of AKI in children admitted to selected hospitals across England, the incidence of AKI was 10.8% with most patients under the age of 6 years and with AKI stage 1. Recognition and management of AKI was seen in just over 25% children. These data highlight the need to improve recognition of AKI in hospitalised children in the UK.

Keywords: acute kidney injury, hospital, epidemiology, alerts, algorithm

INTRODUCTION

Acute kidney injury (AKI) is characterised by a reversible loss of normal kidney function and is recognised by a reduction in urine output and/or an increase in serum creatinine, indicative of a reduction in glomerular filtration rate (GFR). It is also usually accompanied by an inability of the kidneys to maintain water, acid-base and electrolyte balance (1, 2). The definition of AKI has undergone several iterations (3) and to define and stratify the severity of AKI several classification systems have been proposed and include the RIFLE, AKIN, and KDIGO criteria (4). These consensus definitions are to be welcomed as they help to standardise the diagnosis of AKI, stratify AKI severity and allow the development of predictors of outcomes based on the AKI staging. In children AKI is staged using broadly similar criteria to adults modified as pRIFLE (1).

In adults, AKI is commonly seen in hospitalised patients and associated with increased morbidity and mortality, with worse outcomes in the sickest patients and those with co-morbidities (5–8). Over the past decade several studies have described AKI in hospitalised children and although the incidence is lower, children also have increased morbidity and mortality associated with AKI (9). Recently, Sutherland et al., described rates of AKI in hospitalised children across North America and observed 3.9 episodes of AKI per 1,000 admissions (10). Similar data have been reported from other parts of the world (11, 12).

In those admitted to paediatric ICU, recent data from the AWARE study highlights that AKI is common and is associated with poor outcome including increased mortality (13). Early detection of AKI and implementation of strategies to minimise progression is therefore essential for acute management, but is also likely to improve long term outcomes, particularly in those with the most severe illness (e.g., those admitted to PICU, those undergoing cardiac surgery or those with prematurity), although further data are awaited to prove this (14, 15).

In England, a national algorithm, standardising the definition of AKI was agreed and provides the ability to ensure that a timely and consistent approach to the detection and diagnosis of patients with AKI across the National Health Service (NHS) (16), termed the "NHS AKI e-alert algorithm" in the manuscript [online **Supplement**]. Following integration of the algorithm into the Laboratory Information Management Systems the algorithm will identify potential cases of AKI from laboratory data in real time and produce a test result. The laboratory system will then send the test result, using existing IT connections to patient management systems in use in hospitals in England. Full implementation across all NHS England sites was intended by 9th March 2015 but has taken longer and this is often due to individual local hospital issues. Nonetheless, studies in adults have shown the implementation of AKI alerts and using an AKI care bundle improved recognition and management of acute kidney injury in hospitalised patients (17, 18).

The objectives of this study were (i) to investigate the incidence of AKI using the NHS AKI e-alert algorithm as a means of identifying AKI; and (ii) in a randomly selected sub-group of children identified using the algorithm, to evaluate the recognition and management of AKI.

METHODS

This project was a retrospective cross-sectional study including six centres across England including three district general hospitals [Leicester Royal Infirmary hospital (Centre #1–centre sees both secondary and tertiary care patients), North Middlesex Hospital (Centre #2) and Worcestershire Acute Hospitals (Centre #3)] and three tertiary children's hospitals [Bristol Royal Hospital for Children (Centre #4), Evelina London Children's Hospital (Centre #5) and Royal Manchester Children's Hospital (Centre #6)]. The participating hospitals in this study are centres geographically placed across England. They are also representative for level of care, including both secondary and tertiary level hospital-based acute services for children in England. The tertiary level participating centres provide specialist paediatric nephrology services including management of children with chronic kidney disease, dialysis, and transplantation [Centres #4, #5, and #6]. This multi-centre project was supported by the membership of the British Association for Paediatric Nephrology and its Acute Kidney Injury Interest Group.

The study was designed in two parts; in the first part of the study, all plasma creatinine measurements performed over a 6 month study period between 01/07/2012 to 31/12/2012 were electronically retrieved and compared to any creatinine measurements that may have been performed over the previous year, allowing staging of AKI using the NHS AKI e-alert algorithm (16). Patients aged 29 days to 16 years 364 days were included in the study. Plasma creatinine was measured using the enzymatic method.

AKI stage 1 was defined as a rise of 1.5 – $<$ 2x baseline creatinine level; AKI stage 2 a rise of $\geq$ 2.0 and $<$ 3.0; AKI stage 3 a rise of $\geq$ 3.0. The baseline creatinine was defined as the lowest creatinine value in the previous 12 months (if available) or the upper limit of the reference value for the age (19).

The lead centre (Centre #5) requested electronic retrieval of all creatinine measurements performed at participating centres during the study period. In addition to the plasma creatinine measurements a brief dataset associated with each measurement was included: age, gender, date and time of creatinine measurement, stage of AKI and number of AKI alerts. All centres sent data electronically to the lead centre and data analysed by investigators.

In the second part of the study, a subset of children identified to have AKI were randomly selected for case note review from 5 of 6 centres. Study investigators visited participating centres to collect data from paper and electronic case notes. Notes from Centre #2 were unavailable for review.

Data collected included patient demographics, documented evidence of AKI recognition by the clinical team, the stage of AKI, if appropriate investigations and management were carried out, and if a specialist nephrology opinion was sought and outpatient follow up arranged. This was a retrospective analysis evaluating the incidence of AKI from results of clinically indicated investigations and therefore no

consent from patients and ethical approval was required. There was no direct patient or public involvement in this study.

STATISTICAL ANALYSES

Summary statistics are presented as means for continuous data and median and inter-quartile range (IQR) for non-normally distributed data. Comparisons between the two groups in this study were tested as appropriate using the independent samples *t*-test for continuous data, Mann–Whitney *U*-test for non-normally distributed data and the Chi–squared test for categorical data. Statistical analyses were performed using SPSS version 22 (SPSS Inc., Chicago, Illinois). All tests were two-tailed and a $p < 0.05$ was taken to represent a statistically significant result.

TABLE 1 | Number of creatinine measurements per centre analysed over a 6 month period across six participating centres*, including $n = 57,278$ measurements.

Centre	Number of measurements
#1	14,029 (24.5%)
#2	1,775 (3.1%)
#3	965 (1.7%)
#4	7,900 (13.8%)
#5	12,095 (21.1%)
#6	20,514 (35.8%)

Centre #1, Leicester Royal Infirmary hospital; Centre #2, North Middlesex Hospital; Centre #3, Worcestershire Acute Hospitals; Centre #4, Bristol Royal Hospital for Children; Centre #5, Evelina London Children's Hospital; Centre #6, Royal Manchester Children's Hospital.

RESULTS

57278 creatinine measurements over a 6 month period were retrieved from the six centres; measurements from individual centres are shown in **Table 1**. Of these 75.2% measurements [$n = 43,080$] had a baseline creatinine measurement over the previous year to compare with and 24.8% [$n = 14,198$] creatinine measurements had no previous baseline measurements. There were 5,325 [10.8%] creatinine values resulting an AKI alert in 1,112 patients using the NHS AKI e-alert algorithm. The incidence of AKI was higher when restricted to those in whom a previous creatinine measurement 12.4% [5325/43080] was available. Of these AKI alerts, 33,24 (62%) were AKI stage 1, 843 (16%) were AKI stage 2 and 1,158 (22%) were AKI stage 3 alerts. In those with no previous baseline measurement, 7.7% [1088/14198] had creatinine measurement above the upper limit of the reference value for their age and this was significantly lower than those in whom a baseline creatinine was available and who had an AKI alert [$p < 0.0001$]. The age distribution of children with AKI alerts was 222 (20%) <1 year, 432 (39%) 1 ≤ 6 years, 192 (17%) 6 ≤ 11 years, 207 (19%) 11 ≤ 16 years, and 59 (5%) 16–17 years. Their distribution by percentage in each age band by worst stage of AKI is shown in **Figure 1**. AKI stage 1 was the largest group across all ages and 59% of all AKI alerts occurred in those aged <6 years. In those patients with AKI, there were 176, 20, 11, 251, 294, and 360 children with AKI in Centres #1, #2, #3, #4, #5, and #6, respectively, and a statistically significant difference in the incidence of AKI between the centres [$p < 0.0001$]. The incidence of AKI in 1,112 patients shown by percentage of children in each centre by worst stage of AKI is shown in **Figure 2**.

In the second part of the study, 123 of 1,112 (11.1%) patients with AKI alerts were randomly selected for case notes review across 5 of the 6 centres (**Table 2**). There were 68 males (55.2%).

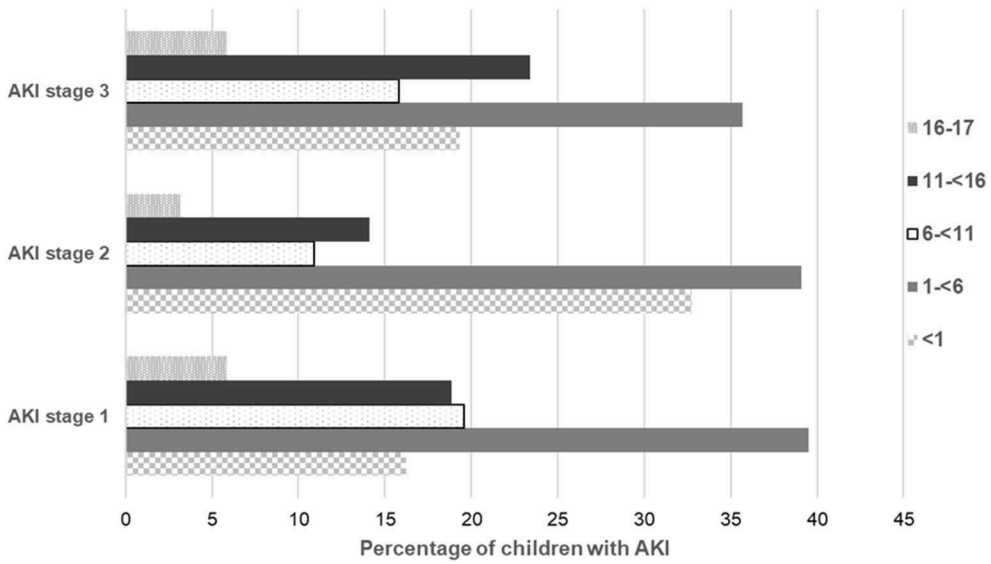

FIGURE 1 | Distribution of $n = 1,112$ children with AKI alerts shown as percentage of children in each age band by worst stage of AKI. Data by worst stage of AKI as indicated by maximal change in creatinine level from baseline using the National Health Service (NHS) AKI e-alert algorithm (16).

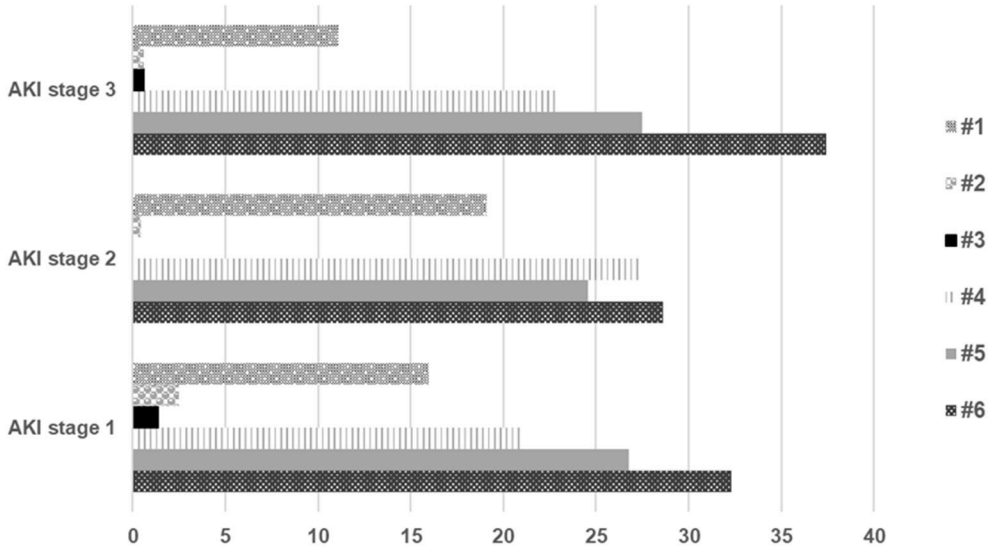

FIGURE 2 | Incidence of AKI alerts in $n = 1{,}112$ patients shown by percentage of children in each centre* by worst stage of AKI. Data by worst stage of AKI as indicated by maximal change in creatinine level from baseline using the National Health Service (NHS) AKI e-alert algorithm (16). *Centre #1, Leicester Royal Infirmary hospital; Centre #2, North Middlesex Hospital; Centre #3, Worcestershire Acute Hospitals; Centre #4, Bristol Royal Hospital for Children; Centre #5, Evelina London Children's Hospital; Centre #6, Royal Manchester Children's Hospital.

TABLE 2 | Clinical characteristics of randomly selected patients with AKI from five centres* identified retrospectively using National Health Service (NHS) AKI e-alert algorithm.

Centres	1	3	4	5	6
Number of patients, *n* (%)	29 (23.5%)	11 (8.9%)	28 (22.8%)	25 (20.3%)	30 (24.3%)
Age in years [median (IQR)], $n = 95$	2.0 (0.5, 3.3)	7.5 (0.6, 12.7)	No data available	2.3 (0.4, 6.6)	3.9 (1.8, 6.4)
Male, *n* (%)	15 (51.8%)	6 (54.6%)	14 (50%)	16 (64%)	17 (56.7%)
Creatinine in μmol/L at presentation [median (IQR)]	45 (36, 61)	46 (36, 85)	71 (51, 141)	44 (33, 86)	27 (21, 46)
Correctly identifed AKI, *n* (%)	3 (10.3%)	1 (9%)	11 (39%)	11 (44%)	6 (20%)
Management plan accounting for AKI					
Yes	4 (13.7%)	2 (18%)	13 (46.4%)	7 (28%)	6 (20%)
No	24	8	13	18	24
No information	1	1	2	0	0

**Centre #1, Leicester Royal Infirmary hospital; Centre #3, Worcestershire Acute Hospitals; Centre #4, Bristol Royal Hospital for Children; Centre #5, Evelina London Children's Hospital; Centre #6, Royal Manchester Children's Hospital.*

The median [IQR] age was 3.1 years (0.5, 6.6). AKI was correctly identified and a management plan following its identification was documented in 32 (26%) of patients. In 87 (71%) patients, AKI was not identified with no clinical notes highlighting this issue or no management plan for AKI; and for 4 (3%) patients there was no information available.

Data for patients in whom AKI was recognised ($n = 32$; AKI-R) was compared to those in whom AKI was not recognised ($n = 87$; AKI-NR) and their management plan following recognition reviewed (**Table 3**).

Median [IQR] age in years was the same in AKI-R and AKI-NR [3.1 (0.7, 6.4) vs. 3.1 (0.5,7.2), $p = 0.56$]. Boys were affected more in both groups AKI-R (59.3%) vs. AKI-NR (53.8%). Median [IQR] creatinine (μmol/L) was significantly higher in the AKI-R group compared to the AKI-NR group [103 (61,239) vs. 39 (29,57), $p < 0.0001$]. The largest number of AKI cases that were not recognised were admitted in General Paediatrics, followed by Cardiology, Intensive Care, and Oncology.

In terms of management of AKI, the risk of possible drug nephrotoxicity was considered in 59% in AKI-R group compared to 1% in AKI-NR group. A specialist nephrology opinion was sought in 37% vs. 1% in the AKI-R vs. AKI-NR group. 90% of the patients in AKI-R group were followed up in clinic compared to 55% in AKI-NR group (**Table 3**). Estimated glomerular filtration rate (eGFR) was rarely measured by non-renal specialties. At the time of discharge, serum creatinine had returned to baseline levels in 78% of patients. There was 1 death (3%) in the AKI- R group and none in the AKI-NR group.

TABLE 3 | Results of case note review for 119 of 123 patients for clinical management of recognised vs. unrecognised AKI during inpatient episode.

Demographics	AKI-Recognised	AKI-Not recognised
Number of patients ($n = 123$)^, *n* (%)	32 (26%)	87 (71%)
Age in years [median (IQR)]	3.1 (0.7,6.4)	3.1 (0.5,7.2)
Boys, *n* (%)	19 (59.3%)	49 (53.8%)
Creatinine in μmol/L at presentation [median (IQR)]^^	103 (61,239)	39 (29,57)
Patients admitted under		
Total	32	87
General Paediatrics	3	29
Cardiology	2	17
Neurology	0	2
Nephrology	13	1
Neonatology	2	1
Intensive care and ECMO	6	8
General surgery	0	2
Oncology	0	6
Specialty not known	6	21
Management		
Daily weight measurements *n* (%)	22 (69%)	13 (14%)
Twice daily weights, *n* (%)	4 (12%)	2 (2%)
Blood pressure measured, *n* (%)	31 (97%)	62 (68%)
Fluid balance, *n* (%)	29 (91%)	39 (43%)
Repeat creatinine measured, *n* (%)	31 (97%)	57 (63%)
Urine dipstick performed, *n* (%)	16 (50%)	13 (14%)
Renal Ultrasound scan performed, *n* (%)	15 (47%)	4 (4%)
Estimated GFR measured, *n* (%)	9 (28%)	1 (1%)
Role of drug related nephrotoxicity and AKI considered, *n* (%)	19 (59%)	1 (1%)
Paediatric Nephrology advice considered, *n* (%)	12 (37%)	1 (1%)
Follow up		
*OPD clinic review, *n* (%)	28 (90%)	50 (55%)
**GP review, *n* (%)	0	1 (1%)
Creatinine returned to baseline levels, *n* (%)		
Yes	25 (78%)	49 (54%)
No	5 (16%)	11 (12%)
Not re-measured	0	18 (20%)
No information	1 (3%)	13 (14%)
Died	1 (3%)	0

All patients identified retrospectively using National Health Service (NHS) AKI e-alert algorithm.

*^no, information available for 4 patients; ^^P < 0.0001; *OPD, outpatient department; **GP, general practitioner.*

DISCUSSION

This is the first multicentre study looking at the incidence and management of AKI in hospitalised children in selected English hospitals using a creatinine-based algorithm and includes a large data set of children over a 6 month period from both district general and teaching hospitals.

The number of studies of AKI in paediatric population are much less than in the adult population (20) and usually include small numbers of patients. Most of the paediatric studies are either single centre or, if multicentre, focused on high risk groups such as children in PICU whereas this study looked at the incidence of AKI among all hospitalised patients.

In this study here, the overall incidence of AKI was 10.8% which was similar to other studies (12, 21). This was less than the incidence reported in meta-analysis including studies across the world, that reported a pooled incidence of AKI in children of 33.7% (20). However, on inspecting the paediatric studies in the meta-analysis, 67% of the studies included patients in critical care or post cardiac surgery, and study numbers were generally small, therefore their results cannot be considered to represent the true incidence of AKI in all childhood admissions worldwide.

AKI stage 1 was the commonest stage across all ages in this study. The incidence of AKI was higher in the younger age group (∼60% of AKI was in children under 6 years of age) which is similar to other studies (11, 21) but contrary to a large epidemiological study by Sutherland et al. where the highest incidence of AKI was seen in 15–18 year olds (10). However, Sutherland et al. used ICD-9-CM codes (International Classification of Diseases, Ninth Revision, Clinical Modification) applied at discharge rather than the creatinine-based criteria of AKIN or pRIFLE criteria for diagnosing AKI (10). Younger children tend to have lower serum creatinine values and therefore a small absolute change in the creatinine value could lead to identification of AKI using the AKIN or pRIFLE criteria but not with the ICD-9-CM coding system (10).

In this study reported here, the pRIFLE or AKIN criteria were not used by clinicians for routinely diagnosing AKI and so patients with a rise in creatinine from baseline but whose creatinine was still within the laboratory specified normal range were less likely to be recognised to have AKI. This includes the group of children who would be at risk of AKI (pRIFLE "R") and in whom appropriate intervention could be anticipated to prevent progression. It is important to note that in the sub-set of randomly selected patients who had case note review, 74% of patients with AKI were not recognised. Even in the group of children where AKI had been recognised (AKI-R), appropriate management was not implemented, highlighting the need for education and training among the medical staff.

The diagnosis of AKI is usually made by identifying increased serum creatinine with or without associated reduced urine output (17). Studies have shown that using a care bundle can improve the recognition of AKI which can lead to early initiation of treatment to reduce progression of AKI and so reduce morbidity and mortality (17, 18). The AKI alert algorithm was introduced across NHS England in 2015 to facilitate early diagnosis. In this study the data set was from a period before the introduction of the algorithm; further studies will be needed to examine if the early identification of AKI as a result of implementation of the NHS algorithm has led to improved recognition and management of AKI with a reduction in AKI related morbidity and mortality.

There are some important limitations to our study findings that need to be considered and include (i) Inherent biases related to the retrospective nature of such data; (ii) electronic alerts of AKI were based on serum creatinine values only and do not have any information regarding GFR or urine output. We recognise that in clinical practice and in widely established AKI criteria, urine output is integrated to facilitate early diagnosis and lack of these data are therefore likely to underestimate AKI, however we wished to examine the application of the NHS AKI e-alert algorithm in routine clinical practice; (iii) Although the algorithm is helpful for identifying AKI, we recognise in clinical practice use of creatinine alone may be misleading particularly in younger children who have physiologically low creatinine values; similar issues are likely to complicate evaluation in children with chronic illness and malnutrition; (iv) only one definition of AKI stage 3 was used. We recognise that sometimes an increase in serum creatinine may be $<3\times$ the baseline but the eGFR for the patient much worse and clinically significant; (v) In those patients in whom no creatinine values were available to compare an increase from a previous baseline, we used the upper limit of reference range as baseline creatinine. In those with a single high value we have not reported this as AKI here as per the algorithm and this may underestimate the true incidence of AKI in our cohort. Previous studies (22–24), have shown increased sensitivity in detecting AKI when using the lower reference limit or using back calculation to estimate the baseline creatinine from the Schwartz formula and defining baseline GFR as 120 ml/min/1.73m^2. Importantly, these approaches may categorise those with CKD as AKI but also overestimate the true incidence of AKI; furthermore back calculation requires the patient height. Despite this as shown in this study, there remain significant shortcomings in the recognition of AKI by non-renal physicians, with poor recording of urine output and/or of eGFR for hospitalised children receiving clinical care. A creatinine based algorithm embedded in hospital reporting systems is likely to be more effective in identifying AKI.

CONCLUSION

The incidence of AKI in this multicentre study was 10.8% with most patients under the age of 6 years and AKI stage 1 was the predominant stage across all ages. There remains an ongoing need for education and training of health professionals to improve recognition and management of patients with AKI. Timely recognition and optimal management of AKI is important to improve long term renal outcomes. Future studies will aim to determine the impact of the NHS AKI e-alert algorithm in the UK.

DATA AVAILABILITY STATEMENT

All relevant data is contained within the article.

ETHICS STATEMENT

The study was registered with the corresponding authors institution as a multi-centre audit. This was a review of historical hospital results/records. No ethical approval was required following audit approval. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SB: data collection, manuscript writing, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. MS and DM: project leads, designing the project, data collection, analysis, manuscript writing, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JS, NM, HM, PH, AH, CS, WH, and RL: data collection from individual centres and approval of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00029/full#supplementary-material

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ORIGINAL RESEARCH
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Positive Response to One-Year Treatment With Burosumab in Pediatric Patients With X-Linked Hypophosphatemia

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X-linked hypophosphatemia (XLH) causes significant burden in pediatric patients in spite of maintained treatment with phosphate supplements and vitamin D derivatives. Administration of burosumab has shown promising results in clinical trial but studies assessing its effect in the everyday practice are missing. With this aim, we analyzed the response to one-year treatment with burosumab, injected subcutaneously at 0.8 mg/kg every 2 weeks, in five children (three females) aged from 6 to 16 years, with genetically confirmed XLH. Patients were being treated with phosphate and vitamin D analogs until the beginning of burosumab treatment. In all children, burosumab administration led to normalization of serum phosphate in association with marked increase of tubular reabsorption of phosphate and reduction of elevated serum alkaline phosphatase levels. Baseline height of patients, from −3.56 to −0.46 SD, increased in the three prepubertal children (+0.84, +0.89, and +0.16 SD) during burosumab treatment. Growth improvement was associated with reduction in body mass index (−1.75, −1.47, and −0.17 SD, respectively), suggesting a salutary effect of burosumab on physical activity and body composition. Burosumab was well-tolerated, mild local pain at the injection site and transient and mild headache following the initial doses of burosumab being the only reported undesirable side effects. No patient exhibited hyperphosphatemia, progression of nephrocalcinosis, worsening of metabolic control or developed hyperparathyroidism. Mild elevation of serum PTH present at the beginning of treatment in one patient 4 was not modified by burosumab administration. These results indicate that in the clinical setting, beyond the strict conditions and follow-up of clinical trials, burosumab treatment for 1 year exerts positive effects in pediatric patients with XLH without major adverse events.

Keywords: X-linked hypophosphatemia (XLH), rickets, burosumab, FGF23, hypophosphatemia, vitamin D, children

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INTRODUCTION

X-linked hypophosphatemic rickets (XLH) is a monogenic disorder that follows a Mendelian dominant inheritance (OMIM 307800) and it is the most common hereditary form of rickets (1).

XLH is caused by loss of function of the gene *PHEX* (2), a phosphate regulating gene with homologies to endopeptidases, leading to elevated circulating levels of fibroblast growth factor

23 (FGF23), renal wasting of phosphate and defective renal production of 1,25-dihydroxyvitamin D (3).

Although XLH is characterized by a broad phenotypical expression, active lesions of rickets, subsequent bone deformities and disharmonic growth retardation are major manifestations of the disease in the pediatric age. Dental abscesses and mineralization defects in teeth are also common findings (1).

Classical medical treatment of XLH is based on the oral administration of phosphate supplements and 1-hydroxy metabolites of vitamin D, given chronically, at least until the end of body growth (4). This therapy usually heals the active radiological lesions of rickets and improves growth but does not normalize serum phosphate, does not avoid major clinical manifestations of the disease, does not result in the achievement of a normal final adult height and entails the risk of potential undesirable side effects such as nephrocalcinosis or hyperparathyroidism (5, 6).

The development of burosumab, a humanized monoclonal antibody for FGF23 is a promising treatment in patients with XLH (7). Preliminary clinical trials indicate that burosumab improves renal tubular phosphate reabsorption, serum phosphorus levels, linear growth, and physical function and reduces pain and the severity of rickets in children with XLH (8, 9). However, very limited data on pediatric patients with XLH treated with burosumab are available. Thus, to know the efficacy and safety of burosumab treatment in the everyday clinical setting we here present five children with genetically confirmed XLH receiving the drug for more than a year.

PATIENTS AND METHODS

Patients younger than 18 years of age with XLH genetically confirmed and on treatment with burosumab for more than a year were identified in three Spanish hospitals. All patients received burosumab subcutaneously at a dose of 0.8 mg/kg every 2 weeks, after an initial dose of 0.4 mg/kg. Phosphate supplements and 1-hydroxy derivatives of vitamin D administration were withdrawn 2 weeks before the beginning of burosumab.

After informed consent, the medical records of these patients were reviewed. The following data were collected from the basal visit, immediately before starting burosumab, and, when available, from each of the outpatient clinic visits during the period of burosumab treatment: height, weight, and clinical manifestations including adverse effects potentially related with the administration of burosumab; fasting serum concentrations of phosphate, alkaline phosphatase, calcium, 1,25-dihydroxyvitamin D and intact parathyroid hormone (PTH); urine calcium/creatinine ratio, tubular phosphate reabsorption (TPR) and tubular maximum reabsorption of phosphate for glomerular filtration rate (TmP/GFR) were calculated. Radiological findings as well as the presence of nephrocalcinosis or dental impairment were also collected.

Somatometric measurements, including height, weight, and body mass index (BMI), were expressed in absolute values and as standard deviation (SD) score of Spanish reference growth charts for age and sex. The profile of the several biochemical variables measured was drawn using absolute values except for alkaline phosphatase whose values were expressed as percentage of the initial one because of the wide dispersion of the results in the different hospitals.

The protocol was approved by *Comité de Ética de la Investigación con Medicamentos del Principado de Asturias (SP no 38/18).* All subjects gave written informed consent in accordance with the Declaration of Helsinki.

RESULTS

Table 1 shows baseline characteristics of the patients before starting burosumab treatment. Hypophosphatemia associated to urinary wasting of phosphate, relatively low values of 1,25-dihydroxyvitamin D and elevated alkaline phosphatase levels all support the diagnosis of XLH, confirmed by the mutation found in the *PHEX* gene. Serum concentrations of calcium and 25-hydroxyvitamin D were normal (not shown). Serum intact PTH levels were also normal except in patient 4 who had a mild elevation (upper normal reference value 65 pg/mL).

Response to burosumab assessed by the biochemical profiles of serum phosphate, serum alkaline phosphatase and renal reabsorption of phosphate is graphically shown in **Figure 1**. The dose of burosumab did not need to be modified and was kept at 0.8 mg/kg every 2 weeks as originally planned. Serum phosphate normalized in all patients, in patient 1 after a transient worsening of hypophosphatemia immediately following the first burosumab dose. The normalization of serum phosphate was associated to greater renal phosphate reabsorption as shown by increases in TRP and TmP/GFR.

Growth of patients is shown in **Table 2**. Height improved in three out of the five children and remained unchanged in two who were pubertal females of 13 and 16 years of age. Weight and BMI decreased, particularly in those children who exhibited greater increase in height SDS, or remained unchanged.

As for the unsuitable effects of burosumab, no patient developed new radiological lesions, nephrocalcinosis or dental problems during the period of burosumab treatment. The patients had been receiving conventional treatment of XLH and did not have active radiological lesions of rickets. As for nephrocalcinosis, it was already present in one patient before starting treatment with burosumab and the kidney ultrasounds did not worsen during burosumab treatment. No patient developed new findings of nephrocalcinosis after 1 year of burosumab treatment. Likewise, no adverse effects potentially related with burosumab were reported except for mild local pain at the injection site (1 patient) and transient and mild headache following the initial doses of burosumab (1 patient). Patient 4 had elevation of PTH at the beginning of treatment. None of the four remaining patients developed hyperparathyroidism, serum PTH concentrations increasing in all of them but within the normal range.

DISCUSSION

This manuscript supports the efficacy and safety of burosumab administered to pediatric patients with XLH in the everyday clinical setting, beyond the strict monitoring conditions of clinical trials (8, 9). This is important because, as stated in a recently published evidence-based guideline for the

TABLE 1 | Clinical, genetic, and biochemical findings in five children with X-linked hypophosphatemia at the moment of starting treatment with burosumab, after withdrawal of administration of vitamin D derivatives and phosphate supplements at least 2 weeks before.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (years)	7	6	16	13	13
Sex	Female	Male	Female	Male	Female
PHEX gene mutation and zigosity	c.871C>T; p.Arg291Ter Heterozygous	c.670C>T; p.Gln224Ter Hemizygous	c. 1936_?del; p.Asp646_?del Heterozygous	c.1079+1G>A; splicing Hemizygous	c.1735G>A; p.Gly579Arg Heterozygous
Serum phosphate (mg/dL)	2.2	2.6	1.7	2.3	1.7
Serum AP (U/L)	504	425	516	1093	605
Serum 1,25$(OH)_2$D (pg/mL)	15.9	36.6	22.0	13.4	23.0
Serum PTH (pg/mL)	34	19	22	83	40
TRP (%)	61	54	32	85	80
TmP/GFR (mg/dL)	0.90	1.40	0.54	1.99	1.36
Height in cm (SD)	119.9 (−0.53)	105.0 (−2.4)	143.5 (−2.89)	129.7 (−3.56)	154.2 (−0.46)
Skeletal findings	Leg bowing Hyperlordosis	Leg bowing	Leg bowing	Leg bowing Scaphocephaly	Leg bowing
Dental abnormalities	No	No	No	Dental abscesses	Dental abscesses

AP, alkaline phosphatase; PTH, parathyroid hormone; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; TRP, tubular reabsorption of phosphate; TmP/GFR, tubular maximum reabsorption of phosphate for glomerular filtration rate.

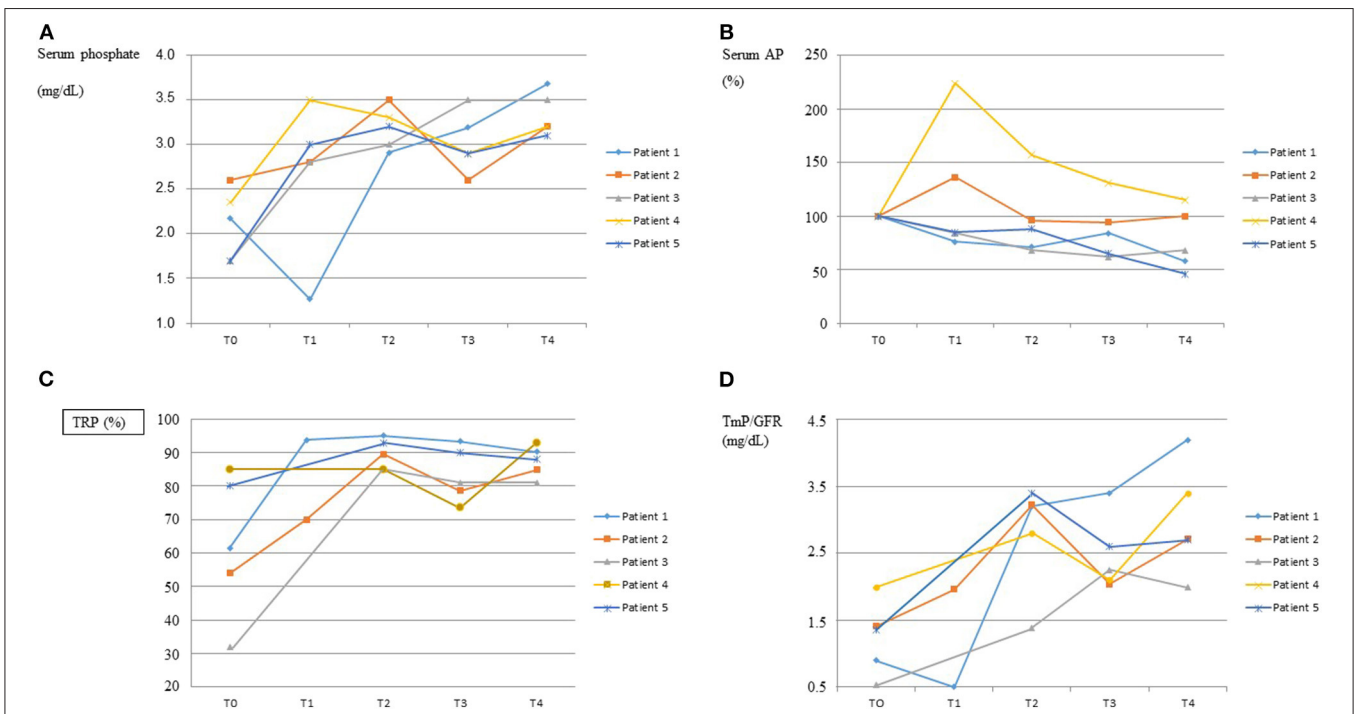

FIGURE 1 | Response to burosumab. The course over time of serum phosphate, serum alkaline phosphatases (AP), tubular reabsorption of phosphate (TRP) and tubular maximum reabsorption of phosphate for glomerular filtration rate (TmP/GFR) are shown in graphs **(A–D)**, respectively, for each patient. On the "x" axis, T0 indicates baseline values, immediately before starting burosumab treatment, and T1–T4 indicate values every 3–4 months on burosumab treatment. X ± SD values for T0 and T4 were as follows. Serum Phosphate (mg/dL): 2.10 ± 0.39 and 3.33 ± 0.24; Serum AP (U/L): 628 ± 267 and 525 ± 419; TRP (%): 62.5 ± 21.3 and 87.4 ± 4.7; TmP/GFR (mg/dL): 1.26 ± 0.53 and 2.99 ± 0.83.

diagnosis and management of XLH (10), recommendations on the use of burosumab cannot be conclusive because the available information is based on the results of trials testing the drug in children with severe XLH (10). We here report that treatment with burosumab for 1 year at a subcutaneous dose of 0.8 mg/kg/14 days results in normalization of serum phosphate, decrease of serum alkaline phosphatase, elevation of serum 1,25-dihydroxyvitamin D, and

TABLE 2 | Effect of 1 year burosumab treatment on growth in five children with X-linked hypophosphatemia.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Height in cm (SD)	130.0 (0.31)	115.4 (−1.51)	144.5 (−2.92)	136.0 (−3.4)	155.6 (−0.76)
Height Δ (SD)	+0.84	+0.89	−0.03	+0.16	−0.30
Weight in kg (SD)	33 (0.79)	27.2 (0.37)	46 (−1.22)	43.7 (−1.14)	53.5 (−0.02)
Weight Δ (SD)	−0.97	−0.39	−0.11	−0.07	−0.18
BMI in kg/m^2 (SD)	19.53 (0.84)	20.42 (1.66)	22.03 (0.14)	23.63 (0.64)	22.27 (0.37)
BMI Δ (SD)	−1.75	−1.47	−0.08	−0.17	−0.02

BMI, body mass index; Δ, SD change during burosumab treatment.

marked improvement of tubular reabsorption of phosphate in five pediatric patients with genetically confirmed XLH after interruption of classical treatment with phosphate supplements and vitamin D derivatives. It is of note that TRP (%) of patients 4 and 5 were within the expected range for a child with normal serum phosphate concentrations but TRP should be much higher (above 90–95%) in the presence of marked hypophosphatemia such as that found in these two patients, below 2.5 mg/dl in both.

Burosumab induced a positive effect on growth in three out of the five children, as demonstrated by a marked increase of height SDS at the end of the 1-year treatment period. In these patients the amelioration of height was noticeably higher than that found by Carpenter et al. (8) in their trial after 64 weeks of treatment. XLH exerts a marked adverse effect on growth (11) and final adult height of pediatric patients with XLH is usually low, even if they have received conventional treatment since diagnosis (12) or growth hormone (13) at infancy or early childhood. Our findings indicate that the growth promoting action of burosumab does not occur in growth retarded pubertal XLH patients, regardless its positive effects on phosphate metabolism. The two patients (patient 1 and 2) with the greater height SD increase during burosumab treatment were those of younger age (7 and 6 years), suggesting the convenience of early administration of burosumab, before puberty. It also supports the assumption that the impairment of growth in XLH is not entirely dependent on hypophosphatemia and rachitic bone lesions (11, 14) so that growth retardation may persist in spite of improvement in mineral metabolism. The characterization of growth response to burosumab needs to be investigated in further studies involving greater number of patients and longer follow-ups.

It is interesting to note that BMI markedly decreased in patients 1 and 2. These patients underwent the greatest improvement in height and, although, unfortunately, our study does not provide quantitatively measured objective data on musculoskeletal function (15), this positive effect on weight and body composition likely resulted from the higher physical activity of children, a fact disclosed by the patients' parents in the clinical interviews. Likewise, families reported the interruption of frequent oral doses of phosphate as an appreciable benefit linked to the new treatment. Burosumab also exerted a beneficial effect on radiological osseous abnormalities although no specific score to quantify bone deformity and/or legs' bowing was used.

Burosumab was well-tolerated and no serious undesirable effects were detected. Headache and local injection-site reactions reported by one of our patients have been notified in the literature in approximately 60% of children treated with burosumab (16). No patient exhibited hyperphosphatemia, progression of nephrocalcinosis, worsening of metabolic control or developed hyperparathyroidism. Mild elevation of serum PTH present at the beginning of treatment in patient 4 was not modified by burosumab administration.

In summary, this study shows the efficacy and safety of burosumab administered for 1 year in the clinical management and follow-up of pediatric patients with XLH. Additional studies are necessary to assess the influence of burosumab on the long-term outcome of these patients and to define the criteria for its use.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética de la Investigación con Medicamentos del Principado de Asturias (SP n° 38/18). 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 minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SM designed the data chart, obtained patient's information from the medical records, analyzed the data, drew the figures, and contributed to writing the manuscript. MG-C, VR, and AC obtained patients' information from the medical records, revised and corrected the data presentation, and the manuscript. FS designed the study, revised and corrected the data presentation and contributed to writing the different versions of the manuscript.

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Human Papillomavirus Vaccination in Male and Female Adolescents Before and After Kidney Transplantation: A Pediatric Nephrology Research Consortium Study

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Background: Kidney transplant (KT) recipients have higher incidence of malignancies, including Human Papillomavirus (HPV)-associated cancers. Thus, HPV vaccines may have an important role in preventing HPV-related disease in this population; however, immunogenicity and safety data are lacking.

Objective: To examine the immunological response and tolerability to HPV vaccination in pediatric KT recipients compared to future KT candidates.

Methods: The quadrivalent HPV vaccine was administered to girls and boys age 9–18 recruited from seven centers part of the Pediatric Nephrology Research Consortium. Subjects were recruited for three groups: (1) *CKD:* chronic kidney disease stages 3, 4, and 5 not on dialysis; (2) *Dialysis;* (3) *KT* recipients. The outcome consisted of antibody concentrations against HPV 6, 11, 16, and 18. Geometric mean titers (GMTs) and seroconversion rates were compared. Vaccine tolerability was assessed.

Results: Sixty-five participants were recruited: 18 in the CKD, 18 in the dialysis, and 29 into the *KT* groups. KT patients had significantly lower GMTs after vaccination for all serotypes. The percentages of subjects who reached seroconversion were overall lower for the *KT* group, reaching statistical significance for HPV 6, 11, and 18. Comparing immunosuppressed subjects (anyone taking immunosuppression medications, whether KT recipient or not) with the non-immunosuppressed participants, the former had significantly lower GMTs for all the HPV serotypes and lower seroconversion rates for HPV 6, 11, and 18. KT females had higher GMTs and seroconversion rates for certain serotypes. There were no adverse events in either group.

Conclusions: HPV vaccine was well-tolerated in this population. Pediatric KT recipients had in general lower GMTs and seroconversion rates compared to their peers with CKD

or on dialysis. Immunosuppression played a role in the lack of seroconversion. Our results emphasize the importance of advocating for HPV vaccination prior to KT and acknowledge its safety post transplantation. Future studies are needed to investigate the effect of a supplemental dose of HPV vaccine in KT recipients who do not seroconvert and to evaluate the long-term persistence of antibodies post-KT.

Keywords: HPV—human papillomavirus, vaccination, kidney transplantation, chronic kidney disease, dialysis, pediatric

INTRODUCTION

Solid organ transplant (SOT) recipients are at increased risk for malignancies in both pediatric and adult populations (1–3), with an elevated risk of dying of their cancer, even after adjustment for stage and treatment (4). Pediatric and adult kidney transplant (KT) recipients carry similar risk and malignancies are third most common cause of mortality within this population (5, 6). Specifically, female KT recipients have an increased risk of cervical cancer up to 14-fold, vulvar cancer up to 50-fold and anal cancers up to 100-fold (7, 8). In addition, male SOT recipients have an increased risk for penile cancers (9). Human papillomavirus (HPV) 16 and to a much lesser extent HPV 18 are responsible for the majority of these cancers (10). In addition, HPV 16 is also responsible for a large proportion of oral pharyngeal cancers (11). Not only that HPV infections are the most prevalent sexually transmitted infections in the US (12), but non-carcinogenic HPV types, HPV 6 and 11, can be associated with genital warts and can be difficult to treat in the face of immunosuppression (13). Immunosuppressed individuals, including transplant recipients, have higher rates of HPV infections (14–16), greater likelihood of persistent infections (17, 18) and higher rates of HPV clinical disease, including cancer (9, 19, 20). Thus, preventing HPV infections in this population is critical. HPV-related vaccine trials have been shown to prevent HPV infection in immunocompetent individuals when the vaccine is given prior to viral exposure (21). In addition, more recent studies have demonstrated a true reduction in abnormal Pap smears and cervical cancers with use of HPV vaccine (22, 23). Because HPV is sexually transmitted and often acquired soon after the onset of sexual activity (24), vaccination should ideally occur before sexual debut.

The efficacy and safety of the HPV vaccine in certain immunocompromised populations, such as pediatric chronic kidney disease (CKD), dialysis and KT recipients, has not been well-studied. This is a population at increased risk for infections and HPV related sequelae; in addition, they appear to also be at high risk for not getting vaccinated due to lack of data and less immediate concerns about sexual activity when ill and awaiting transplant, as has been shown in other patients with severe chronic illnesses (25, 26). The goal of this study was to examine the immunological response and tolerability of the quadrivalent HPV vaccine in both female and male pediatric patients with CKD, on dialysis and with a KT. The primary hypothesis was that all these populations will mount an immune response to the vaccine. Our secondary hypothesis was that pediatric patients with CKD 3, 4, 5 and on dialysis, therefore future KT candidates, will respond to the HPV vaccination better than the KT recipients.

Abbreviations: SOT, solid organ transplant; KT, kidney transplant; HPV, human papilloma virus; CKD, chronic kidney disease; GFR, glomerular filtration rate; eGFR, estimated GFR calculated using the modified Schwartz formula (0.413 × height in cm/serum creatinine in mg/dL); T1, time 1 (day 1 of enrollment prior to dose 1 of the vaccine administration); T2, time 2 (one month after vaccine series was completed); VRC, vaccination report card; AEs, adverse events; SD, standard deviation; IQR, interquartile range.

MATERIALS AND METHODS

Study Population

Males and females ages 9–18 years were recruited from seven collaborating large medical centers in the Pediatric Nephrology Research Consortium: Riley Hospital for Children in Indianapolis, IN, Primary Children's Hospital in Salt Lake City, UT, Medical University of South Carolina Children's Hospital in Charleston, SC, University of Minnesota Masonic Children's Hospital in Minneapolis, MN, Kentucky Children's Hospital in Lexington, KY, C.S. Mott Children's Hospital in Ann Arbor, MI and Nationwide Children's Hospital in Columbus, OH. Subjects were recruited in three cohorts:

- Group 1 (CKD) included patients with CKD stages 3 [glomerular filtration rate (GFR) 30–59 mL/min per 1.73 m^2], 4 (GFR 15–29 mL/min per 1.73 m^2) or 5 not on dialysis (GFR $<$15 mL/min/1.73 m^2, but not on dialysis).
- Group 2 (Dialysis) included patients on either chronic hemodialysis or peritoneal dialysis.
- Group 3 (KT) included patients who had received a KT at least 6 months prior to enrollment, in keeping with the recommendations of the American Society of Transplantation that inactivated vaccines may be resumed 3–6 months after a SOT (27).

Exclusion criteria were: pregnancy (self-reported), post-transplant lymphoproliferative disorder, active infectious diseases, fever, bleeding disorders, having received blood products in the past 6 months and hypersensitivity to any of the vaccine components.

Study Design

All participants who met eligibility criteria and who had not been vaccinated against HPV were approached within the dialysis units or clinics by a study coordinator at each site. Study

coordinators discussed the study and consent was obtained from the parent/guardian and assent from the participant. Medical records were used to abstract age, gender, race, and estimated GFR (eGFR) using the modified Schwartz formula (0.413 × height in cm/serum creatinine in mg/dL). For the CKD 3, 4, 5 and dialysis patients, it was documented whether they were on immunosuppression medications for their respective primary kidney diseases. The rationale for doing so was to perform sub-analyses to determine whether patients on immunosuppression medications (including KT recipients, but also patients with CKD 3, 4, and 5 taking immunosuppression medications to treat their primary disease) had a weaker immune response to the HPV vaccine compared to the rest. In addition, for KT patients, type of donor (living vs. deceased), number of transplants and time since most recent transplant were recorded. HPV quadrivalent vaccine (Gardasil®, Merck & Co., Inc.) was administered to each study participant per protocol according to the Advisory Committee on Immunization Practices recommendations at the time (28). Dose 1 of the HPV vaccine was administered to each study participant on day 1 of enrollment, after obtaining a baseline blood sample for antibody testing. Dose 2 was targeted to be administered at month 2 and dose 3 at month 6. The minimum interval between dose 1 and 2 of the vaccine was 4 weeks. The minimum recommended interval between dose 2 and 3 of the vaccine was 12 weeks. No subject received dose 2 or 3 more than 4 weeks later than initially targeted date. The Institutional Review Board of each participating center approved the study.

Outcomes

The primary outcome was the antibody response to each of the four HPV serotypes contained within the vaccine (6, 11, 16, 18). Serum was collected from all subjects on day 1 of enrollment prior to dose 1 of the vaccine administration [Time 1 (T1)] and at month 7 (or 1 month after vaccine series was completed) [Time 2 (T2)]. Anti-HPV responses were measured using a competitive Luminex Immunoassay performed by PPD Vaccines and Biologics (Wayne, PA) and expressed as geometric mean titers (GMTs). A participant was considered to have seroconverted if no antibody titers were found at enrollment and antibody levels post immunization were above the sero-status cutoffs for HPV types 6, 11, 16, and 18, as determined by the assay developer (GMTs $\geq$ 20, $\geq$16, $\geq$20, and $\geq$24 mM units/mL, respectively).

Secondary analyses evaluated the potential effect of other variables on antibody response to the HPV vaccine, such as age, gender, race, eGFR and use of immunosuppression medications. Within the transplanted group, donor type (living vs. deceased), number of transplants and time from transplant were also evaluated.

In addition, although the sample size was small, data was collected regarding safety of the HPV vaccine in this patient population, since it has not been well-studied. All subjects received a vaccination report card (VRC) after each vaccine dose administration. On the VRC, the parent/guardian was asked to record the subject's oral evening temperature daily for 5 days. In addition, injection-site and systemic adverse events (AEs) for a total of 15 days after each vaccination were recorded by parent/guardian. For injection-site erythema and swelling, subjects were instructed by the VRC to measure an injection-site reaction at its greatest width ("maximum size") from edge to edge in maximum units ranging from 0 to >7 inches (17.5 cm) on the VRC, rounding up to the next unit if in between 2 units [each unit on the VRC measured ∼1 inch (2.5 cm)]. For all AEs, subjects were instructed by the VRC to estimate the severity of AEs as mild (awareness of symptom but easily tolerated), moderate (discomfort enough to cause interference with usual activities), or severe (incapacitating with inability to work or do usual activity). Serious AEs were collected for the whole duration of the study regardless of causality and were followed for outcome. In addition, for KT recipients, data was specifically collected on any biopsy-proven acute rejection episodes after T1 up to 6 months after the last dose of the vaccine administration.

Statistical Analyses

Basic demographic and clinical characteristics are presented as means [standard deviations (SD)] for age, median [interquartile range (IQR)] for eGFR due to non-linearity, and frequencies (percentages) for categorical variables (sex, race, use of immunosuppression medications), with analyses comparing groups performed with ANOVA, Kruskal–Wallis, and Chi–Square tests, respectively. The two main outcomes of serotiter and seroconversion were assessed to determine if there were significant differences between groups at T2. Serotiter values were analyzed using Kruskal–Wallis non-parametric tests and seroconversion proportions using Fisher's Exact tests, with descriptive statistics being given as medians (IQRs) and frequencies (percentages), respectively. Pairwise comparisons were made for serotiter levels using a Bonferroni adjusted p-0.0167 to control for inflated type I error rates. Similar analyses, for demographics and the two outcome variables, were also performed comparing those who were on immunosuppression medications vs. those who were not, with similar analyses, using Student's t-tests, Wilcoxon rank-sum tests, and Fisher's Exact tests. All analyses were performed using SAS v9.4 (SAS Institute, Cary, NC) and all analytic assumptions were verified. Non-inferiority analyses were also performed, but all were non-significant due to the extremely low power due to our small sample size (results not presented).

RESULTS

A total of 72 subjects were enrolled: 28 from Riley Hospital for Children in Indianapolis; 15 from Primary Children's Hospital in Salt Lake City; 9 from University of Minnesota Masonic Children's Hospital in Minneapolis; 9 from Medical University of South Carolina Children's Hospital in Charleston; 5 from Kentucky Children's Hospital in Lexington; 5 from C.S. Mott Children's Hospital in Ann Arbor; 1 from Nationwide Children's Hospital in Columbus. A total of 65 participants completed the study: 18 participants in the CKD group, 18 participants in the dialysis group and 29 participants in the KT group (7 subjects were excluded for either not receiving all three doses of the vaccine or for not doing both serology collections). The average age of enrollment was 13.6 years (SD 2.6) and little

TABLE 1 | Demographics and renal status at enrollment*.

	Overall ($n = 65$)	CKD ($n = 18$)	Dialysis ($n = 18$)	Transplant ($n = 29$)	*p*-value**
Age [years; mean (SD)]	13.6 (2.6)	12.9 (2.7)	14.9 (2.5)	13.2 (2.4)	0.055
Sex [Male; *N* (%)]	34 (54.8)	12 (70.6)	4 (25.0)	18 (62.1)	0.018
Race [*N* (%)]					
Asian	2 (3.2)	0 (0)	2 (12.5)	0 (0)	0.006
Black	11 (17.5)	2 (11.1)	2 (12.5)	7 (24.1)	
Hispanic	9 (14.3)	2 (11.1)	6 (37.5)	1 (3.5)	
White	41 (65.1)	14 (77.8)	6 (37.5)	21 (72.4)	
Use of immunosuppression medications [*N* (%)]	38 (61.3)	1 (5.9)	8 (50.0)	29 (100)	<0.001
eGFR [mL/min/1.73 m^2] [median (IQR)]	31.6 (12.8, 61.9)	30.0 (20.6, 31.6)	9.6 (6.6, 11.2)	63.1 (45.7, 85.6)	<0.001

*Values are means (standard deviation) for age, frequencies (percentages) for categorical variables, and median (IQR) for eGFR; **p-values are from ANOVA, Fisher's Exact, and Kruskal–Wallis tests, respectively. Frequencies may not add to column totals due to missing data.*

TABLE 2 | Antibody responses to HPV vaccine (GMTs)—continuous variable by groups*.

	CKD ($n = 18$)	Dialysis ($n = 18$)	Transplant ($n = 29$)	*p*-value**
HPV 6[#]	869 (128, 1739); 539.67	217.5 (137, 326); 205.37	115 (12, 590); 113.16	0.016
HPV 11	1694.5 (622, 2740); 1208.30	431.5 (180, 996); 370.08	83 (13, 675); 110.13	0.001
HPV 16	5639.5 (934, 9189); 4390.79	1581.5 (436, 3404); 1709.62	436 (74, 4316); 508.43	0.011
HPV 18	1406.5 (150, 5121); 1039.62	331.5 (69, 622); 266.45	52 (9, 497); 91.30	0.004

*Values are medians (IQRs); mean GMTs at T2. **p-values are from Kruskal–Wallis non-parametric tests. [#]HPV 6 analyses had sample sizes of 17, 16 and 29, respectively, as those who were not naïve at baseline were excluded for that particular outcome's analysis. Post-hoc pairwise comparisons were made with Wilcoxon rank-sum tests, using a p-value of 0.017 for significance to control for inflated type I error rate; pairwise comparisons indicate the significant associations are driven by the difference between CKD and KT groups, although CKD and dialysis groups were also different for HPV type 11.*

over one half were males ($N = 34$, 54.8%). The majority of participants were white ($N = 41$, 65.1%) or black ($N = 11$, 17.5%). **Table 1** compares the demographics of the three groups with differences demonstrated among the three groups in regards to sex, race, eGFR and use of immunosuppression medications. All the KT patients were taking immunosuppression medications; in addition, some of the patients in the other two groups were also taking immunosuppression medications to treat the immune-mediated diseases leading to CKD or dialysis). Within the KT group ($n = 29$) the median (range) time since first transplant was 3.9 years (3.6, 10.5), about half (51.9%) had received a kidney from a living donor, one individual had had two transplants; two KT recipients reported events of rejection during the study enrollment.

All participants were seronegative for all four of the vaccine HPV types at enrollment, except for three participants who were not naïve for HPV 6, and thus excluded from serotiter and seroconversion analyses for that particular HPV type only. Serotiters for all HPV types were detected post-vaccination at T2, with KT patients having significantly lower GMTs for all serotypes; pairwise comparisons indicated the significant associations were driven by the difference between CKD and KT groups, although CKD and dialysis groups were also different for HPV type 11 (**Table 2**). Examination of the seroconversion rates based on manufacturer cutoffs showed significant heterogeneity between the groups (**Table 3**). The seroconversion rates post-vaccination were significantly lower for the transplanted group for three of the serotypes (HPV 6, 11, and 18). Although not statistically different, seroconversion rates were also lower for the HPV 16 serotype.

All variables found to be different among the three treatment groups were examined directly with the two outcome variables, GMTs at T2 and seroconversion. Age, sex, race, and eGFR were found to have no association with GMTs at T2 or actual seroconversion. However, when only the KT population was examined, being a female KT-recipient was associated with greater GMTs at T2 for HPV 6 and 11 only ($p = 0.020$ and 0.046, respectively) and with higher seroconversion rates for HPV 18 only (90.9 vs. 44.4% for females and males, respectively; $p = 0.020$). Although statistically significant, this is difficult to interpret clinically with the inconsistencies among the various HPV types and small numbers for multiple analyses.

Comparing subjects who were using immunosuppression medications (including KT recipients, as well as CKD 3,

TABLE 3 | Seroconversion rates—categorical variable by groups*.

	CKD ($n = 18$)	Dialysis ($n = 18$)	Transplant ($n = 29$)	*p*-value**
HPV 6#	17 (100)	15 (93.8)	21 (72.4)	0.017
HPV 11	18 (100)	16 (88.9)	20 (69.0)	0.010
HPV 16	18 (100)	17 (94.4)	26 (89.7)	0.570
HPV 18	17 (94.4)	15 (83.3)	18 (62.1)	0.032

*Values are frequencies (percentages) of participants who seroconverted, defined as being HPV naïve at T1 and having GMTs ≥20, ≥16, ≥20 and ≥24 milli-Merck units/mL for HPV types 6, 11, 16, and 18, respectively at T2. **p-values are from Fisher's Exact tests. #HPV 6 analyses had sample sizes of 17, 16, and 29, respectively as those who were not naïve at baseline were excluded for that particular outcome's analysis.

TABLE 4 | Comparison between subjects who used immunosuppression medications vs. subjects not on immunosuppression medications*.

	Immuno suppression medications use ($n = 38$)	No Immuno suppression medication use ($n = 24$)	*p*-value**
Age [years; mean (SD)]	13.5 (2.4)	13.6 (3.1)	0.904
Sex [Male; *N* (%)]	19 (50.0)	14 (60.9)	0.440
Race [*N* (%)]			
Asian	1 (2.6)	1 (4.2)	0.030
Black	9 (23.7)	2 (8.3)	
Hispanic	2 (5.3)	7 (29.2)	
White	26 (68.4)	14 (58.3)	
eGFR [mL/min/1.73 m^2] [median (IQR)]	56.7 (20.4, 69.4)	21.4 (9.8, 31.1)	<0.001
GMTs at T2 [median (IQR)]			
HPV 6#	120 (19, 564)	362.5 (140.5, 1216)	0.005
HPV 11	121 (13, 675)	1247 (522, 2028.5)	<0.001
HPV 16	436 (89, 3404)	4751.5 (1829, 9091)	<0.001
HPV 18	50 (9, 497)	963.5 (396, 4243.5)	<0.001
Seroconversion [*N* (%)]			
HPV 6#	26 (74.3)	24 (100)	0.008
HPV 11	27 (71.1)	24 (100)	0.004
HPV 16	34 (89.5)	24 (100)	0.151
HPV 18	24 (63.2)	23 (95.8)	0.005

*Values are means (standard deviation) for age; frequencies (percentages) for sex, race, and seroconversion; median (IQR) for eGFR and for GMTs. **p-values are from Student's t-test, Fisher's Exact tests, and Wilcoxon tests, respectively. Frequencies may not add to column totals due to missing data. The total number of subjects in this analysis was 38 + 24 = 62, as opposed to 65 subjects in the other analyses. This is due to missing medication information from the data collection forms on three subjects. #HPV 6 analyses had sample sizes of 35 and 24, respectively, as those who were not naïve at baseline were excluded for that particular outcome's analysis.

4, and 5 on immunosuppression medications to treat their primary kidney conditions) vs. not, the immunosuppression medication-using group had significantly lower serotiters for all four HPV serotypes at T2 and lower seroconversion rates for HPV 6, 11, and 18 (**Table 4**). The immunosuppression medications used in the transplant population included prednisone, tacrolimus, cyclosporine A and mycophenolate mofetil. The immunosuppression medications used to treat the primary kidney diseases in the CKD and dialysis patients included prednisone, tacrolimus, mycophenolate mofetil and azathioprine. Unlike in the KT population, when the male vs. female differences were examined specifically in the immunosuppression medication-using group, no differences were seen.

Within the transplant population, neither the donor type, neither time since transplant, nor the number of transplants, made any difference in terms of GMTs at T2 or seroconversion rates.

As for the AEs, they were minimal, limited to only mild local reactions. Two patients underwent acute rejection episodes within 6 months following the last dose of the vaccine. However, upon review of chart data, these events were considered to be likely related to medication non-adherence.

DISCUSSION

American Society of Transplantation recommends that HPV vaccine be given to all SOT candidates and recipients in the recommended age-group (27). However, the efficacy and tolerability of the HPV vaccine in such populations, as well as the optimal timing to do so, are not completely understood. Our results demonstrated that the HPV vaccine was well-tolerated and an adequate immune response for all four HPV types was observed with CKD and dialysis patients. However, the immune response was slightly lower within the transplanted population and immunosuppressant medications had an effect on lack of seroconversion. HPV vaccine acceptance by patients and their parents/guardians has always been problematic even in the general population (29, 30) and probably more so in these specific groups, where data is scant (26). Our study provides further evidence that patients who are likely to need a KT in the future respond best to the HPV vaccine pre-transplantation, while with CKD or on dialysis. As the majority of causes of end-stage renal disease in pediatrics are congenital diseases in which the kidney function slowly deteriorates over time, pediatric nephrologists often have the advantage of being able to better plan a KT (whether preemptive or not) considering multiple aspects, including vaccinations, compared to their adult nephrologist colleagues (26). This study showed that girls and boys with CKD and on dialysis had stronger immunologic responses to the HPV vaccine compared to those who were already transplanted. Therefore, this study should help pediatric nephrologists and pediatricians in their work with patients and families to pursue and complete the administration of HPV vaccine ideally prior to transplant whenever possible, but also post-transplant in cases where it was not given prior to KT. Is is to be noted that in 2015 the Advisory Committee on Immunization Practices (ACIP) recommended the 9-valent HPV vaccine (9vHPV) (Gardasil 9, Merck and Co., Inc.) as an alternative to the quadrivalent vaccine (31). The 9-valent vaccine was found to be safe, provided more extensive serotype coverage and it was even found to be cost-saving when compared with the quadrivalent vaccine, therefore it is currently more widely used (31). Our study was initiated well before 2015, hence the use of the quadrivalent vaccine.

It is important for the HPV vaccine to be given before HPV infection is acquired, meaning before the onset of sexual activity (28). This is the rationale behind the fact that the HPV vaccine is recommended to be given at 11–12 years of age, although it can be administered starting at age 9 (28). In the US, the proportion of males who reported having sexual intercourse before age 13 years varied from 5 to 25% across metropolitan sites (32), hence the need to vaccinate at a young age, designed to capture most teenagers before becoming sexually active. In our study, the onset of sexual activity was not assessed out of concerns that in a multisite trial, the investigators could not assure patient confidentiality relative to their parents/caregivers for all participants. However, obtaining this information in future studies would be helpful in assessing clinical risk and outcomes in patients with chronic diseases (33, 34). In children with CKD and on dialysis, contrary to what was believed in the past, recent cohort studies have shown that growth and sexual maturation are only slightly delayed compared to healthy subjects (35). Although there is lack of specific data, given the circumstances, it is plausible that adolescents with CKD or on dialysis are not far behind from their peers in terms of their onset of sexual activity. It is therefore of concern that in our study the mean age of subjects was 13.6 (SD 2.6), meaning that our patient population was vaccinated on average later than the recommended age.

Our study showed for the first time both in boys and girls that overall, transplant patients had a decreased response to the HPV vaccine compared to CKD and dialysis patients, likely related to their state of medication-induced immunosuppression. However, specifically for HPV 16, seroconversion rates were not found to be significantly lower for KT patients and neither were they found to be lower for patients taking immunosuppression medications. Although power may have biased this result, it is also somewhat reassuring since the majority of HPV cancers are associated with type 16. At the very least, the data supports ongoing vaccination in this group due to the fact that the majority of individuals did effectively respond to the vaccine. Review of literature revealed that the first studies to look into HPV vaccine responses in immunosuppressed populations was in HIV-positive cohorts, which showed promising results, as 95–100% of the subjects demonstrated seroconversion (36–38). Thus, seroconversion was not much lower than in healthy populations (39). Surprisingly, the first study of the immunogenicity of the HPV vaccine in SOT recipients was done in a cohort of 47 adults (females and males) and reported much lower rates of seroconversion: 63.2, 68.4, 63.2, and 52.6% for HPV 6, 11, 16, and 18, respectively (40). Somewhat similarly, in pediatrics, a study of a cohort of 57 patients (females only) aged 9–21 years old with CKD, on dialysis, or post-KT, showed that among patients with transplants, the percentages achieving seropositivity were significantly lower compared to CKD and dialysis patients: 63.6, 63.6, and 72.7% for HPV 6, 11, and 18, respectively (41). Our study included both girls and boys age 10 to 16 with CKD, on dialysis, or post-KT and showed lower seroconversion rates: 72.4, 69.0, and 62.1% for HPV 6, 11, and 18, respectively, in transplant patients, thus demonstrating similar results for the first time in boys, as well.

In this study, it is likely that the entire population was affected by varying levels of immunosuppression, regardless of the study group. Indeed, patients with CKD and more so the ones on dialysis have been shown to have various degrees of immunosuppression, whether caused by defects in neutrophil function, antigen processing, cell-mediated immunity, or antibody-mediated immunity (42, 43). Antibody responses to certain vaccines have been found to be attenuated in this patient population for certain vaccines, such as the measles, mumps and rubella (44). On the contrary, for other vaccines immune responses have been found to be similar to the general population (45). In the case of HPV vaccine, a study on pediatric and adult patients with CKD 4, 5, and on dialysis showed 98.2, 100, 100, and 98.2% seroconversion for HPV genotypes 6, 11, 16, and 18, respectively (46). These values are comparable to the data generated in healthy adult women large populations, which reported 99.7, 99.5, 99.7, and 99.2% seropositivity for HPV genotypes 6, 11, 16, and 18, respectively (39). Our study also reported robust immune responses in the CKD and dialysis pediatric patients. Although there was a non-statistically significant tendency to less robust responses for dialysis patients compared to CKD, this was probably explained by the fact that half of the dialysis patients were on immunosuppressive medications for their kidney conditions. In addition, we have found no difference in terms of HPV vaccine responses between patients on hemodialysis vs. peritoneal dialysis in our study population (data not presented). It is likely that being on anti-rejection medications creates a more profound state of immunosuppression in transplant recipients than the immune dysregulation seen in CKD or while on dialysis, since KT patients had less robust immune responses to the HPV vaccine in our study. Likewise, patients with CKD or on dialysis who were on immunosuppression medications for their primary kidney diseases, which are in fact similar to anti-rejection medications, also demonstrated poorer seroconversion rates.

It is possible that girls and boys who are KT recipients respond differently to the HPV vaccine. The quadrivalent HPV vaccine was approved in the US in 2006 for use in girls/women only. Subsequently, in 2009, the indication was extended to boys/men as well. The few studies that compared the seroconversion rates to HPV vaccine between males and females in the general population have not shown any differences (47, 48). Our study showed that overall there were no differences between males and females in terms of HPV antibody responses. However, when data was analyzed specifically for the KT recipients, male subjects had lower GMTs at T2 for HPV 6 and 11 (but not 16 or 18) and lower seroconversion rates for HPV 18 (but not for HPV6, HPV11, or HPV16) compared to females. Although statistically significant, this is difficult to interpret clinically with the inconsistencies among the various HPV types and small numbers for multiple analyses. Interestingly enough, unlike in the KT population, when the male vs. female differences were examined specifically in the immunosuppression medication-using group, no differences were seen. Sex-based differences in immune function and responses to vaccination have been described before, where females typically developed higher antibody responses and experienced more adverse reactions following vaccination than males for bacillus Calmette-Guerin, measles, mumps, rubella, yellow fever and influenza vaccines

(49, 50). In addition, men SOT recipients have a higher incidence of malignancies, also suggesting the fact that SOT men could become more immunosuppressed than women when treated with anti-rejection medications (51). In any event, the fact that male KT recipients tend to have lower antibody titers and seroconversion rates for certain serotypes following HPV vaccination, while of uncertain significance, still raises questions about optimal vaccination strategies specifically in this patient population, which has been already shown to also have lower vaccination rates compared to females (52).

AEs reported were similar to those noted in prior studies, limited to minor vaccine local skin reactions. Two patients underwent acute rejection episodes within 6 months following dose 3 of the HPV vaccine. In 2014, a study initiated HPV vaccination in 14 pediatric KT recipients; unfortunately six of them developed acute rejection shortly after the initiation of the vaccine series, which lead to an early termination of the study (53). In contrast, in 2016, only two of the 23 pediatric KT recipients of HPV vaccination were reported to have acute rejection episodes (41). In our patient population, we report a similar acute rejection burden; upon chart review, it was felt that our two cases of acute rejection were related to medication non-adherence, as opposed to vaccine administration.

We acknowledge that our study has certain limitations. Like in many pediatric SOT studies, our study was limited by its small sample size. Unfortunately three subjects were missing information on medication use in the data collection form and some were not complete, thus impairing our ability to analyze the effect of specific immunosuppresion medications. Indeed, for specific vaccinations, for example influenza vaccination in KT recipients, it has been demonstrated that different imunosuppression regimens can influence the immune response (54). In addition, our study lacked long-term follow up, as we did not look into long-term HPV-vaccine induced antibody persistence post-transplantation; other studies suggested that there was a decline in titers over time (40). Lastly, we were unable to determine factors beyond immunosuppression that may play a role in preventing seroconversion in transplant recipients. However, this is the largest and the only multicenter study that examined the humoral response to the HPV vaccine in both girls and boys with KTs, compared to CKD and on dialysis. It is also the first study that compared the humoral response between girls and boys in these populations. In addition, we included only data for the subjects who were HPV naïve at baseline, had complete data in terms of antibody titers and completed the entire three dose vaccination series.

In conclusion, this study addresses the immune response to the HPV vaccine in pediatric KT recipients, CKD and dialysis patients, as well as its safety. The HPV vaccine was well-tolerated in these populations. Pediatric KT recipients had in general lower GMTs and seroconversion rates compared to their peers in the CKD and dialysis groups. In addition, immunosuppression (either due to the need to address immune-mediated diseases of the native kidneys or to prevent rejection in KT) played a role in the lack of seroconversion. Our results emphasize the importance of advocating for HPV vaccination prior to KT and acknowledge its safety post transplantation. However, questions still remain. Specifically, studies to identify factors associated with the lack of seroconversion in KT recipients are needed for targeted interventions. In addition, future studies should investigate the effect of a supplemental dose of HPV vaccine in KT recipients who do not seroconvert. It would also be important to evaluate the long-term persistence of antibodies post-KT and the possible need for re-immunization post-KT.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Indiana University Office of Research Administration Institutional Board Review. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CN generated the hypothesis, obtained funding, designed the study, recruited subjects at her institution, interpreted data, and wrote the manuscript. RN, PV, KT, AC, MM, and JM had solutions for improving design, recruited subjects at their respective institutions, and corrected the manuscript. JS analyzed and presented data, while also helping CN and MS write the statistics paragraph and interpret data. MS mentored and helped CN in all of the above endeavors.

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frontiers in Pediatrics

SYSTEMATIC REVIEW
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Prevalence of Bladder and Bowel Dysfunction in Toilet-Trained Children With Urinary Tract Infection and/or Primary Vesicoureteral Reflux: A Systematic Review and Meta-Analysis

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Introduction: Urinary tract infection (UTI) in children leads to renal scarring in 10–15% of patients. Urinary tract anomalies and bladder and bowel dysfunction (BBD) are documented risk factors for recurrent UTIs. Estimates of baseline prevalence of BBD in children with UTI will help the clinician in the management strategy. Hence, a systematic review and meta-analysis was conducted to estimate the pooled prevalence of BBD.

Methods: MEDLINE, EMBASE, and CENTRAL (Cochrane Central Register of Controlled Trials) databases were searched for articles related to UTI, primary vesicoureteral reflux (VUR), and BBD. We included studies that provided prevalence of BBD in toilet-trained patients aged 1–18 years with UTI and/or VUR. BBD was defined based on clinical history or questionnaire or urodynamic studies. Two authors independently reviewed, assessed, and abstracted data from studies. Pooled prevalence was calculated based on a random effects model.

Results: Forty-three studies fulfilling the eligibility criteria were selected from a total of 1,731 studies. Among patients presenting with UTI without primary VUR, pooled prevalence of BBD was 41% (95% CI: 26–55; nine studies, 920 patients, $I^2 = 96.0\%$), whereas its prevalence in patients with primary VUR was 49% (43–56; 30 studies, 5,060 patients, $I^2 = 96.0\%$). Weighting by the study design and quality did not affect the prevalence. In patients with primary VUR, prevalence of BBD was higher in females (53%; 42–65) than in males (44%; 15–73). In studies where urodynamic study was used for the diagnosis of BBD, prevalence was 63%. The presence of BBD in patients with primary VUR increased risk of recurrent UTIs [relative risk (RR): 2.1; 1.7–2.5]. In five studies that reported separate data on constipation, pooled prevalence of constipation was 27% (16–37).

Conclusion: Almost half of the patients with primary VUR have BBD, and its presence increases the risk of recurrent UTIs. Trends of high BBD prevalence were also observed in patients presenting with UTI without VUR. These prevalence estimates suggest that all toilet-trained children presenting with UTI with or without VUR should be assessed for BBD, which will help in their further management.

Keywords: dysfunctional elimination syndrome, vesicoureteral reflux, voiding dysfunction, constipation, urinary tract infection

INTRODUCTION

Rationale

Urinary tract infections (UTIs) are one of the most commonly encountered infections in childhood and may lead to long-term sequelae in a proportion of patients (1, 2). Whereas, presence of urinary tract anomalies is a known risk factor for recurrent UTIs in children, risk of recurrence is also influenced by age, gender, and bladder and bowel dysfunction (BBD) (3, 4). The term BBD is used to describe the spectrum of lower urinary tract symptoms accompanying bowel disturbance in the form of constipation and/or encopresis (4). BBD has also been reported as one of the important risk factors for recurrent UTIs in children. The risk of UTI is higher in patients with BBD and primary vesicoureteral reflux (VUR) than in patients with only VUR (5). Presence of BBD delays resolution of VUR and increases risk of UTI following reimplantation (6, 7). BBD has also been reported to impact the rate of breakthrough UTIs in patients with VUR who are on continuous antibiotic prophylaxis (5). As the presence of BBD in patients with UTI affects long-term outcomes, early recognition, and treatment are essential (6). In patients with UTI, variable prevalence (18–54%) of BBD has been reported in previous studies. Knowledge about the baseline prevalence of BBD in toilet-trained children presenting with UTI with or without primary VUR will help clinician in planning the management strategy for these patients (8). We performed a systematic review and meta-analysis to provide pooled estimates of prevalence of BBD in patients presenting with UTI and/or primary VUR.

Objective

The aim of this study was to determine the prevalence of BBD in toilet-trained children with UTI with or without primary VUR.

Research Question

What is the prevalence of BBD in toilet-trained children with UTI with or without primary VUR?

MATERIALS AND METHODS

Study Design

A systematic review and meta-analysis was performed by review of observational and interventional trials published between January 1980 and December 2018.

Participants, Interventions, and Comparators

All published data during 1980–2018 were searched for prevalence of BBD in toilet-trained children with UTIs with or without primary VUR. No interventions or comparators were assessed.

Search Strategy

Protocol for the study was published (PROSPERO: CRD42019127086) and conducted in accordance with the Meta-analysis Of Observational Studies in Epidemiology guidelines (9). Two authors (JM and GM) independently performed literature search in MEDLINE, EMBASE, and CENTRAL (Cochrane Central Register of Controlled Trials) for original articles published, between January 1980 and December 2018. Search strategy design included patients aged 1–18 years with UTI and/or primary VUR. Search strategy was based on four basic groups of terminology: study population (pediatric/children/adolescent) and terms related to or describing the BBD, UTI, and VUR. Terminologies used for literature search were as follows: Bladder bowel dysfunction, dysfunctional elimination syndrome, dysfunctional voiding, lower urinary tract dysfunction, enuresis, urinary incontinence, urgency, overactive bladder, constipation, encopresis, fecal incontinence, vesicoureteral reflux, urinary tract infection, pyelonephritis, cystitis, pediatric, children, adolescent, prevalence, and incidence. Specific search strategies were created for each search engine by using MeSH term and terms described above (**Supplementary Table 1**). Electronic search was also supplemented by hand search of bibliographies of the included studies and relevant review articles.

Data Sources, Study Selection, and Data Extraction

Predefined criteria were used for final selection of studies included in the review. All observational studies and controlled trials were included in this review if they (i) reported data on BBD prevalence in patients aged 1–18 years with UTI and/or primary VUR and (ii) defined BBD based on clinical history or questionnaire or urodynamic studies (UDSs). Conference abstracts were also included if they provided sufficient information on sample size, methods of data collection, case definition, and prevalence of BBD. Studies were excluded if they reported (i) BBD prevalence in 10 or less patients; (ii) patients with neurological abnormalities that affect normal

functioning of bladder and bowel, or secondary VUR; (iii) non-toilet-trained children; and (iv) in languages other than English. A well-structured, standardized proforma was used for data extraction. Data included information for risk of bias assessment of the study, prevalence of BBD, author name, year of publication, journal, study setting and design, study population, baseline demographic characteristics, details of intervention, and control group (in case of randomized controlled trials), case definition of BBD, and recurrence of UTI. Any disagreement between two reviewers was resolved through discussion with the third author (PH).

Statistical Analysis and Quality Assessment

The authors independently assessed the quality of articles using the Cochrane risk bias tool for randomized controlled trials. Quality of observation studies was assessed by using a risk of bias assessment tool developed by Hoy et al. for prevalence studies (10). We reviewed full-text articles to determine the following: (i) whether study participants are a close representation of true population; (ii) whether the method used for selection of the study participants was appropriate; (iii) whether data were directly collected from patients and their response rate; (iv) whether acceptable case definition and tool were used for defining BBD and UTI; and (v) whether appropriate numerator and denominator were used for calculating prevalence of BBD. Disagreement between two authors in assessment of risk of bias was resolved by the third author (PH).

Meta-analysis was performed using Stata version 14. We pooled data from individual studies using random effects model with assumption that BBD prevalence would be variable across the studies. Forest plots represent studies in order of year of publication. Heterogeneity in studies was explored by inspection of forest plot as well as using chi-square test on Cochran's Q statistics. Study heterogeneity was assessed by using the Higgins and Thompson I^2 method (11). The I^2 heterogeneity was categorized as follows: 0–50% low, 50–75% moderate, and >75% considerable heterogeneity. Sensitivity analyses were undertaken to investigate the individual study influence and the studies using only low risk of bias. A subgroup analysis was performed to explain heterogeneity and calculate prevalence of BBD by sex, study design (controlled trials and prospective and retrospective observation studies), and method of assessment.

RESULTS

Search Results

A total of 1,731 articles were identified through the search strategy in all databases (PubMed 667, EMBASE 525, and CENTRAL 539). There were 1,319 articles after removing 412 duplicates, and 105 of these articles were assessed as potentially relevant, for the systematic review, by screening through the title and abstract. Among these, 80 were full-text original articles, whereas the rest 25 were conference abstracts (**Figure 1**). We also screened the reference list of the full-text articles, but no additional article was identified through this process. Finally, 43 studies comprising 6,627 patients were selected for this review (**Table 1**).

Study Selection and Characteristics

On the basis of patients enrolled, we categorized studies into two groups: (i) nine studies of patients with UTI and without primary VUR (5, 17, 24–26, 28, 31, 43, 53) and (ii) 30 studies of patients with primary VUR (12–16, 18–23, 27, 29, 30, 32–35, 37–42, 44–47, 49–52, 54). Three studies were included in the final systematic review as they reported data on rates of recurrence of UTI (15, 22, 45), and one study by Chung et al. provided data on constipation (50).

Prevalence of Bladder and Bowel Dysfunction Among Patients With Urinary Tract Infection Without Primary Vesicoureteral Reflux

A total of nine studies comprising of 920 patients reported prevalence of BBD in toilet-trained children presenting with UTI without VUR (5, 17, 24–26, 28, 31, 43, 53). The pooled prevalence of BBD in patients with UTI was 41% (95% CI: 26–55%) (**Figure 2**). Owing to high heterogeneity ($I^2 = 95.99\%$), random effects estimates were used. Three studies reported separate data on prevalence of BBD in girls, at 41% (95% CI: 25–58%). None of the studies provided separate information on prevalence of BBD in boys. On a subgroup analysis, the prevalence of BBD was higher (51%, 10–93%) in prospective studies as compared with retrospective studies (35%, 21–49%).

Prevalence of Bladder and Bowel Dysfunction Among Patients With Primary Vesicoureteral Reflux

Thirty studies comprising 5,060 patients reported prevalence of BBD in patients with primary VUR (14–16, 18–23, 27, 29, 30, 32–35, 37–42, 44–47, 49–52, 54–58). The pooled prevalence of BBD was 49% (95% CI: 43–56%) (**Figure 3**). In this group of studies, the heterogeneity was also high ($I^2 = 96\%$). Separate data for prevalence of BBD for boys were reported in five studies, with pooled prevalence of 44% (95% CI: 15–73%). Seven studies reported data for girls with BBD prevalence of 53% (95% CI: 42–65%). In studies with high risk of bias, the prevalence of BBD was high (71%), as compared with that in studies with low-to-moderate risk of bias (45% and 48%). No difference in BBD prevalence was observed when comparing prospective with retrospective studies. In eight studies that used UDS, the pooled prevalence of BBD was 63% (95% CI: 56–70%; $I^2 = 52.8\%$).

Risk of Recurrence of Urinary Tract Infection in Patients With Bladder and Bowel Dysfunction

Seven studies of patients with primary VUR explored the relationship between recurrence of UTI and BBD (15, 18, 19, 22, 37, 45, 47). A meta-analysis of these studies using random effects estimate showed that in the presence of BBD, the risk of recurrent UTIs was increased two-fold, as compared with that in patients without BBD (RR: 2.01; 95% CI: 1.47–2.74, $I^2 = 57.3\%$)

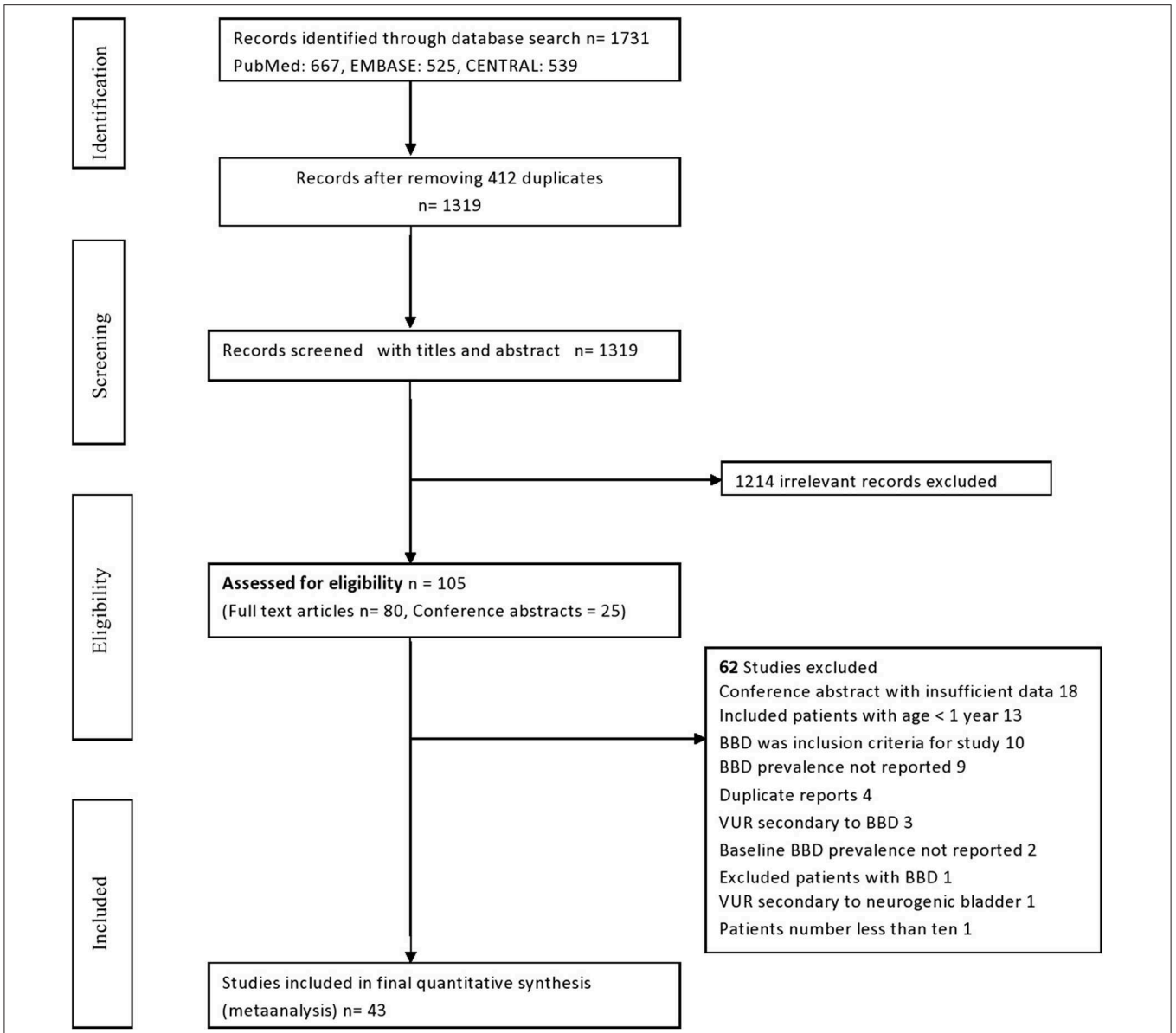

FIGURE 1 | Systematic review flow diagram for selection of studies.

(**Figure 4**). One study in patients with UTI without primary VUR reported that the presence of BBD did not significantly increase the risk of UTI (RR: 1.07, 95% CI: 0.51–2.23).

Prevalence of Constipation

Two studies on patients with UTI showed that the pooled prevalence of constipation was 30% (95% CI: 25–36%) (24). Data for constipation were reported in 972 patients with primary VUR across seven studies (18, 21, 40, 47, 49, 50, 52). Pooled prevalence of constipation from these seven studies was 27% (95% CI: 16–37%).

Risk of Bias

Quality of studies was assessed based on Cochrane risk bias tool for randomized trials, and a modified tool by Hoy et al. was used for observational studies (10). Based on these tools, seven studies were at high risk of bias, 17 at moderate risk of bias, and 15 at low risk of bias, whereas in four studies, risk of bias could not be assessed owing to insufficient information (**Table 1**).

DISCUSSION

Summary of the Main Findings

Patients with bladder dysfunction are at increased risk of bowel dysfunction and vice versa. Anatomical and function interaction that leads to this increase risk has been well-established. BBD is an important risk factor for UTI in children, more so in the ones who are toilet trained. In a meta-analysis, the American Urological Association (AUA) guideline for the management of

TABLE 1 | Basic characteristics of studies included in the systematic review.

References	Country	Study design	Risk of bias assessment	Study group	Sample size
Taylor et al. (12)	USA	Prospective	3	VUR	37
Seruca (13)	Portugal	Prospective and retrospective	3	VUR	43
Snodgrass et al. (14)	USA	Cross sectional	1	VUR	70
van Gool et al. (15)	USA	Prospective	1	VUR	310
Lipski et al. (16)	USA	Retrospective	2	VUR	30
David et al. (17)	France	Retrospective	3	UTI	30
Koff et al. (18)	USA	Prospective	1	VUR	143
Snodgrass et al. (19)	USA	Prospective	1	VUR	128
Trsinar et al. (20)	Slovenia	Prospective	2	VUR	94
Soygür et al. (21)	Turkey	Prospective	1	VUR	62
Willemsen et al. (22)	Netherlands	Prospective	1	VUR	102
Capozza et al. (23)	Italy	Prospective	1	VUR	180
Barroso et al. (24)	Brazil	Prospective	1	UTI	45
Mazzola et al. (25)	Switzerland	Retrospective	3	UTI	141
Vlajkovic et al. (20)	Yugoslavia	Prospective	1	VUR	74
Shaikh et al. (26)	USA	Prospective	1	UTI	123
Chen et al. (27)	USA	Retrospective	1	VUR	1721
Mingin et al. (28)	USA	Retrospective	2	UTI	12
Lavelle et al. (29)	USA	Prospective	2	VUR	52
Im et al. (30)	Korea	Retrospective	2	VUR	56
Colen et al. (31)	USA	Retrospective	2	UTI	132
Szymanik-Grzelak et al. (32)	Poland	Not clear	Unclear	VUR	150
Higham-Kessler et al. (33)	USA	Retrospective	2	VUR	80
Yucel et al. (34)	USA	Retrospective	2	VUR	92
Izquierdo and Luque Mialdea (35)	Spain	Prospective	2	VUR	63
Williams (36)	USA	Retrospective	Unclear	VUR	82
Sillén (37)	Sweden	Prospective	1	VUR	148
Whittam et al. (38)	USA	Retrospective	2		295
Altobelli et al. (39)	Italy	Retrospective	1	VUR	138
Hong et al. (40)	USA	Prospective	1	VUR	298
Palcic et al. (41)	Croatia	Retrospective	Unclear	VUR	92
Giuseppe et al. (42)	Italy	Retrospective	3	VUR	78
Öztürk et al. (43)	Not known	Not clear	Unclear	UTI	192
Akhavan et al. (44)	USA	Retrospective	2	VUR	78
Alexander et al. (45)	USA	Retrospective cohort	2	VUR	225
Heckler et al. (46)	USA	Prospective	2	VUR	169
Hoberman et al. (47)	USA	Randomized controlled trial	1	VUR	126
Cetin (48)	Turkey	Not clear	3	UTI	188
Chang et al. (49)	Taiwan	Retrospective	2	VUR	34
Chung et al. (50)	Korea	Retrospective	3	VUR	90
Arlen et al. (51)	USA	Retrospective	2	VUR	222
Shaikh et al. (5)	USA	Prospective	1	UTI	57
Sharif-Rad et al. (52)	Iran	Retrospective	1	VUR	225

Risk of bias assessment (Low = 1, moderate = 2, High = 3).

primary VUR showed that the presence of BBD significantly delayed resolution of VUR (7). In children presenting with UTI, who already possess a risk factor like primary VUR, the presence of BBD further increases risk of breakthrough UTI even while on antibiotic prophylaxis (5). Two recent studies support the notion that BBD predisposes patients for recurrence of UTI and increases risk of renal scarring as well (3, 5). Recently, a reanalysis of data from the RIVUR trial by Wang et al. showed that antibiotic prophylaxis is more beneficial in the group of patients with BBD compared with those without it (8). Hence,

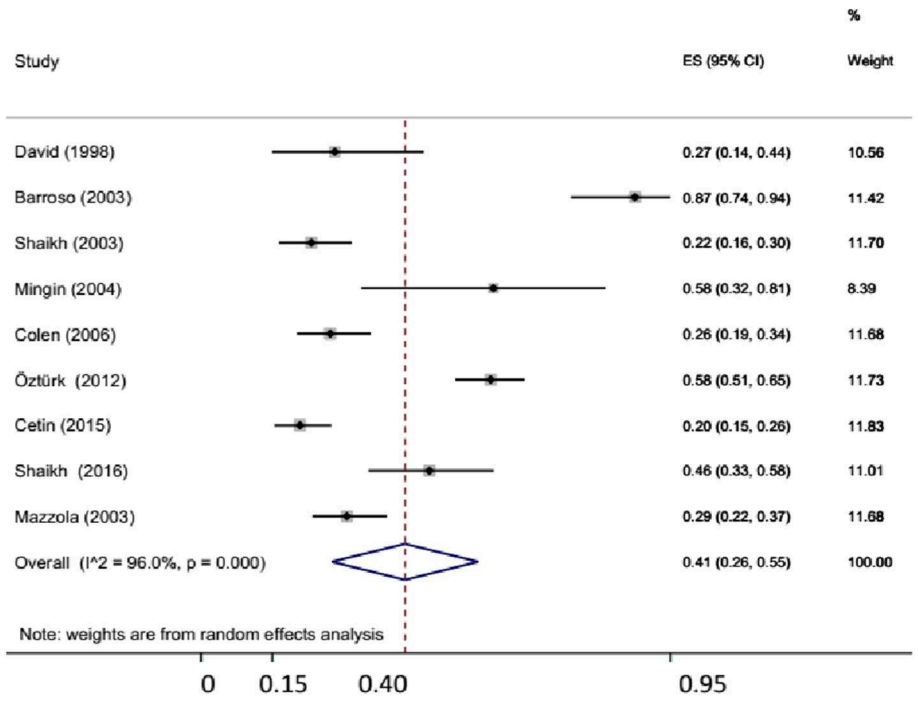

FIGURE 2 | Prevalence of bladder dysfunction in children with urinary tract infection (UTI) without primary vesicoureteral reflux (VUR).

it is of paramount importance to assess children presenting with UTI for BBD even in the presence of other anatomical risk factors before deciding management strategy. In this review, we found that prevalence of BBD is slightly higher in patients with primary VUR than in patients with UTI without VUR. Within primary VUR, cohort girls had higher prevalence of BBD than boys. When BBD is assessed by more invasive tools like UDS, almost two-thirds of patients with primary VUR were detected to have BBD. In the present meta-analysis, we also found that the presence of BBD increases risk of recurrence of UTI by almost two times in patients with primary VUR. Functional constipation was documented in almost one-third of the patients with either VUR or UTI. Prevalence of BBD in patients with UTI without VUR is clearly higher, in the present meta-analysis, than in the general population of school-going children (20%) (55). This higher prevalence of BBD in children with UTI than the general population might point toward a strong association between BBD and UTI.

We found higher prevalence of BBD in patients with primary VUR than did a meta-analysis in the 2010 guideline for management of VUR by the AUA. In the meta-analysis by AUA, pooled prevalence of BBD in 15 studies was 31%. We used a predefined strategy for selection of the studies, which resulted in inclusion of different studies compared with those included in the meta-analysis by AUA. We also found that BBD is more common in girls with VUR, which could explain higher risk of breakthrough UTI in girls. Gaither et al. also reported higher risk of BBD in girls (56). Prevalence of BBD in patients with primary VUR, in the present meta-analysis, has varied from 18 to 91% (14–16, 18–23, 27, 29, 30, 32–35, 37–42, 44–47, 49–52, 54–58). This large variation in prevalence is likely due to multiple factors, which include characteristics of study population, study design, intervention, and assessment tool used for BBD. The largest randomized trial (RIVUR) in patients with primary VUR reported almost similar prevalence of BBD (56%) as in the present meta-analysis.

The relationship between VUR, BBD, and recurrent UTIs is complex and not so well-understood. A previous report from Shaikh et al. showed that patients with both VUR and BBD have the highest rate of recurrent UTIs than have patients with only VUR or BBD (5). This meta-analysis underscores the same fact and showed almost two-fold higher risk of recurrent UTIs in patients with coexisting VUR and BBD than in patients with VUR alone. Reanalysis of RIVUR trial showed that a subgroup of patients with both VUR and BBD had the most benefit from antibiotic prophylaxis. Significant reduction in recurrent UTIs following successful management of BBD with urotherapy has been reported, which again suggests a strong role of BBD in recurrence of UTI (57, 58). Hence, evaluation for BBD is essential while planning management for patients with primary VUR.

Prevalence of functional constipation was reported to be 9.5% in healthy children in a recent systematic review (59). In our review, prevalence of constipation was almost three times higher in children with or without VUR, suggesting that BBD is an important risk factor for UTI in toilet-trained children. A systematic review reported that 37–64% patients with functional constipation have lower urinary tract symptoms, hinting toward the association of BBD.

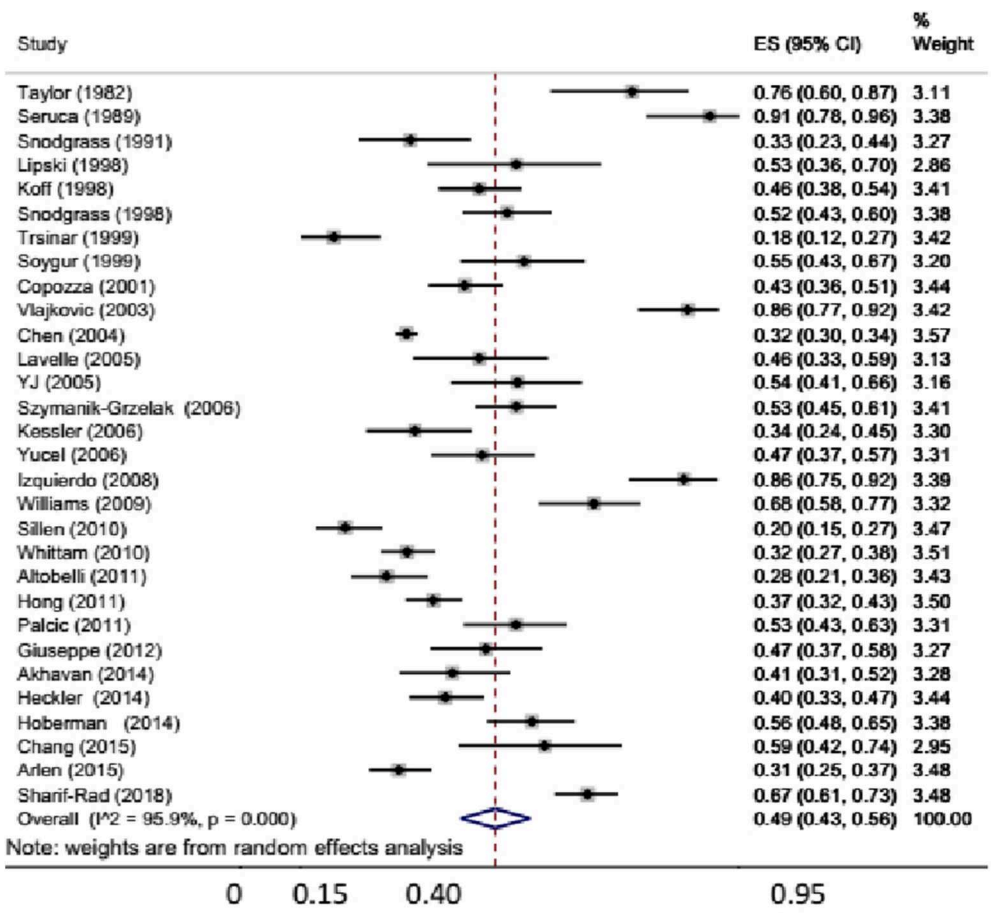

FIGURE 3 | Prevalence of bladder dysfunction in children with primary vesicoureteral reflux (VUR).

Limitations and Strengths

There are few limitations of our systematic review. Large heterogeneity for final pooled prevalence could be considered a limitation; however, because there is no existing standardized diagnostic criterion to define BBD in children, we had to use all previous studies that provided data for BBD prevalence using various definitions. Second, the large heterogeneity could be because studies with all kinds of study design have been used in the present review, although in the sensitivity analysis, we could not find any major difference in various subgroups. Third, we had to exclude many studies that included infants, as these children aged < 1 year are likely to be non-toilet trained and because diagnosing BBD in them is difficult. This exclusion criterion was defined *a priori*. Finally, we had limited our search to English-language databases only; hence, it might have resulted in exclusion of few studies published in non-English languages.

This systematic review has several strengths. First, we followed a rigorous methodology that included a comprehensive search of three major databases of medical literature, predefined protocol for study selection process, data extraction, and a statistical analysis that was registered in PROSPERO. We provided estimated pooled prevalence of BBD separately, in children with UTI only without VUR and other cohort of patients with primary VUR. We also showed that in patients with VUR, prevalence of BBD is higher in girls, which could explain higher number of recurrent UTIs in girls. Finally, we also assessed relative risk of recurrence of UTI in patients with both VUR and BBD and with VUR alone.

CONCLUSIONS

In summary, this systematic review of currently available literature shows that BBD is common in toilet-trained children presenting with UTI with or without primary VUR. A subgroup meta-analysis also shows that functional constipation is common in these children, with almost every third child affected with it. We also found that the presence of both BBD and VUR doubles the risk of recurrence of UTI; hence, all children presenting with UTI should be carefully evaluated for presence of BBD and managed accordingly. As BBD is an important risk factor for UTI recurrence, in future, intervention trials for patients with primary VUR should be stratified as per presence of BBD.

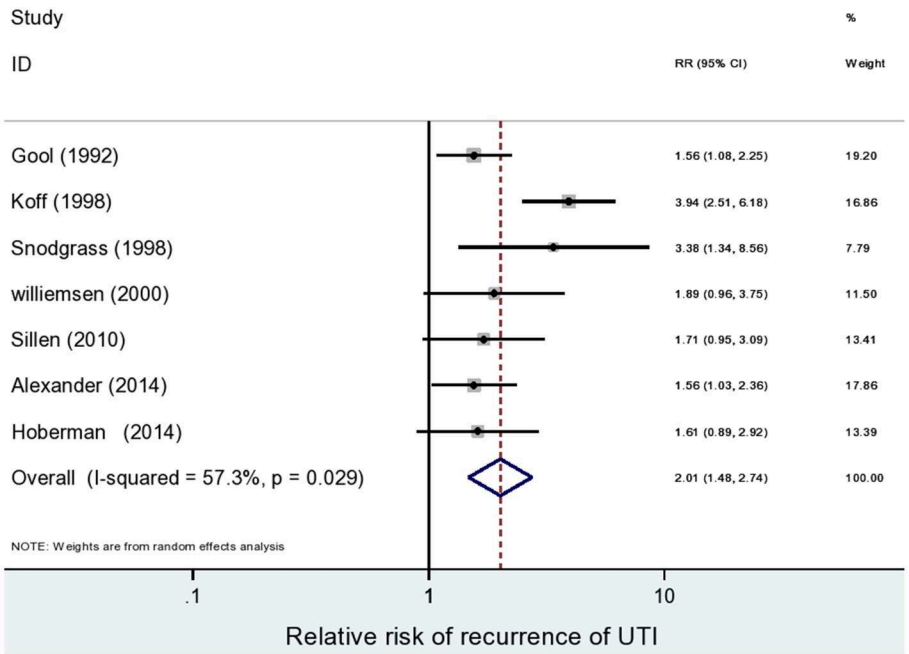

FIGURE 4 | Risk of recurrence of urinary tract infection (UTI) in patients with vesicoureteral reflux (VUR) and bladder and bowel dysfunction (BBD) compared with patients without BBD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Materials**.

AUTHOR CONTRIBUTIONS

JM conceived of the presented idea, formulated the protocol, and wrote the manuscript. JM and GM did independent data collection and analysis. PH decided on conflicting data interpretation. AS, AB, and PH provided critical feedback. All authors discussed the result and helped shape the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2020.00084/full#supplementary-material

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Adrenal Insufficiency in Children With Nephrotic Syndrome on Corticosteroid Treatment

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Background: Adrenal insufficiency can result from impaired functions at all levels of hypothalamic-pituitary-adrenal (HPA) axis. We here studied risk factors associated with adrenal insufficiency in children receiving prolonged exogenous steroid treatment for nephrotic syndrome.

Method: We performed low-dose Synacthen tests (LDSTs, 0.5 μg/m^2) in children with steroid-sensitive nephrotic syndrome 4–6 weeks after discontinuation of the corticosteroid therapy. We measured early morning serum cortisol levels at baseline and at intervals of 10, 20, 30, and 60 min following the stimulation test. We defined normal HPA axis stimulation responses as those with peak cortisol cut-off values >550 nmol/L.

Result: We enrolled 37 children for this study research. All children enrolled had normal early morning cortisol levels. However, 13 (35.1%) demonstrated HPA axis suppression (by LDST) 4–+6 weeks after discontinuation of oral prednisolone. Nephrotic syndrome diagnosed before 5 years of age (OR, 0.75; 95% CI, 0.57–0.99; $p = 0.043$), and steroid-dependence [OR, 5.58; 95% confidence interval (CI), 1.06–29.34; $p = 0.042$] were associated with increased risk of developing adrenal suppression after steroid discontinuation.

Conclusion: HPA axis suppression, may go unnoticed without proper screening. A normal early morning cortisol level (275–555 nmol/L) does not exclude adrenal insufficiency in children with steroid-sensitive nephrotic syndrome. Further screening with LDSTs, particularly in children younger than 5 years at diagnosis, may be warranted.

Keywords: adrenal insufficiency, steroid withdrawal, nephrotic syndrome, HPA axis, cortisol, low-dose Synacthen test, adrenal suppression

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INTRODUCTION

The most common glomerular disease in childhood is idiopathic nephrotic syndrome (NS), for which corticosteroids are the first line of treatment. Almost 80% of children with NS demonstrate steroid responsiveness, achieving complete remission within 4 weeks. However, ∼40–50% of these children are steroid dependent and require a long course of steroids, leaving them vulnerable to the adverse effects of chronic steroid usage, such as stunted growth, hypertension, obesity, secondary diabetes, vitamin D deficiency and secondary osteoporosis, cardiomyopathy, and

hypothalamic-pituitary-adrenal (HPA) axis suppression. Most of these conditions are well known, but HPA axis suppression has to date been understudied and under-reported.

The recommended treatment regimen for NS at diagnosis, according to the KDIGO guidelines (1), is with induction of prednisolone at 60 mg/m^2/day, or 2 mg/kg/day, (maximum 60 mg/day) for 4–6 weeks. This will be followed by alternate-day prednisolone given in single doses at 40 mg/m^2 or 1.5 mg/kg (maximum 40 mg on alternate days), for 2–5 months with tapering of the dose. This is a longer duration of steroid therapy compared to the original ISKDC guideline (2) which consisted of 4 weeks induction and 4 weeks of 40 mg/m^2 3 days a week. Recently, PREDNOS trial (3) compared the pattern of disease relapse in patients receiving 8 weeks vs. extended 16 weeks of corticosteroids. There was no significant difference in time to first relapse in these two groups. They found that extended corticosteroid treatment reduced the healthcare resource use and only made a small improvement in quality of life. Treatment of relapse is different from initial presentation. Alternate day dosing of 0.1–1 mg/kg for at least 3 months is recommended in frequently relapsing and steroid dependent NS. The steroid weaning regimen may vary based on the individual patient's previous experiences with relapses, steroid dependency, frequency of relapses, steroid toxicity and the use of steroid sparing agent. Close monitoring is imperative to detect complications associated with prolonged use of high-dose corticosteroids.

The physiological secretory rate of endogenous steroids in the intact HPA axis was ∼6 mg/m^2/day. Exogenous steroid doses were adjusted above this secretory rate (8–10 mg/m^2/day) as their bioavailability is reduced by gastric acids and first-pass metabolism in the liver. High-dose steroids are defined as supraphysiological steroid levels. HPA axis suppression is dependent on the total duration, total cumulative dose, and potency of the steroid used (4).

Adrenal insufficiency can be primary, secondary, or tertiary. Primary adrenal insufficiency results from intrinsic adrenal cortex diseases. Secondary and tertiary adrenal insufficiencies are caused by impaired production or action of corticotrophin, and are collectively known as central adrenal insufficiency. Overall, the prevalence of secondary adrenal insufficiency is 150–280 per million inhabitants in a population (5) and the condition can occur in children on high-dose and prolonged steroid prescriptions (4).

Various stimuli may activate the HPA axis to maintain normal body homeostasis and to combat stress, such as that induced by infections and endotoxins (6),absence or reduced glucocorticoid negative feedback (7), and hypoglycemia. Suppression of this critical mechanism via endogenous or exogenous pathways may alter the body's adaptive processes, triggering a pathogenic cascade. Adrenal insufficiency is associated with significant morbidity and mortality when inadequately treated during periods of intercurrent illness (8).

Different methods of assessing the HPA axis function exist. The insulin tolerance test and the Standard Short Synacthen test (SSST) were frequently used, but their limited reliability, tediousness, and associated adverse events led to the low-dose Synacthen test (LDST) being the preferred test nowadays. Moreover, LDST can detect mild degrees of adrenal insufficiency missed by SSST (9).

MATERIALS AND METHODS

Study Population

We identified 119 patients from the admissions list of the Pediatrics Institute of the Hospital Kuala Lumpur (Malaysia) between January 2017 and January 2018. We identified 119 patients as having steroid-sensitive NS, and enrolled 37 patients who fit the study inclusion criteria. Eligible patients had: (i) steroid-sensitive idiopathic NS; (ii) achieved remission; and (iii) been off steroids for 4–6 weeks. We excluded patients with: (i) steroid-resistant NS; or (ii) secondary NS. **Figure 1** summarizes the selection process (**Table 1**).

Data Collection

We used a pre-defined standardized data collection sheet for extracting data that contained information on patients' socio-demographic information (gender, age, ethnicity), anthropometric measurements, age at diagnosis, total number of relapses, and total duration of steroid use before LDST.

We collected blood samples for morning serum cortisol and ACTH levels, and for the LDST after steroid discontinuation during a planned visit. We then examined the patients every 3 months for a year to monitor their disease progress, their growth parameters, and to detect relapses or steroid toxicity.

LDST

Synacthen was given intravenously (0.5 μg/m^2) and serum cortisol levels were measured before and after the administration. The peak cortisol response at any time within 60 min should exceed 550 nmol/L (10). We used a lower Synacthen dose 0.5 μg/m^2 than the conventional 250 μg/m^2 dose based on the observation that the low-dose ACTH stimulation test using 1 μg of tetracosactrin can be used to detect mild secondary adrenal insufficiency (9). A cortisol increment at 30 min above the basal level is a measure of adrenal reserve and a low absolute level indicates adrenal insufficiency.

We educated parents about hydrocortisone replacement at times of illness or stress during transient adrenal insufficiency periods (11, 12). We informed parents to give oral hydrocortisone at three times the physiological replacement dose (30 mg/m^2/day) divided into three equal doses) during moderate illnesses such as fever and minor injury (this is called hydrocortisone "stress dosing"). During severe illnesses such as serious injury or trauma, or for anesthesia for surgery, we prescribed intravenous hydrocortisone at 10 times the physiological replacement dose (100 mg/m^2/day divided into four equal doses) (11).

For study subjects who relapsed during the study period, treatment was instituted as per International Study of Kidney Disease in Children (ISKDC) guideline. Patients who developed an intercurrent illness during the study period were treated as per other children with NS. Assessment of the volume status, the need for antibiotics and steroid replacement therapy was on the discretion of the treating physician.

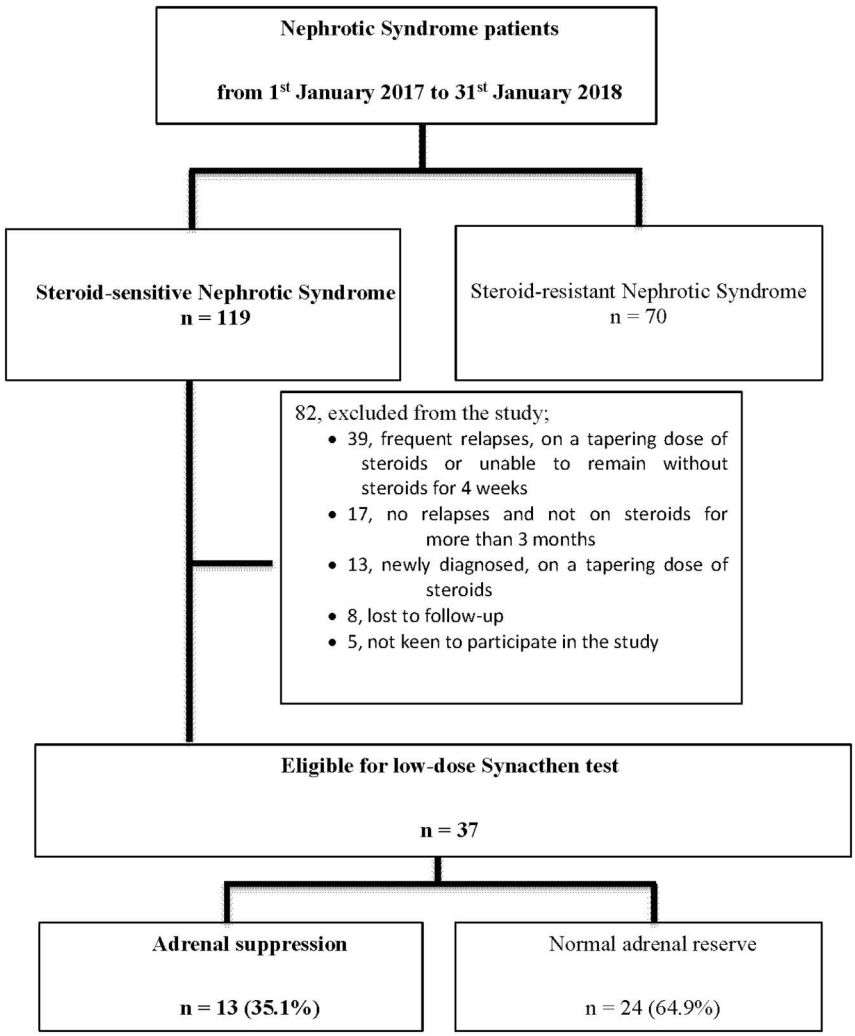

FIGURE 1 | Overview of the study flow.

TABLE 1 | Common definitions in nephrotic syndrome.

Remission	Urine albumin nil or trace for three consecutive days
Relapse	Urine albumin 3+ or more for three consecutive days
Frequent relapses	Two or more relapses in the initial 6-month period or more than three relapses in any 12 months
Steroid dependence	Two consecutive relapses when on alternate day steroid therapy or within 14 days of its discontinuation
Steroid resistance	Absence of remission despite therapy with daily prednisolone at a dose of 60 mg/m^2/day

Ethical Considerations

We registered this study with the National Medical Research Register (NMRR) under the identification number NMRR-16-1427-30586 (IIR). The Medical Research Ethics Committee (MREC), the University Malaya Medical Centre (UMMC) approved the study under the registration number MREC UMMC ID: 2016816-4142. This study was supported by a grant from the University Malaya Research Fund Assistance (BKP034-2015). We obtained written informed consent from all parents or guardians of the patients prior to starting the study. All children enrolled were also required to sign an assent form or to agree verbally. We performed all tests in accordance with approved guidelines.

Statistical Analysis

We used descriptive statistics to compare baseline characteristics between children with adrenal suppression and those with normal adrenal function. We expressed numerical data as means and standard deviations, and tabulated categorical variables as predicted numbers and percentages. We first used simple logistic regression analyses to determine unadjusted associations of factors of interest, and then applied multivariate analyses using the logistic regression model to identify predictors of HPA

insufficiency. We performed data analyses using the Statistical Package for Social Science software version 23.0. The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

RESULTS

Between 1st January 2017 and 31st January 2018, a total of 189 patients received treatment for NS in the Pediatric Institute of the Hospital Kuala Lumpur in Malaysia. Of 189 patients, 70 (37%) were steroid-resistant and 119 patients were steroid-sensitive, but only 31% of the patients (37 of 119) fit the eligibility criteria for enrolment; we excluded 69 patients, lost eight to follow-up, and encountered five patients who chose not to participate in the study (**Figure 1**).

Our study population showed a greater preponderance of males, with a male to female ratio of 3.6:1. The majority of patients were of Malay ethnicity and consisted of 26 patients (70.3%) and the rest were Chinese and Indians (in almost equal proportion). The mean age at diagnosis of NS was 3.38 years (±2.28), and the mean age at LDST was 9.47 years (±3.73). Thirty-four out of 37 patients (91.9%) were 5 years old or younger. The mean duration of the NS was 6.09 years (±3.44), with 21/37 (43.2%) having the illness for more than 5 years (**Table 2**).

Almost two thirds of the patients, 62.2% (23/37), had evidence of steroid toxicity. However, only 54% (20/37) had received a steroid-sparing agent (SSA). We possess no data that can explain this discrepancy. The SSAs prescribed were cyclophosphamide (13), levamisole (12), cyclosporine (7), rituximab (3), mycophenolic acid (1), and tacrolimus (1). Four patients received three or more types of SSA, six patients received two types of SSA, and the remaining 10 patients received a single agent. We categorized the steroid toxicities according to the affected system: immunosuppression; cardiovascular (hypertension); eye involvement (cataract, glaucoma); and musculoskeletal (short stature, cushingoid features, and skin striae). Eight out of 37 patients (21.6%) had frequent relapses and 20 (54.1%) patients were steroid dependent.

Thirteen out of 37 patients (35.1%) had HPA axis suppression. All of them were younger than 5 years of age and 76.9% were boys ($n = 10/13$). The mean durations of steroid usage were 66 (±121.25) weeks in those who developed HPA axis suppression and 30 (±35.31) weeks in those who did not. Ten of those patients with HPA axis suppression (76.9%) were steroid dependent.

We found that children with a younger age (mean age at diagnosis of 2.54 years) were more likely to develop HPA axis suppression than older children (95% CI, 0.567–0.990). Steroid-dependent patients were six times more likely to develop HPA axis suppression than to maintain normal HPA axis function (95% CI, 1.06–29.34) (**Table 3**).

The mean basal plasma cortisol concentration was significantly higher by 40% in patients with a normal adrenal response than in patients with a suppressed adrenal response [303.92 (±133.99) vs. 215.69 (±102.61) nmol/L, $p = 0.012$]. After the injection of 0.5 μg/m^2 Synacthen, both groups of patients demonstrated serum cortisol peaks at 20 min. The mean 20-min peak serum cortisol concentration was lower in the children with suppressed responses than in those with

TABLE 2 | Demographic and clinical characteristics of patients with normal HPA axis and HPA axis suppression.

Characteristics		Normal HPA axis ($n = 24$) (64.9%)	HPA axis suppression ($n = 13$) (35.1%)
Gender	Male	19 (79.2)	10 (76.9)
	Female	5 (20.8)	3 (23.1)
Race	Malay	16 (66.7)	10 (76.9)
	Chinese	5 (20.8)	1 (7.7)
	Indian	3 (12.5)	2 (15.4)
Age at diagnosis, years, mean ± SD*		3.83 ± 2.69	2.54 ± 0.80
Age at diagnosis	0–5 years	21 (87.5)	13 (100)
	6–11 years	2 (8.3)	0 (0.0)
	12–18 years	1 (4.2)	0 (0.0)
Age at LDST, years, mean ± SD*		10.44 ± 4.25	7.67 ± 1.32
Age at LDST	0–5 years	5 (20.8)	2 (15.4)
	6–11 years	10 (41.7)	11 (84.6)
	12–18 years	9 (37.5)	0 (0.0)
Duration of illness, years, mean ± SD*		6.61 ± 3.99	5.13 ± 1.86
Duration of illness	≤ 5 years	10 (41.7)	6 (46.2)
	> 5 years	14 (58.3)	7 (53.8)
Duration of steroid use, weeks, mean ± SD*		30.54 ± 35.31	66.05 ± 121.25
Duration of steroid use	< 20 weeks	13 (54.2)	7 (53.8)
	≥ 20 weeks	11 (45.8)	6 (46.2)
The dose of steroid use, mg/m^2/days, mean (SD)*		22.37 ± 8.30	25.63 ± 11.36
Steroid-dependent	No	14 (58.3)	3 (23.1)
	Yes	10 (41.7)	10 (76.9)
Frequent relapse	No	20 (83.3)	9 (69.2)
	Yes	4 (16.7)	4 (30.8)
Steroid toxic	No	8 (33.3)	6 (46.2)
	Yes	16 (66.7)	7 (53.8)
Use of steroid-sparing agent (SSA)	No	9 (37.5)	8 (61.5)
	Yes	15 (62.5)	5 (38.5)
Immuno-suppression prior to LDST	Prednisolone alone	19 (79.2)	11 (84.6)
	Combination with SSA	5 (20.8)	2 (15.4)
Comorbid diseases	No	17 (70.8)	11 (84.6)
	Yes	7 (29.2)	2 (15.4)
ACTH, pmol/L, mean ± SD		20.52 ± 14.20	40.92 ± 85.14
ACTH Result	Normal	22 (91.7)	10 (76.9)
	Abnormal	2 (8.3)	3 (23.1)

SD, standard deviation.

normal responses; this difference, however, was not statistically significant [467.54 (±69.16) and 630.33 (±97.86), respectively, $p = 0.869$] (**Figure 2**).

DISCUSSION

We analyzed cortisol levels in patients with steroid-sensitive NS using LDST and confirmed that 13 of them (35.1%) had HPA axis suppression. Multivariate analyses showed that younger age at diagnosis and steroid dependency were the two significant factors associated with HPA axis suppression. We defined HPA axis suppression as cortisol levels <550 nmol/L despite stimulation. This definition may help to anticipate adverse outcomes associated with inappropriate cortisol responses during "stress periods" in children on steroid therapy.

The duration of steroid usage in children with NS varies with the frequency of relapses, steroid dependency, presence of steroid toxicity, and different medical practices. In our center, we administer steroids as per the ISKDC guideline. Case-to-case evaluations are frequent as some patients maintain remission states and avoid relapses when given prolonged steroid courses.

The HPA axis functions as a complex neuroendocrine feedback mechanism crucial during stress adaptation (14); it is influenced by circadian and ultradian rhythms (15). Animal studies have shown that the HPA axis regulates the secretion of glucocorticoids, which have dominant effects in regulating cardiovascular, metabolic, cognitive, and immunological states (13, 16, 17). The paraventricular nuclei (PVN) of the hypothalamus initiate axis activation (18, 19). Exposure to stressors stimulates the secretion of corticotrophin-releasing hormone (CRH), and this hormone eventually stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH in turn binds to its receptors on the adrenal gland and induces cortisol secretion (20, 21), which inhibits ACTH and CRH production (negative feedback mechanism), in a manner similar to that in children with excess exogenous glucocorticoids (22).

Children with NS receive supraphysiologic doses of steroids that may potentially inhibit cortisol production. When this inhibition lasts longer than the duration of the corticosteroid exposure, it is called adrenal suppression. Although the effect may be transient, it can result in significant morbidity during periods of physiologic stress (23). In children receiving high-dose steroids, adrenal suppression is not apparent and clinicians need to be aware that abrupt withdrawal may trigger adrenal crises (4, 22).

The suppression of the HPA axis can occur after a single dose of steroid, but it typically recovers quickly. However, the long-term use of systemic corticosteroids may take a long time to recover (24). Studies have looked at the effects of corticosteroid use on the HPA axis and at the corresponding recovery times. The recovery time depends on the glucocorticoid potency and its types, the duration of therapy, and the weaning protocols; therefore, estimations are difficult, and comparisons between studies are complex also because of the different diagnostic tests used to assess the adrenal function.

In our study, the local prevalence of adrenal suppression in children with NS 4–6 weeks after steroid discontinuation was 35.1%. This figure is lower than that reported in a study by Abeyagunawardena et al. (25), which showed that 20 of 32 (62.5%) children on alternate-day prednisolone for

TABLE 3 | Multivariate analysis for children with nephrotic syndrome on corticosteroid treatment.

Variables		ß*	Df**	*p*-value	Adjusted $^{+}$OR (95% $^{++}$CI)
Age at diagnosis		−0.288	1	0.043	0.749 (0.567–0.990)
Steroid-dependent	No	1.719	1	0.042	5.58 (1.06–29.34)
	Yes				

**ß, beta; **Df, degrees of freedom; $^{+}$OR, odd ratio; $^{++}$CI, confidence interval; LDST, low-dose Synacthe n test.*

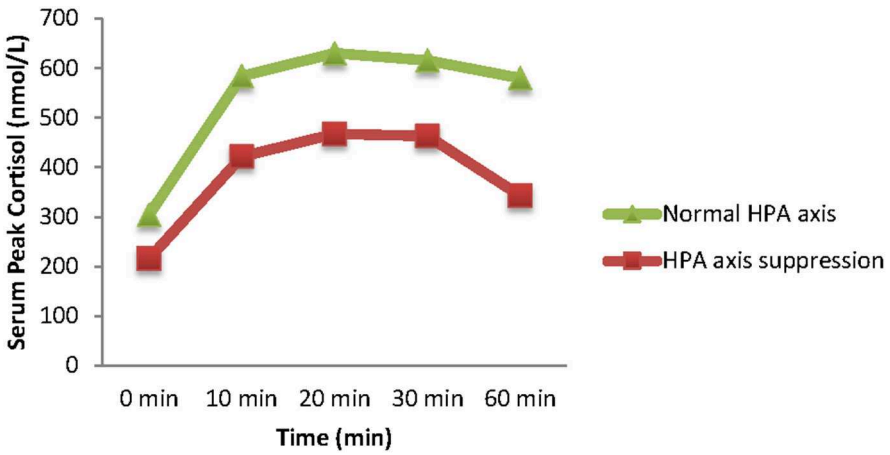

FIGURE 2 | Serum peak cortisol concentrations (means) from baseline (time 0) following stimulation with 0.5 μg/m^2 Synacthen (10, 20, 30, and 60 timepoints).

steroid-dependent NS had HPA axis suppression. Furthermore, their cut-off cortisol level to diagnose adrenal suppression was found to be <500 nmol/L. Mantan et al. (26) also studied children on low dose steroids and 28 of 70 children had adrenal suppression. Diagnosis is based on a single early morning dose of serum cortisol. Those at a risk of acquiring adrenal suppression in his study were mainly frequent relapsers and steroid resistant NS.

The variety of investigations used and different cut-off values to examine the HPA axis are potential caveats concerning the validity of the assessment results. Recently, Mongioi et al. (27) studied the accuracy of LDST in 103 adults. All of them had primary AI or hypothalamic-pituitary diseases. Those receiving steroids were excluded. The receiver operating characteristic curve showed 100% sensitivity and 67.3% specificity when 500 nmol/L was used as the serum cortisol cut-off value. Our study, however, is based on the study done by Tordjman et al. (9), which used 550 nmol/L of serum cortisol as the cut-off value in LDST, resulting in 100% sensitivity and 89% specificity.

We found steroid toxicity in 62.2% of our cohort, suggesting that predicting which patients are at risk of developing adrenal crisis is impossible based on steroid toxicity alone. In a resource limited setting, conducting screening tests for HPA axis suppression in every patient on corticosteroid treatment is additionally unfeasible. Therefore, predicting patients who warrant screening for adrenal suppression is important. Based on our study findings, patients younger than 5 years, and who are steroid-dependent, should be screened for adrenal suppression by measuring both a morning serum cortisol level and an LDST. Identifying children at risk for adrenal suppression early would reduce the burden of illness in terms of morbidity, mortality, and cost of care.

We acknowledge the limitations of our study. We had no data on the frequency of hospital admissions, severity of intercurrent illnesses, or recovery rates to compare between patients with HPA axis suppression and those without HPA axis suppression. We also failed to examine the recovery periods in children with HPA axis suppression.

In conclusion, a life-threatening HPA axis suppression may go unnoticed without proper screening. A normal early morning cortisol level (275–555 nmol/L) does not rule out adrenal insufficiency in children with steroid-sensitive NS. Further screening with LDSTs may be warranted, particularly in children younger than 5 years of age at diagnosis.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to Karmila Abu Bakar, karmila@um.edu.my.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by NMRR. 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

All authors worked together in designing the study, getting ethics approval, recruiting patients, performing the low-dose synacthen test, analyzing the data, and writing up the paper.

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