Causal and Putative Pathogenic Mutations Identified in 38.6% of Children with Primary SRNS in South Africa

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Abstract

The aim was to identify causal mutations in genes implicated in steroid resistant nephrotic syndrome (SRNS) within a South African population. We enrolled 119 children with primary NS; 71 SRNS and 48 steroid-sensitive NS. All children with SRNS underwent kidney biopsy. We first genotyped the *NPHS2* gene for the p.V260E variant in all NS cases (n= 119) and controls (n= 219). To further identify additional variants, we performed whole-exome sequencing and interrogated ten genes (*NPHS1, NPHS2, WT1, LAMB2, ACTN4, TRPC6, INF2, CD2AP, PLCE1, MYO1E*) implicated in SRNS/FSGS in 56 SRNS cases and 29 controls; we also performed exome sequencing on two patients carrying the *NPHS2* p.V260E mutation as positive controls. The overall detection rate of pathogenic mutations in children with SRNS was 27/70(38.57%): 15(21.43%) carried the *NPHS2* p.V260E mutation and 12(17.14%) carried a pathogenic mutation in the heterozygous state in *INF2* (n=8), *CD2AP* (n=3) or *TRPC6* (n=1) genes. *NPHS2* p.V260E homozygosity was specifically associated with biopsy-proven FSGS, accounting for 23.81% of Black children (15 of 63) with SR-FSGS. No causal mutations were identified in *NPHS1, WT1, LAMB2, PLCE1, MYO1E* and *ACTN4.* We report four novel variants in *INF2, PLCE1, ACTN4* and *TRPC6.*

Conclusion: The *NPHS2* p.V260E mutation is a prevalent cause of SR-FSGS among Black South African children occurring in 23.81% of children with SRNS. Screening all Black African children presenting with NS for *NPHS2* p.V260E will provide a precision diagnosis of SR-FSGS and inform clinical management.

What Is Known

- The high rate of steroid resistance in Black South African children with focal segmental glomerulosclerosis (FSGS) compared to other racial groups is partially explained by the founder variant *NPHS2* p.V260E.

What Is New

- We report putative causal missense variants predicted to be pathogenic in *INF2, CD2AP* and *TRPC6* among SR-FSGS children, demonstrating the utility of genetic testing for precision diagnosis of SR-FSGS to inform family planning and clinical management.

Introduction

Primary nephrotic syndrome (NS) is one of the most frequent glomerular diseases in childhood characterised by massive proteinuria, hypoalbuminemia, hyperlipidaemia and oedema[1, 2]. Most children with primary NS have a gratifying response to conventional corticosteroid therapy with a good prognosis long-term. However, 10-20% of children show partial, late or complete resistance to corticosteroid therapy and are labelled as steroid-resistant NS (SRNS)[3, 4]. SRNS continues to be one of the most common intractable causes of chronic kidney disease (CKD) in children with 50-70% of these children developing progressive loss of kidney function leading to end-stage kidney disease (ESKD) within 5-10 years of diagnosis[5]. The therapeutic options in SRNS are often ineffective in inducing complete remission and complicated by significant toxicity adding to the morbidities, high cost, and sometimes even mortality associated with these therapies[6].

Previous studies emphasize the considerable influence of racial and geographical factors on steroid response, histopathological pattern and outcome of primary NS[7–9]. The two most common histopathological features noted in childhood NS are focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD)[10]. The incidence of FSGS is higher in children of African ancestry compared to those of European ancestry[11–13]. In South Africa, the most common histological diagnosis in Black children is FSGS, whereas MCD is more frequently diagnosed in White and Indian children[14–16]. In a recent review of primary NS in the New Millennium at a tertiary/quaternary referral centre in Durban, KwaZulu-Natal, South Africa, the prevalence of FSGS in children was reported to be 70.1% of 209 children undergoing kidney biopsy; the prevalence in Black African children being as high as 73.5%[17]. The higher prevalence rate of biopsy-proven SR-FSGS in children and adults, among certain ethnic groups, suggests the role of genetic susceptibility to disease[18].

Mutations in several genes that are highly expressed in the glomerular filtration barrier and podocytes have been reported as causative agents in paediatric NS[19]. To date, more than 50 monogenic causes of SRNS (predominantly FSGS) have been identified displaying autosomal recessive (AR) or autosomal dominant (AD) modes of inheritance[19]. Mutations in AR genes tend to present early in childhood whereas AD genes present later in childhood or adulthood and tend to be less penetrant[20, 21]. Numerous genes encoding slit diaphragm proteins expressed by podocytes have recently been discovered and have been shown to be associated with primary SRNS[22]; including mutations in *NPHS1, NPHS2, PLCEl*, and *MYO1E*, showing AR inheritance and in *ACTN4, TRPC6, IFN2* and *CD2AP* showing AD inheritance[23–27]. Genes encoding proteins related to transcriptional activity in podocytes, such as *WT1* (AD inheritance) are also critical for podocyte integrity and function[28, 29]. Additionally, AR mutations in *LAMB2* (AR) encoding laminin beta 2 are a cause of SRNS; *LAMB2* is secreted by podocytes and contributes to the configuration and spatial architecture of the glomerular basement membrane[22, 30].

A precise genetic diagnosis in patients with SRNS is more conclusive than a histopathologic classification enabling a better prediction of prognosis to mediate clinical management of the child[31]. Like many genetic disorders, SRNS shows ethnic and geographic differences that may arise from founder or population-specific mutations. In South Africa, there are limited genetic studies on childhood-onset NS. Asharam *et al.* identified a common founder mutation *NPHS2* p.V260E variant accounting for a third or more of SR-FSGS in Black South African children living in the KwaZulu-Natal province[18]; this association was extended to other black ethnic groups[32]. However, further research is required to identify additional mutations that account for the high rate of SRNS in South African Black children. This study aimed to screen for causal mutations in ten genes (*NPHS1, NPHS2, WT1, LAMB2, ACTN4, TRPC6, INF2, CD2AP, PLCE1, MYO1E*) previously associated with SRNS, in a South African population with primary SRNS seen at a tertiary hospital in KwaZulu-Natal, South Africa.

Material And Methods

Study participants

This study protocol was reviewed and approved by Biomedical Research Ethics Committee of the University of KwaZulu-Natal and Department of Health, KwaZulu-Natal, South Africa, approval number BE374/17. Children 1-18 years old with primary NS (n= 118) and biopsy-proven FSGS treated between January 2000 to February 2019 at Inkosi Albert Luthuli Central and King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa, were enrolled in the study. Children that were HIV negative, with no kidney disease and similar geographic and ethnic backgrounds, were enrolled as the control group (n= 219). Patients with incomplete data, indeterminate histopathology, congenital NS, secondary forms of NS, and those lost to follow-up (defined as a patient not having contact with the hospital/clinic for 180 days or more since their last recorded expected date) or who refused to participate in the study, were excluded.

Data capture

All patient data was extracted from the hospitals electronic records using MEDITECH®. Hypertension was defined by the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents as follows: normal if <90th percentile; prehypertension if ≥90th to <95th percentile or >120/80 mmHg in adolescents; Stage 1 HPT if between ≥95th to 99th percentile plus 5 mmHg; Stage 2 HPT if >99th percentile plus 5 mmHg[33]. Patients estimated glomerular filtration rate (eGFR) was calculated using the modified Schwartz formula[34, 35] and children were classified according to Kidney Disease Outcomes Quality Initiative (KDIGO) guidelines for CKD[36].

Treatment

All children with primary NS were given oral prednisone at a dose of 2 mg/kg (maximum 60 mg) for six weeks, followed by the same dose on alternative days for another six weeks, and reduced to none over a two and a half-month period. Failure to respond to oral steroids after six weeks was taken as steroid resistance in accordance with standard criteria[4]. Following a kidney biopsy, treatment for children with SRNS included *inter alia* intravenous pulse methylprednisolone and cyclophosphamide, calcineurin inhibitors (cyclosporin A, or tacrolimus) and mycophenolate mofetil in combination with low dose oral steroid and angiotensin-converting enzyme antagonists. All patients also received multivitamins and folic acid supplements.

Kidney biopsy

Examination of kidney biopsies was performed by light, fluorescent and electron microscopy. Histologic criteria for MCD were normal glomeruli by light microscopy, including the absence of segmental sclerosis and thickening of the glomerular basement membrane, and absence of significant deposits by immunofluorescence study. FSGS was defined as a histopathological lesion characterized by the presence of sclerosis in parts (segmental) of some (focal) glomeruli. FSGS was classified histopathologically according to the Columbia classification into the following categories: (i) classical FSGS or FSGS not otherwise specified, (ii) collapsing variant, (iii) tip variant, (iv) perihilar variant and (v) cellular variant.

DNA Extraction and Sequencing

Genomic DNA was extracted from whole blood using the ThermoFisher Scientific GeneJet DNA Purification Mini Kit as per manufacturers guidelines (Waltham, MA). We first genotyped the *NPHS2* gene for the p.V260E variant in SSNS (n= 48), SRNS (n=70) cases and controls (n= 219) (Figure 1) using TaqMan assay (ThermoFisher Applied Biosystems, Waltham, MA), since previous studies identified this mutation as the most frequent Mendelian cause of SRNS in Black South African children with both familial and sporadic disease[18, 32]. To identify additional variants, we performed whole-exome sequencing (WES) in 56 SRNS cases and 29 controls; we also performed exome sequencing on two patients carrying the *NPHS2* p.V260E mutation as positive controls. WES was performed by LC Sciences (Huston, TX). Extracted DNA underwent library prep according to the Agilent SureSelect XT (All Exon V5 +UTR) protocol and sequenced on Illumina HiSeq4000 device using paired-end sequencing to an average sequencing depth of > 80x. Image analysis and base calling were performed with the pipeline software using default parameters. Mapping was done using the human reference genome assembly (GRCh37/hg19).

WES variant filtering analysis

We limited our analysis of the WES sequencing results to variants in ten genes (*NPHS1, NPHS2, WT1, LAMB2, ACTN4, TRPC6, INF2, CD2AP, PLCE1, MYO1E*) implicated in SRNS/FSGS. We followed standard guidelines of investigating variants for Mendelian disorders from WES data[37, 38]. Alignment and variant calling was performed with the Centre for Cancer Research Collaborative Bioinformatics Resource (CCBR) pipeline (https://github.com/CCBR/Pipeliner). Due to our small sample size, we interrogated the top ten genes associated with childhood SRNS using analytical methodologies from Gee *et al*[39] and Sperling *et al*.[40]. Variants present in the homozygous or heterozygous state in control samples were excluded. Only protein-altering (missense) variants, insertion/deletion (indel), or splice-site variants were selected. In further filtering, we included single heterozygous variants that were not present in controls. The relatedness of samples was calculated using the kinship coefficient.

Statistical analysis

Data analysis was performed using Graph pad prism 5 software (Graph Pad Software, San Diego, CA, USA). The descriptive analysis was performed by calculating the means and standard deviations for continuous variables or numbers and percentages for categorical variables. All the data were normally distributed and tested using the Kolmogorov Smirnov test. A comparative analysis between two groups was calculated using a two-sample t-test for comparing the means and a Chi-square test for comparing categorical variables. Comparison of the distribution of age of onset in children carrying the mutation was calculated using one-way analysis of variance. A p-value <0.05 was considered statistically significant, except in the case of multiple comparisons where we corrected for gene number.

Results

Clinical characteristics

One hundred and eighteen children with primary NS were enrolled in this study; 70 children had SRNS and 48 children had steroid-sensitive (SSNS) (Figure 1). Pairwise inbreeding coefficient tests in PLINK showed no evidence of consanguinity among the 70 children and no evidence of relatedness among cases carrying causal or putative mutations. Sixty-three (90.00%) children with SRNS were Black African and seven (10.00%) were Indian. Nine (18.75%) children with SSNS were Black African and 39 (81.25%) were Indian. SRNS was much more frequent among Black children with NS; 87.50% Blacks *vs.* 15.22% Indians (95% CI: 0.52-0.91; $p< 0.0001$). FSGS was the most common histopathological finding present in 67 (95.71%) children. MCD was present in three (4.29%) children. According to the Columbian classification of the 67 FSGS cases, 40 (59.70%) children had a histopathological finding characterised as not otherwise specified, 20 (29.85%) had cellular variant, two (2.99%) collapsing variant, one (1.49%) had a tip variant and four (5.97%) were not categorizable as there was a mixed histology.

Clinical and laboratory data of children presenting with SRNS and SSNS are presented in Table 1. Children with SRNS (mean 99.79; 95% CI: 88.48-111.10 months) presented at a much older age than children with SSNS (mean 63.71; 95% CI: 49.66-77.75 months; $p= 0.001$). At disease presentation, mean urinary protein creatinine ratio was significantly increased in children with SRNS (mean 2.35; 95% CI: 2.19-4.01 g/mmol) compared to SSNS (mean 0.88; 95% CI: 0.64-1.13 g/mmol; $p= 0.017$). A significantly higher mean serum cholesterol levels were also noted in children with SRNS (mean 7.57; 95% CI: 6.66-8.48 mmol/l) *vs.* SSNS (mean 6.04; 95% CI: 5.51-7.23 mmol/l; $p= 0.013$). Serum albumin levels were increased in children with SRNS (mean 29.58; 95% CI: 21.97-28.33 g/l) when compared to SSNS (mean 28.53; 95% CI: 25.70-31.36 g/l), albeit non-significant ($p= 0.22$). Among children with SRNS, the mean period of follow-up was 25.11 months (95% CI: 19.12-31.10 months). At last hospital visit, 41(57.74%) patients had a normal eGFR, 22(30.99%) patients progressed to CKD stages II–IV, and 8(11.27%) progressed to ESKD. Three children received a kidney transplant from a related living donor and five were on maintenance dialysis. None of the children who were transplanted had a recurrence of the disease in the transplanted kidney.

Table 1
Clinical and demographic characteristics of primary steroid resistant nephrotic syndrome and steroid sensitive nephrotic syndrome cases

Clinical and biological data	**SSNS (mean±SD)**	**SRNS (mean±SD)**	***p* value**
N	48	70	
Female	16	36	
Male	34	34	
Ethnicity			**< 0.0001**
Black	9 (18.75%)	63 (90.00%)	
Indian	39 (81.25%)	7 (10.00%)	
Age at onset (months)	63.71 ± 48.37	99.79 ± 46.75	**0.001**
BMI (kg.m^2)	15.68 ± 7.84	20.35 ± 4.82	**0.013**
Blood pressure (mmHg)			
Normotensive	24 (48%)	34 (48.57%)	
Stage 1	14 (28%)	21 (30%)	
Stage 2	8 (16%)	15 (21.43%)	
Presence of oedema	19 (38%)	31 (44.29%)	
Histology			
FSGS		67 (95.71%)	
MCD		3 (4.29%)	
Other histology		0	
uPCR (g/mmol)	0.88 ± 1.01	2.35 ± 4.97	**0.017**
Serum albumin (g/L)	28.53 ± 11.33	29.58 ± 10.78	0.22
Serum cholesterol (mmol/L)	6.04 ± 2.76	7.57 ± 3.31	**0.013**
Duration of follow up (months)	45.36 ± 37.20	25.11 ± 25.68	**0.0078**
ESKD	0	8	
Time to ESKD (months)	0	30.00 ± 14.34	

SSNS: steroid sensitive nephrotic syndrome; SRNS: steroid resistant nephrotic syndrome; BMI: body mass index; uPCR: urinary protein; ESKD: end–stage kidney disease

Gene Mutations Detected In Four Genes

The overall detection rate of causal and putative causal mutations in children with SRNS was 27/70(38.57%): 15(21.43%) carried the *NPHS2* p.V260E mutation in the homozygous state and 12(17.14%) SRNS cases carried a pathogenic mutation in the heterozygous state in genes *(INF2, CD2AP*, and *TRPC6)* known to have AD inheritance mode. Mutations in the *NPHS2* (15/70, 21.13%) gene were most common, followed by mutations in *INF2* (8/70,11.43%), *CD2AP* (3/70, 4.29%) and *TRPC6* (1/70, 1.43%).

NPHS2 p.V260E homozygosity was specifically associated with biopsy-proven FSGS, accounting for 23.81% of Black children (15 of 63) with SR-FSGS but was present in none of the Indian children (0/7). In the heterozygous state, *NPHS2* p.V260E was present in one of 206 (MAF= 0.24%) Black healthy controls (gnomAD allele frequency- 0.00013). WES was performed on seven Indian and 49 Black SRNS cases; ten genes implicated in monogenic SRNS in the SRNS children were interrogated. Autosomal dominant putative causal mutations are shown in Table 2. For the *INF2* gene mutations, eight Black SRNS children carried the *INF2* p.P516L (c.1547C>T; p.Pro516Leu) variant in the heterozygote state; this variant was absent in controls. Whole exome sequencing of 100 Black South Africans found the frequency of *INF*2 to be 0.03 (personal communication from Dr. M. Ramsay, University of Witwatersrand, South Africa). Assuming that the population frequency is 3%, the probability that 8 or more out of 49 Black SRNS with WES data carry the *INF2* p.P516L variant by random chance is 1.4×10^{-5}; however, more population typing will be required to get a robust estimate of the population frequency of this African-specific variant. With a Bonferroni correction for 10 genes tested, $p = 1.4 \times 10^{-4}$. The three Black children with SR-FSGS with mutations of the *CD2AP* gene were carriers of the p.K633R mutation. A novel mutation, p.G162V in the *TRPC6* gene, predicted to be 'possibly damaging' by PolyPhen was identified in one Indian child with SR-FSGS and was absent in the controls.

Table 2
Description of variants identified in children with steroid resistant nephrotic syndrome

Gene, Amino acid change	Nucleotide change	RS number	Prediction		MAF according to GenomAD	Prevalence in control population	Reference PMID or ClinVar ID
			PolyPhen-2	SIFT			
Known causal variants							
NPHS2 V260E	c.3032-21A>T	775006954	Probably Damaging	Damaging	0.00013	1	PMID: 25349199; PMID:28658201; PMID: 26420286 PMID:23595123; PMID:22565185; PMID:21415313 PMID:20947785; PMID:15253708; PMID:14675423 PMID:30450462; PMID:31754646; PMID:26211502
Putative causal variants							
CD2AP K633R	c.1898A>G	116754410	Probably Damaging	Damaging	0.00755	0	RCV000398184.1; RCV000250382.1
TRPC6 G162V	c.485G>T	Novel	Probably Damaging	Damaging	Not reported	0	Present study
INF2 P516L	c.1547C>T	111589086	Probably Damaging	Damaging	0.00739	0	RCV000591895.2
Novel non-causal variants							
INF2 S409T	c.1226G>C	Novel	Benign	Tolerated	Not reported	0	Present study
PLCE1 A757E		Novel	Benign	Tolerated	Not reported	0	Present study
ACTN4 A58A	c.174C>T	Novel	N/A	N/A	Not reported	0	Present study

SIFT: sorting intolerant from tolerant; N/A: not available; gnomAD: Genome Aggregation Database

No pathogenic mutations were identified in *NPHS1, WT1, LAMB2, PLCE1, MYO1E* and *ACTN4*. However, we report four novel variants in *INF2, PLCE1, ACTN4* and *TRPC6* (Table 2).

Genotype-phenotype Associations

Cases with causal or putative pathogenic mutations in the homozygous or heterozygous state, respectively, had a histology of FSGS and none responded to first-line corticosteroid therapy (Table 3). As shown in Figure 2, age-of-onset was younger in children homozygous for *NPHS2* p.V260E compared to those carrying heterozygous mutations (mean 98.07; range 62-141 months *vs.* mean 156; range 119-202 months, respectively) (p= 0.03). Among the 12 children carrying heterozygous mutations, seven were treated with 2nd line immunosuppressive therapy. Partial remission with 2nd line immunosuppression was achieved for the child EN300232 heterozygous for *TRPC6* p.G162V and for child EN300052 heterozygous for *CD2AP* p.K633R; a second child with *CD2AP* p.K633R did not respond to 2nd line treatment. Five of 15 children (30%) with the *NPHS2* p.V260E progressed to ESKD whereas none of the children with *INF2* p.P516L progressed to ESKD over the mean 25.11 months of follow-up.

Table 3
Clinical characteristics of steroid-resistant nephrotic syndrome children with identified mutations

Patient No.	Sex	Ethnicity	Gene	Mutation	Age at onset (Months)	Dipstick analysis	Serum albumin	Histology	Response to corticosteroids (1st line)	Response to immunosuppressants (2nd line)	ESK
EN300027	Male	Black	*NPHS2*	V260E (hom)	86	3+	25	FSGS	No response	No response	YES
EN300041	Female	Black	*NPHS2*	V260E (hom)	80	2+	21	FSGS	No response	Not given	NO
EN300044	Female	Black	*NPHS2*	V260E (hom)	127	2+	29	FSGS	No response	No response	YES
EN300045	Female	Black	*NPHS2*	V260E (hom)	62	3+	19	FSGS	No response	Not given	NO
EN300047	Female	Black	*NPHS2*	V260E (hom)	90	2+	22	FSGS	No response	Not given	NO
EN300048	Female	Black	*NPHS2*	V260E (hom)	157	1+	39	FSGS	No response	No response	YES
EN300050	Male	Black	*NPHS2*	V260E (hom)	24	2+	25	FSGS	No response	Not given	NO
EN300055	Female	Black	*NPHS2*	V260E (hom)	25	2+	18	FSGS	No response	Not given	NO
EN300057	Female	Black	*NPHS2*	V260E (hom)	178	3+	19	FSGS	No response	No response	NO
EN300072	Female	Black	*NPHS2*	V260E (hom)	101	3+	15	FSGS	No response	No response	NO
EN300139	Female	Black	*NPHS2*	V260E (hom)	64	2+	16	FSGS	No response	Not given	NO
EN300305	Female	Black	*NPHS2*	V260E (hom)	61	2+	28	FSGS	No response	No response	YES
EN300310	Female	Black	*NPHS2*	V260E (hom)	141	2+	19	FSGS	No response	No response	YES
EN300329	Male	Black	*NPHS2*	V260E (hom)	99	3+	18	FSGS	No response	No response	NO
EN300355	Male	Black	*NPHS2*	V260E (hom)	176	3+	23	FSGS	No response	Not given	NO
EN300144	Male	Black	*INF2*	P516L (het)	81	4+	11	FSGS	No response	No response	NO
EN300022	Male	Black	*INF2*	P516L (het)	131	3+	26	FSGS	No response	Not given	NO
EN300156	Male	Black	*INF2*	P516L (het)	167	1+	45	FSGS	No response	No response	NO
EN300157	Female	Black	*INF2*	P516L (het)	220	1+	35	FSGS	No response	No response	NO
EN300312	Female	Black	*INF2*	P516L (het)	115	1+	72	FSGS	No response	Not given	NO
EN300313	Male	Black	*INF2*	P516L (het)	211	1+	17	FSGS	No response	Not given	NO
EN300345	Male	Black	*INF2*	P516L (het)	168	2+	43	FSGS	No response	No response	NO
EN300363	Female	Black	*INF2*	P516L (het)	169	3+		FSGS	No response	Not given	NO
EN300052	Female	Black	*CD2AP*	K633R (het)	62	4+	18	FSGS	No response	Partial remission	NO
EN300302	Female	Black	*CD2AP*	K633R (het)	175	2+	21	FSGS	No response	No response	NO
EN300322	Male	Black	*CD2AP*	K633R (het)	165	2+	35	FSGS	No response	Not given	NO

ESKD: end stage kidney disease; hom: homozygous; het: heterozygous; FSGS: focal segmental glomerulosclerosis; *participant underwent kidney transplant

Patient No.	Sex	Ethnicity	Gene	Mutation	Age at onset (Months)	Dipstick analysis	Serum albumin	Histology	Response to corticosteroids (1st line)	Response to immunosuppressants (2nd line)	ESK
EN300232	Male	Indian	*TRPC6*	**G162V (het)**	216	2+	38	FSGS	No response	Partial remission	NO

ESKD: end stage kidney disease; hom: homozygous; het: heterozygous; FSGS: focal segmental glomerulosclerosis; *participant underwent kidney transplant

Differences in clinical features between children with mutations and without detected mutations

Clinical and laboratory data of children with monogenic cause of FSGS *vs* no mutations are presented in Table 4. Although the mean age at onset of proteinuria was lower in the group with mutations (mean 124.10 months; 95% CI: 101.50-146.70) than in children with no mutations (mean 138.70; 95% CI: 124.30-153.00 months) this was not statistically significant (p= 0.37). No significant difference was noted in the clinical presentation (oedema and blood pressure), proteinuria, serum albumin and cholesterol levels between the groups. Children with mutations presented with a higher serum creatinine (mean 94.85; 95% CI: 25.45-164.2 umol/l) than children with no mutation (mean 43.36; 95% CI: 31.52-55.19 umol/l) although this was not statistically significant (p= 0.17). Nine (20.45%) children with non-genetic forms of SRNS achieved complete remission and six (13.64%) were in partial remission following treatment with additional immunosuppressants compared with none (100%) of the children with genetic forms of SRNS. The proportion of patients who progressed ESKD was higher in children with genetic mutations than in children without genetic mutations, albeit not statistically significant (p= 0.13). The duration from first presentation and progression to ESKD was significantly longer in children with no genetic mutations (mean 47.00; 95% CI: 37.06-56.94 months) than in children with genetic mutations (mean 19.80; 95% CI: 16.97-22.63 months) (p= 0.01).

Table 4
Genotype-phenotype correlations in paediatric patients with steroid-resistant nephrotic syndrome.

Phenotype	Positive mutation detected (n= 27)	No mutation detected (n= 43)	Mutation vs no mutation *p* value
Sex			0.33
Male	11 (40.74%)	23 (53.48%)	
Female	16 (59.26%)	20 (46.51%)	
Ethnicity			0.24
Black	26 (96.30%)	37 (86.04%)	
Indian	1 (3.70%)	6 (13.95%)	
Age of onset (months)	124.10 ± 57.10	138.69 ± 47.09	0.37
Presence of oedema at first presentation	15 (55.56%)	16 (37.21%)	0.12
Hypertension			0.50
Stage 1	8 (29.63%)	13 (29.55%)	
Stage 2	8 (29.63%)	7 (15.91%)	
Proteinuria	3.79 ± 4.21	3.65 ± 4.51	0.87
Serum Albumin (g/l)	26.81 ± 12.93	29.41 ± 10.14	0.13
Creatinine (umol/l)	94.85 ± 71.80	43.36 ± 36.51	0.17
Cholesterol (mmol/l)	8.01 ± 3.16	7.15 ± 3.43	0.90
Kidney biopsy			0.28
FSGS	27 (100%)	40 (93.02%)	
MCD	0	3 (6.98%)	
ESKD	5 (18.52%)	3 (6.98%)	0.13
Time at ESKD (months)	19.80 ± 2.28	47.00 ± 4.00	**0.01**
Recurrence after kidney transplant (n= 3)	0	0	

FSGS: focal segmental glomerulosclerosis; MCD: minimal change disease; ESKD: end stage kidney disease

Discussion

The high rate of steroid resistance in Black South African children compared to other racial groups prompted the need to further investigate the genetic basis for this racial disparity. Asharam *et al*. identified the *NPHS2* p.V260E mutation as a frequent cause of AR SR-FSGS in Black children with NS, potentially

sparing these children the use of steroids and other immunosuppressive drugs as well as the need to undergo kidney biopsy[18, 32]. As the *NPHS2* p.V260E is not infrequent in the general South African population (MAF 0.2-1%) and explains approximately 30% of SR-FSGS in Black South African children; therefore, we first genotyped all cases and controls for the p.V260E mutation.

In this study, we detected *NPHS2* p.V260E homozygosity in 21.43% of Black children with a general diagnosis of SRNS and 23.81% with a specific diagnosis of SR-FSGS. The detection rate of 23.81% for the homozygous p.V260E mutation-specific to SR-FSGS in our study was lower than the two previous reports from South Africa that reports rates of 33% (Asharam *et al*., 2018) and 50%[32]. The *NPHS2* gene, encoding podocin, is an integral structural protein of the podocyte. Although the *NPHS2* p.V260E variant likely arose in Africans, it was first identified in several consanguineous families from the previous Omani empire[18, 41, 42]. Like the European founder variant p.R138Q, the p.V260E variant disrupts the transport of the modified podocin protein from the endoplasmic reticulum to the plasma membrane [43–45].

A variable and earlier age of onset (range 24 to 178 months) in children was observed in children homozygous for the *NPHS2* p.V260E mutation. In an earlier report, Black SR-FSGS cases homozygous for p.V260E had an earlier age of onset compared to SRNS cases homozygous for the p.V260 reference allele (median onset of age, 34 months vs. 78 months, p= 0.01)[18]. The findings of our study in children carrying the p.V260E mutation highlight the variability of the phenotype seen in SR-FSGS. That said, it is important to consider *NPHS2* p.V260E mutation as a cause for SRNS in all paediatric age groups. In contrast to the findings in SRNS, we did not detect any homozygous or compound heterozygous mutations for *NPHS2* p.V260E among patients with SSNS, consistent with previously published reports showing that this mutation is specifically associated with SR-FSGS[18, 32, 46–48].

NPHS2-mediated disease is resistant to corticosteroids and other immunosuppressants[21, 42, 46, 49]. Additionally, several studies have highlighted a high risk of progression to ESKD in monogenic SRNS, with many patients requiring kidney transplantation and a low risk of disease reoccurrence post-transplant when a genetic aetiology has been confirmed[19, 42, 50–53]. In keeping with published reports, none of the children in our study homozygous for the *NPHS2* p.V260E variant responded to any second-line treatment. We also found that individuals who did not have the *NPHS2* p.V260E variant progressed more slowly to ESKD. Moreover, three of the five children that progressed to ESKD underwent a kidney transplant and no disease recurrence occurred. Screening for the *NPHS2* p.V260E mutation has the potential to provide a precision diagnosis of SR-FSGS thereby informing the differential diagnosis, prognosis, and treatment in 20-30% of Black African children presenting with NS.

In those children lacking the *NPHS2* p.V260E mutations, we observed 12 children with sporadic SR-FSGS carrying putative causal mutations in the following AD genes [*INF2* (n=8), *CD2AP* (n=3) and *TRPC6* (n=1)]. Autosomal dominant SRNS typically presents later in life, in adolescence or adulthood, and has significant phenotypic variability[20]. In our study, a later age of onset in children carrying an AD mutation (range 62-220 months) was observed when compared to AR mutation (range 24-178 months). Two children carrying an AD mutation in *CD2AP* and *TRPC6* were in partial remission following treatment with calcineurin inhibitors in contrast to all 15 children with an *NPHS2* mutation who did not respond to any additional immunosuppressants.

Recently, mutations in *INF2* were reported as the most common cause of adolescent-onset autosomal dominant SR-FSGS[54, 55]. Only three *INF2* mutations have been previously reported in a total of 436 sporadic SR-FSGS cases (<1%), all of which occur in the N-terminus[54–57]. In this study, a putative causal variant *INF2* p.P516L in the *INF2* gene was present in the heterozygote state in eight Black SRNS cases with biopsy-proven FSGS with an age of onset ranging from 6 to 18 years. The clinical characteristics of the children carrying the p.P516L variant in this study are in corroboration with previous reports which show *INF2* mutations as a common cause of FSGS with incomplete penetrance and variable age of onset ranging from 10-70 years[27, 54, 57]. The finding that 16% of Black children with SRNS undergoing WES carried the mutation compared to 3% of the South African population indicates that this predicted pathogenic variant merits further investigation to determine penetrance and causality.

Despite the clear association of *CD2AP* defects with a glomerular pathology suggestive of idiopathic FSGS in animal models, data is sparse in humans[58, 59]. In our study, we identified three Black children with SR-FSGS harbouring a heterozygous putative causal mutation (p.K633R) in the *CD2AP* gene. The first report by Kim *et al*. described one heterozygous nucleotide variant resulting in an aberrant *CD2AP* splicing in two patients with idiopathic FSGS[58]. More recently, Asharam *et al*. reported on a heterozygote *CD2AP* p.K346N variant in an Indian child with SR-FSGS[18]. Notably, the only child with *CD2AP*-associated nephropathy was in partial remission at last hospital visit after treatment with cyclosporin A. This treatment reduced the proteinuria by ~50%, whilst the remaining two patients failed to respond. A case report by Tsvetkov *et al*. showed that ciclosporin A is a treatment option for *CD2AP*-associated nephropathy[60]. However, further studies and clinical trials are required on the use of cyclosporin A as a therapeutic option for patients with *CD2AP*-associated nephropathy.

The *TRPC6* gene encodes the transient receptor potential cation channel, which is located on the podocyte membrane where together with podocin, it regulates mechano-sensation at the slit diaphragm[61]. autosomal dominant SR-FSGS, with variable age of onset from early childhood through adulthood[62–65]. In our study, we report a novel putative causal mutation, *TRPC*6 p.G162V in one SRNS Indian child with biopsy-proven FSGS with an age of onset of 18 years. The clinical phenotype of this patient improved after treatment with calcineurin inhibitors (tacrolimus). At last hospital visit, the patient was in partial remission. Our findings together with previous reports suggest that calcineurin inhibitors could open up new avenues on the treatment of *TRPC6*-associated FSGS[65, 66].

This study has several limitations. Family members were not available for segregation studies to determine the penetrance of the heterozygous variants. Due to the small number of controls typed for *INF2* P.516L, and the limited number typed for the population frequency estimate of this variant the statistical significance of its association with disease is not clearly established. Causality of *INF2* p.P516L, *CD2AP* p.K346N, and TRC6 p.G162V has not been demonstrated in animal models or in vitro cell studies.

This is the largest study in a paediatric South African population to perform sequence analysis of ten candidate genes (*NPHS1, NPHS2, WT1, LAMB2, ACTN4, TRPC6, INF2, CD2AP, PLCE1, MYO1E*) associated with SR-FSGS. We have confirmed that the *NPHS2* p.V260E mutation is a prevalent cause of SR-FSGS among Black South African children occurring in 23.81% of children with SRNS. Given the large number of children with *NPHS2* mutations, sufficiently powered clinical trials may be possible on testing different treatment therapies on these children and comparing outcomes. We also report putative causal missense variants in *INF2, CD2AP* and *TRPC6*. Screening all Black African children with NS for *NPHS2* p.V260E and possibly *INF2* p.P516L, if causality is shown, has the potential to be highly predictive of a diagnosis of SR-FSGS and multi-resistant forms of NS. Detection of pathogenic mutations will obviate the need for a kidney biopsy and the use of additional immunosuppressant drugs thus avoiding complications of an invasive procedure and the serious adverse effects of treatment. Precision genetic diagnosis will enable clinicians to provide genetic counselling, detect carriers, and propose prenatal diagnosis to couples at risk.

List Of Abbreviations

AD: autosomal dominant; AR: autosomal recessive; CKD: chronic kidney disease; ESKD: end stage kidney disease; FSGS: focal segmental glomerulosclerosis; MCD: minimal change disease; NS: nephrotic syndrome; SRNS: steroid resistant nephrotic syndrome; SSNS: steroid sensitive nephrotic syndrome; WES: whole exome sequencing

Declarations

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Ethics approval: This study protocol was reviewed and approved by Biomedical Research Ethics Committee of the University of KwaZulu-Natal and Department of Health, KwaZulu-Natal, South Africa, approval number BE374/17.

Consent to participate: Written informed consent was obtained from the parent/guardian of each child and children over seven years provided assent for participation in the study.

Consent for publication: All authors named consent to the publication of this manuscript.

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Figures

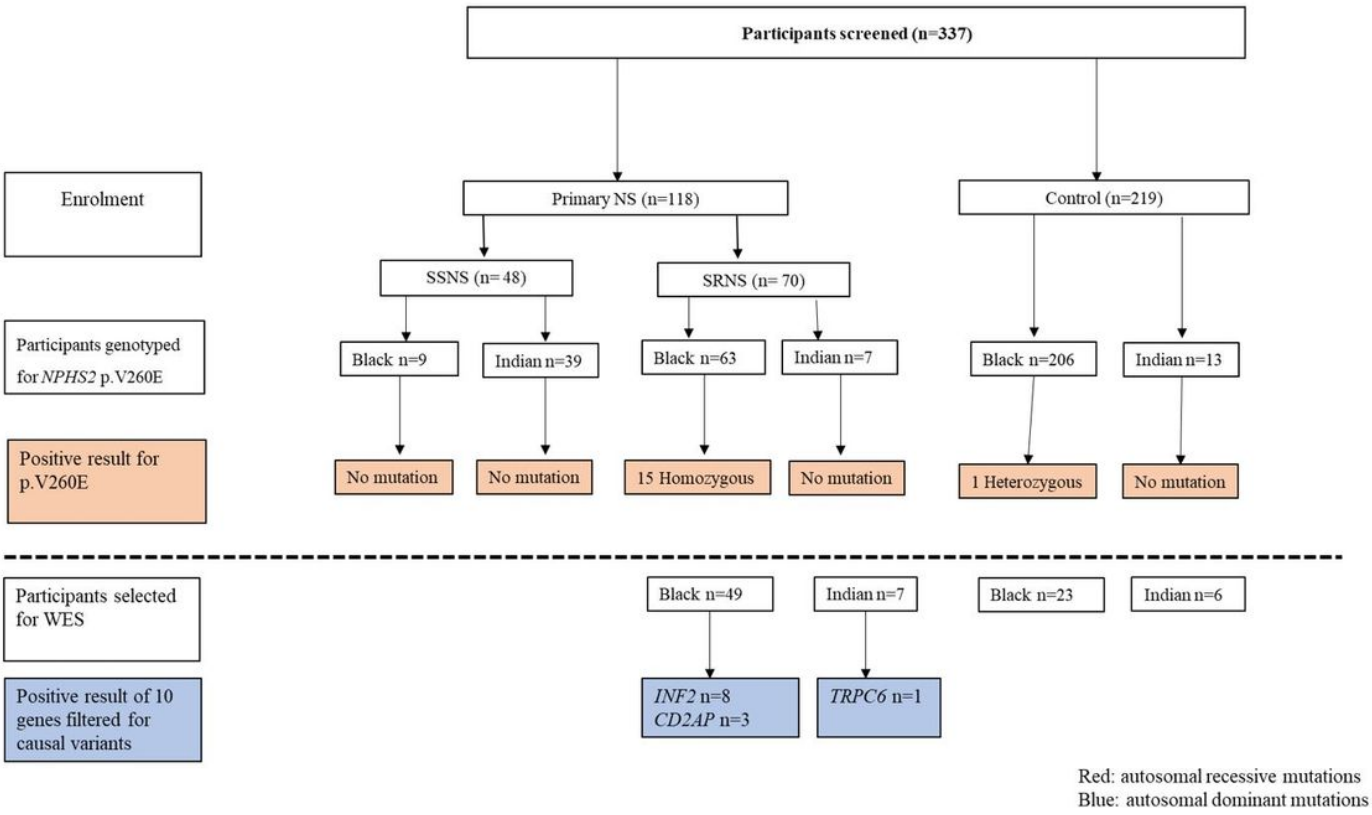

Figure 1

Study population

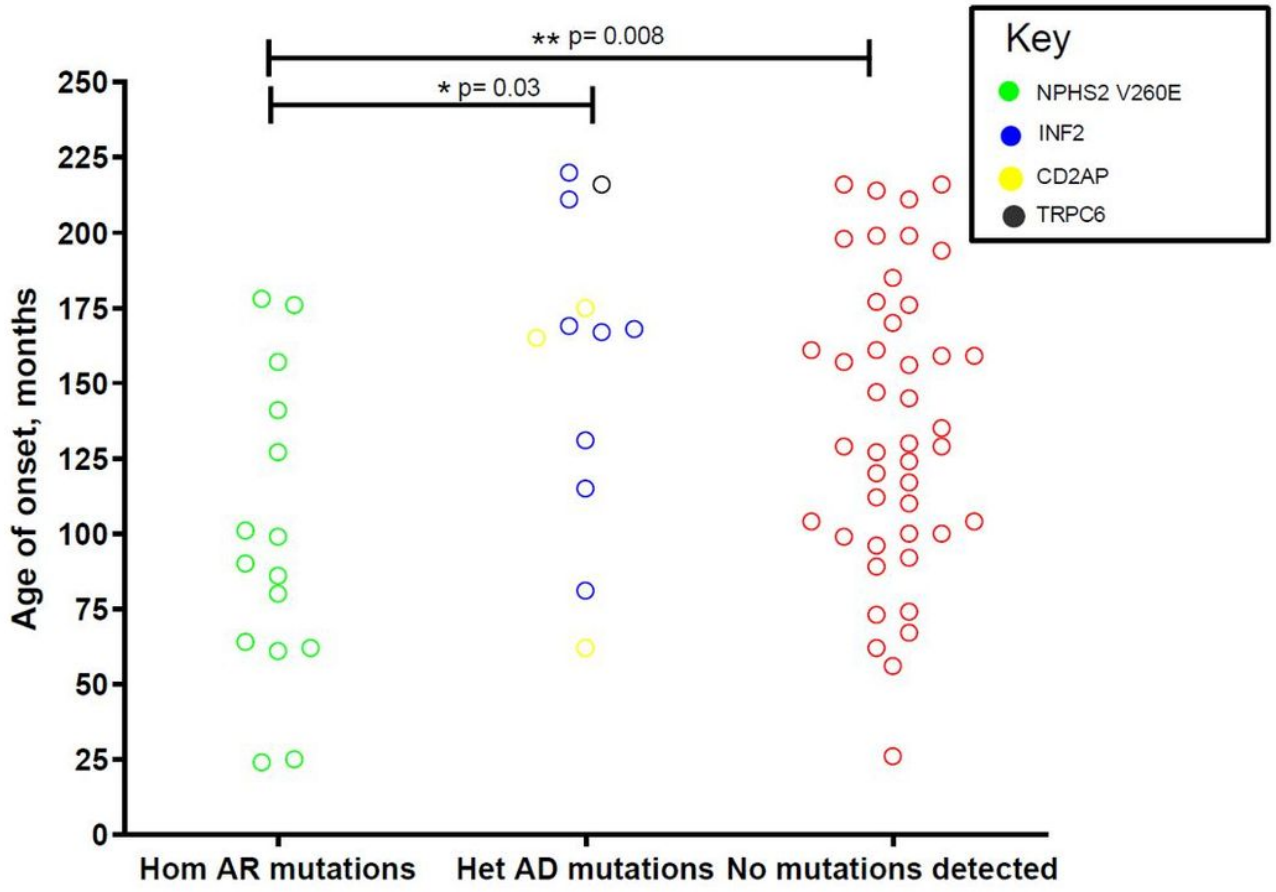

Figure 2

Comparison of distributions of ages of onset of steroid resistant nephrotic syndrome between carriers of the *NPHS2* p.V260E, *INF2* p.P516L and other (*CD2AP* p.K633R and *TRPC6* p.G162V) variants. Distribution of age of onset was significant in *NPHS2* p.V260E *vs* heterozygote autosomal dominant mutations (p = 0.03) and *NPHS2* p.V260E *vs* no mutations detected (p= 0.008). The p value was calculated using one-way analysis of variance.