Genetic associations with onset and progression of focal segmental glomerulosclerosis (FSGS) in black South African children

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Dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree

of

Master of Science in Medicine

DECLARATION

I, Melanie Ann Govender declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine, in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature of candidate: _____

18 April, 2019

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

1. 11th Conference of African Society of Human Genetics (Oral Presentation)
   19 to 21 September 2018
   Kigali, Rwanda (3rd Prize)
   Title: Genetic associations with onset and progression of focal segmental glomerulosclerosis (FSGS) in black South African children
   Presenting author: Melanie Govender

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   Title: Genetic associations with onset and progression of focal segmental glomerulosclerosis (FSGS) in black South African children
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PUBLICATION ARISING FROM THIS RESEARCH PROJECT

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Title: Common African-specific *NPHS2* V260E mutation in steroid resistant nephrotic syndrome in black South African children with biopsy proven focal segmental glomerulosclerosis

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ABSTRACT

Introduction - In children who present clinically with nephrotic syndrome (NS), the most common type of primary glomerular disease causing end stage kidney disease (ESKD) is focal segmental glomerulosclerosis (FSGS). Internationally, there is evidence of a more rapid progression of FSGS to ESKD and higher rates of steroid resistance in black patients compared to other ethnic groups and few studies have been performed in black South African children. The aim of this study was to determine genetic associations with *apolipoprotein L1* (A*POL1*) risk variants and *podocin* (*NPHS2*) variants in black children with FSGS.

Methods – Thirty-two black South African children with biopsy proven FSGS from 30 families were recruited from two clinics in Johannesburg. Three *APOL1* risk variants were genotyped and the exons of the *NPHS2* gene sequenced in the cases and healthy ethnically matched controls. Genetic association analyses with *APOL1* risk variants and *NPHS2* variants were performed. The *NPHS2* V260E variant was correlated with kidney function and treatment in all FSGS cases. Two families were examined for allelic segregation with FSGS.

Results – There was a weak association between FSGS cases without V260E and *APOL1* risk alleles for the dominant model (OR=2.97; 95% CI, 1.01 – 8.75; p= 0.048), but not the recessive model. Steroid resistant nephrotic syndrome (SRNS) and steroid sensitive nephrotic syndrome (SSNS) was present in 22 (69%) (including two sib-pairs) and 10 (31%) of the cases respectively. The OR of having SRNS increased from 36.8 (95% CI, 3.4 - 396.7; p=0.002) when carrying one copy of *NPHS2* V260E to 101.2 (95% CI, 10.7 - 955.9; p=7.76e-8) when carrying two copies. The presence of the *NPHS2* V260E variant was associated with a more rapid decline in kidney function (p= 0.026).

Conclusion - The *NPHS2* V260E variant is African-origin and is strongly associated with SRNS in black South African children, segregating as an autosomal recessive trait. Genotyping the V260E variant in black children with FSGS could provide useful information in a clinical setting to guide treatment, may prevent the need for renal biopsy and avoid side effects of glucocorticoid and other immunosuppressive therapies, and facilitate genetic counselling in families.

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AD	autosomal dominant
AR	autosomal recessive
CKD	chronic kidney disease
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ESKD	end stage kidney disease
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
HIVAN	HIV-associated nephropathy
INS	idiopathic nephrotic syndrome
MAF	minor allele frequency
MCD	minimal change disease
MN	membranous nephropathy
NS	nephrotic syndrome
OR	odds ratio
RIM	renin-angiotensin-aldosterone inhibition monotherapy
SR-FSGS	steroid-resistant focal segmental glomerulosclerosis
SRNS	steroid-resistant nephrotic syndrome
SSNS	steroid-sensitive nephrotic syndrome

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1. Literature review

1.1 Nephrotic syndrome in children

Nephrotic syndrome (NS) is the clinical syndrome that develops as a consequence of increased permeability of the glomerular filtration barrier (Andolino and Reid-Adam, 2015). It is generally characterized by excessive proteinuria (protein excretion greater than 50mg/kg per 24 hours) with associated hypoalbuminaemia (serum albumin levels below 3 g/dl), oedema and hyperlipidaemia (Bagga and Mantan, 2005). The first two features are used diagnostically because the last two features may not be present in all patients. NS is classified based upon whether or not there are signs of systemic disease. Primary NS refers to NS in the absence of an identifiable systemic disease. Within primary NS are patients with idiopathic nephrotic syndrome (INS). Secondary NS can be characterized by renal manifestation of an identifiable systemic disease (Kodner, 2009).

The incidence of NS is approximately 4.4 (ranging from 1.15 to 16.9) per 100 000 children worldwide (Banh *et al.*, 2016). The most common form of childhood NS is idiopathic nephrotic syndrome (INS), with approximately 90% of children presenting between the ages of 2-10 years (Niaudet and Boyer, 2016). Vogt and Avner (2007) report childhood INS to be 15 times more common than adult INS. In children, the incidence of INS is 16 per 100 000 children, with a rate of 2-7 new cases per 100 000 children (Skrzypczyk *et al.*, 2014).

Clinically, INS is further classified by the response or lack of response to oral corticosteroid therapy as 'steroid-sensitive nephrotic syndrome' (SSNS) versus 'steroid-resistant nephrotic syndrome' (SRNS). The majority of children with INS are steroid sensitive, while approximately 10% of all children with INS do not respond to oral corticosteroid therapy (Bagga and Mantan, 2005). Approximately 40% of SSNS cases have either no relapse or a single relapse making the prognosis more favourable in this group (Sinha and Bagga, 2012). The clinical management of SRNS is problematic, because resistance to corticosteroids requires use of alternative immunosuppressive agents which may/not control the glomerular injury and have deleterious side effects for the affected children. A patient is considered to have SRNS if there is continuing proteinuria after taking oral prednisone for 4 to 8 weeks (Iijima *et al.*, 2017; Li *et al.*, 2018). This is established on knowledge that 95% of patients with SSNS go into

remission within 4 weeks of steroid treatment, and that adverse effects are associated with prolonged therapy (Sinha and Bagga, 2012).

It is now well known that African children with INS have a different clinical and epidemiological profile compared to children of other ethnic groups. In many parts of the world, INS is dominated by steroid responsiveness with predictable outcomes and good prognosis. However, INS among African children differs from patterns observed on other continents; with steroid resistance being the more common outcome (Asharam *et al.*, 2018; Bakhiet, 2016; Bhimma *et al.*, 1997). Therefore, black ethnicity is frequently seen as an indication for kidney biopsy in children with NS (Ladapo *et al.*, 2014).

1.2 Histopathological diagnosis of INS and clinical implications of FSGS

Histopathological assessment of the kidney biopsy is vital for diagnosis and for guiding treatment options. The histopathological classification of INS includes (i) MCD which shows normal glomeruli on light microscopy; no signs of glomerular deposits of immunoglobulins under immunofluorescence examination; and changes in podocyte architecture that are only visible with scanning electron microscopy (Jefferson *et al.*, 2011). (ii) MN which shows sub-epithelial immune complex deposition with thickening of the glomerular basement membrane (iii) FSGS is characterized by damaged or scarred glomeruli (**Figure 1.1**), where the following refers: “focal” - only some of the glomeruli are involved; “segmental” - only a part of each affected glomerulus is involved; “glomerulosclerosis” - scarring of the affected part of glomerulus (Andolino and Reid-Adam, 2015).

According to Kriz (2003) the structure and function of normal glomeruli are altered in FSGS. **Figure 1.2** shows a histological image of a normal glomerulus by light microscopy. Three main elements of the glomerular filtration barrier are essential for regular functioning of the kidneys; podocytes (highly specialized epithelial cells found in the Bowman’s capsule) (**Figure 1.1**), endothelial cells (line the surface of blood and lymphatic vessels), and glomerular basement membrane (GBM) (derives from the fusion of endothelial cells and podocytes). Together, a selectively permeable filtration barrier is provided by these three intact components (Reidy and Kaskel, 2007). The slit diaphragm is created through specialized tight

connections between podocyte foot processes and is integral in the prevention of protein entering the urinary space (Asanuma and Mundel, 2003).

Even though FSGS often clinically presents as a varied group of features, proteinuria is a defining feature, which indicates the loss of the glomerular filtration barrier (Kim *et al.,* 2016). The alteration (or effacement) of the normal structure of the foot processes of podocytes is shown by electron microscopy in FSGS cases (D'Agati, 2003; D'agati *et al.*, 2011). The connection between the GBM and foot processes of the epithelial layer can be disturbed, resulting in the loss of proteins into the filtrate (Asanuma and Mundel, 2003).

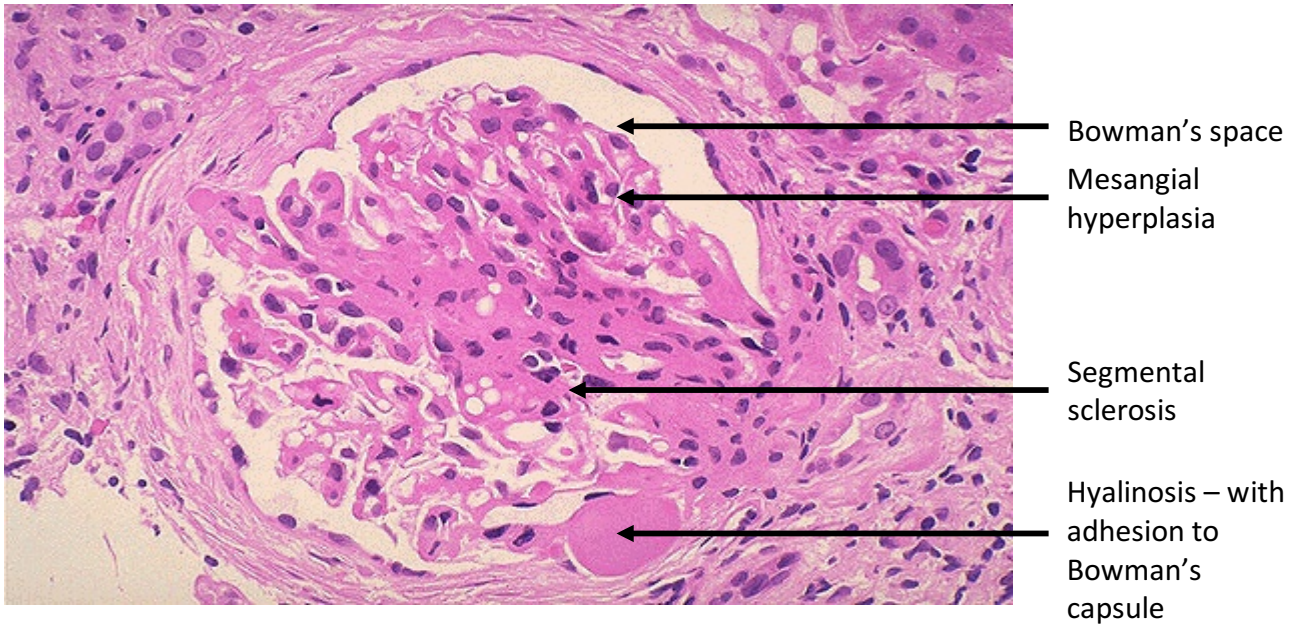


Figure 1.1: Imaging showing histological section of the kidney of a patient with FSGS (https://library.med.utah.edu/WebPath/RENAHTML/RENAL082.html)

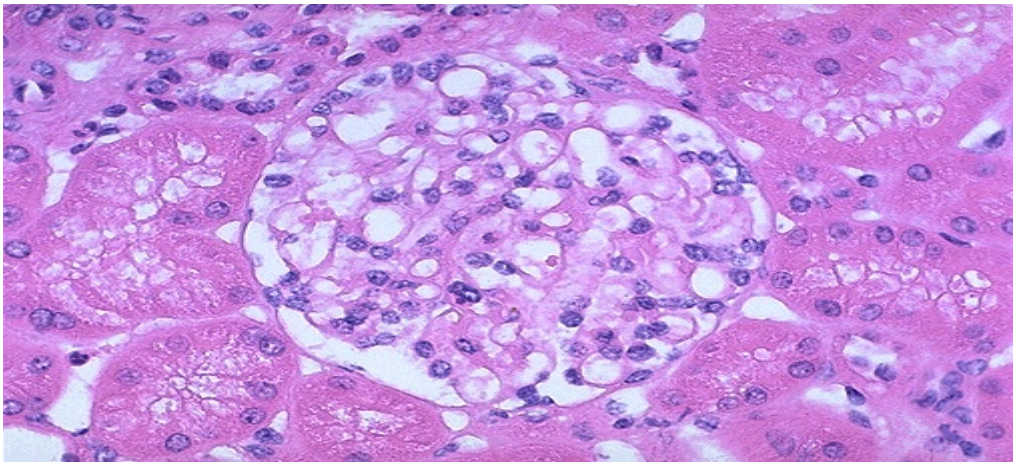


Figure 1.2: Histological image of a normal glomerulus by light microscopy (https://library.med.utah.edu/WebPath/RENAHTML/RENAL101.html)

If there is progression of the initial insult and glomerular scarring occurs, as can be seen in FSGS (**Figure 1.1**), there is a risk of progression to ESKD (Kiffel *et al.*, 2011; Schnaper, 2003). At this stage of kidney disease, the damage is irreversible and support in the form of chronic dialysis or a kidney transplant is required in order to sustain life. However, patients that undergo a kidney transplant for FSGS are at risk of recurrence in the new graft, as FSGS has been shown to recur in 30-50% of cases following transplantation (Kiffel *et al.*, 2011; Sinha and Bagga, 2012).

1.3 Genetic predisposition to FSGS

In common forms of complex diseases such as hypertension, diabetes and kidney disease; genetic variation plays a significant role in susceptibility and can vary according to ethnicity (Kasembeli *et al.*, 2015). The contributing role of genetics in the aetiology of kidney disease was supported by the finding that a higher incidence of FSGS is reported among children with African ancestry compared to those with European ancestry (Chanchlani and Parekh, 2016). In addition, there have been studies showing a more rapid progression of FSGS to ESKD in African individuals (Boyer *et al.*, 2007; Sorof *et al.*, 1998). The higher occurrence of biopsy proven FSGS in children, among certain ethnic groups, therefore suggests the role of genetics in susceptibility to disease.

Ancestry informative population variation data has been used in an attempt to explain ethnic differences in the presentation of FSGS and ESKD. The discovery of variants in the *non – muscle myosin heavy chain 9* (*MYH9*) gene located on chromosome 22 that were genetically associated with non – diabetic FSGS and ESKD in African Americans was published in Nature Genetics in 2008 by two different groups (Kao *et al.*, 2008; Kopp *et al.*, 2008). The *MYH9* gene codes for a protein (myosin IIA) that is expressed in podocyte cells, broadly distributed and vital for the rearrangement of the cytoskeleton, cell division, cell motility, and cell to cell adhesion (Winkler *et al.*, 2010). As a result, the *MYH9* gene was regarded as a good biologically relevant candidate for association with chronic kidney disease (CKD). Hence, it was suggested that *MYH9* variants played a contributing role in disease pathology by increasing susceptibility to CKD (Kopp, 2010). The strongest associations to CKD were with three *MYH9* haplotypes referred to as E, S and F (Behar *et al.*, 2010; Oleksyk *et al.*, 2010). The E1 haplotype was found to be greatly correlated with CKD in individuals with African ancestry

and was therefore defined as the risk haplotype (Oleksyk *et al.*, 2010). Even though the E1 haplotype seemed to explain some of the heritability of CKD in African Americans with a 70% attributable risk for FSGS, no mutations were identified to have a distinct predicted functional effect on kidney function (Kopp *et al.*, 2008).

The *MYH9* gene is tightly linked to the *APOL1* gene on chromosome 22, hence, additional fine mapping studies revealed that the risk variants are actually in the *APOL1* gene and not in the *MYH9* gene, as previously thought (Genovese *et al.*, 2010; Tzur *et al.*, 2010).

1.3.1 *APOL1* risk variants and FSGS

The *APOL1* risk variants include two non-synonymous variants collectively referred to as the G1 risk allele ($G1^{GI}$= rs73885319 and $G1^{GM}$= rs60910145) and a 6 bp in-frame deletion (rs71785313) defined as the G2 risk allele (**Figure 1.3a**). The two variants that define the G1 risk allele are in almost complete linkage disequilibrium (r^2 ~ 1.0) with each other in most populations studied and strongly correlated with the predisposition of CKD. The two non-synonymous variants result in two amino acid substitutions (Ser342Gly and Ile384Met,) while the 6 bp deletion results in the loss of two amino acids (Asn388-Tyr389del).

Figure 1.3 shows the markers that make up the G1 and G2 risk alleles and their frequencies in a black South African population (Kasembeli *et al.*, 2015). The G1 and G2 risk "alleles" are actually defined by a haplotype, and the variants that comprise the G1 and G2 risk "alleles" appear to have occurred independently as they are present on different chromosomal backgrounds and have not yet been observed on the same chromosome (Limou *et al.*, 2015). In previous studies on adults, the odds ratio (OR) for having FSGS with 2 *APOL1* risk alleles was 17 in African Americans and 6.03 in South Africans (Kasembeli *et al.*, 2015; Kopp *et al.*, 2011). In comparison to the *MYH9* E1 risk haplotype (referred to as G3)(Figure 1.4), the *APOL1* risk variants were found to be more highly correlated with the risk for CKD in individuals of African ancestry.

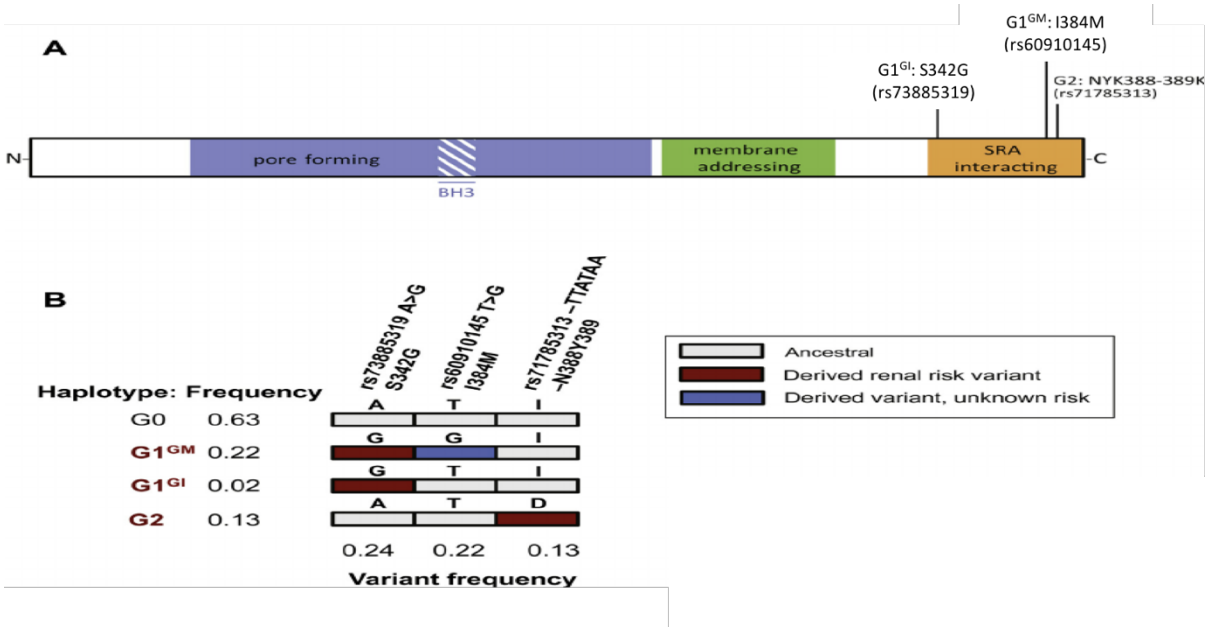


Figure 1.3: *APOL1* gene showing the position of the risk variants (A) (Limou *et al.*, 2015) and haplotypes with haplotype allele frequencies in a black South African population (B) (Kasembeli *et al.*, 2015) (Modified)

Individuals carrying the *APOL1* risk variants are known to be protected against trypanosomiasis, also referred to as sleeping sickness, a disease that affects millions of Africans (Kasembeli *et al.*, 2015). The G1 and G2 risk allele frequencies are distinctly distributed among African and African ancestry populations (Kopp *et al.*, 2011). The G1 and G2 risk alleles arose by chance and were subjected to positive selection and hence increased in frequency in regions where trypanosomiasis was endemic. The frequency for the G1 risk allele is approximately 20% in African Americans but greater than 45% in Yoruba from Nigeria in West Africa (**Figure. 1.4**).

There are 3 *Trypanosoma* species; *Trypanosoma brucei brucei, Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense.* Due to human serum containing *apolipoprotein A1,* high density lipoprotein (HDL), and trypanolytic factor (TLF) containing *apolipoprotein L1, Trypanosoma brucei brucei* is incapable of infecting humans. However, the other two *Trypanosoma* species evade this host defense mechanism by expressing a serum resistance associated (SRA) protein (Brun *et al.*, 2010; Stephens and Hajduk, 2011). The SRA protein carried by *T. b. rhodesiense* and *T. b. gambiense* binds to and inactivates the wild type *APOL1* protein. However, the ability of *APOL1* to lyse *T. b. rhodensiense* is restored by the presence of the G1 and G2 variants, providing protection against trypanosomiasis (Pays *et al.*, 2014). These variants have therefore conferred protection to the host, in both the

heterozygote and homozygote states, and their frequencies increased in areas where trypanosomiasis is or was endemic.

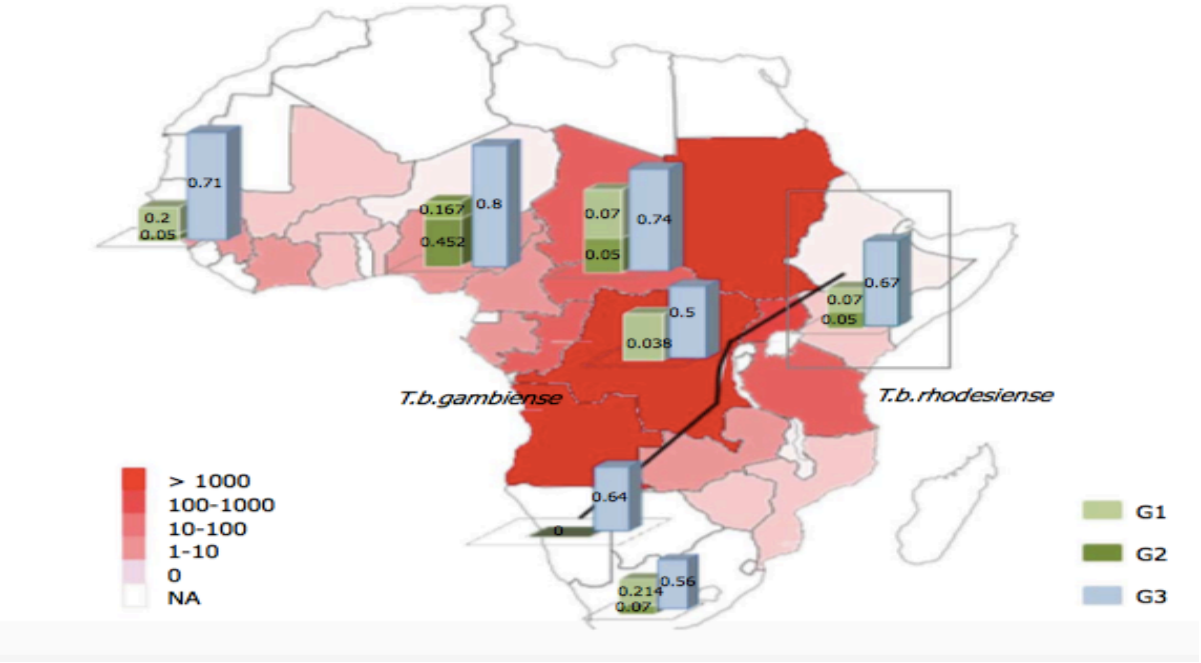


Figure 1.4: Distribution of human sleeping sickness (*T.b gambiense and T.b rhodesiense*), *APOL1* G1 and G2 alleles frequencies, and *MYH9* E1 risk haplotype (referred to as the G3 haplotype) in different African populations (Kasembeli *et al.*, 2015).

Genovese *et al.* (2010) suggest a recessive model of the G1 and G2 risk alleles in the FSGS cases, suggesting that you need 2 risk alleles for increased susceptibility. Though, in larger cohorts, there have been observations of mild dominant effects (OR= 1.26 for 1 allele and OR =7.3 for 2 alleles). Hence, this mild dominant model suggests that a single allele can contribute to susceptibility (Kasembeli *et al.*, 2015). Moreover, since extended linkage disequilibrium occurs in this region due to selective pressure, other rare variants in *APOL1* or other adjacent genes may also be implicated in predisposition to disease (Freedman *et al.*, 2010).

1.4 Pathogenesis and genes associated with monogenic forms of NS and FSGS

Studies have described many podocyte genes that are correlated with severe forms of NS that progress to ESKD (**Figure 1.5**). Loss or injury of podocytes is significant in the pathogenesis of FSGS (D'Agati, 2008). Four mechanisms lead to damage to podocytes; interference with its architecture or distortion of the slit diaphragm, modification of the GBM, dysregulation of the actin cytoskeleton, or adjustment of the podocyte negative surface charge (Reidy and Kaskel,

2007). The removal of podocytes from the GBM through apoptosis is triggered by damage to podocytes (Kwoh *et al.*, 2006). The resultant decrease in the number of podocytes is considered to play a vital role in the pathogenesis of FSGS (Fogo, 2003). Podocyte cells generally have restricted proliferative ability in reaction to damage/injury, however further injury is facilitated by mechanical stress, additional loss of polarity, and cytokine release, causing scarring and sclerosis of the glomeruli (Fogo, 2003; Kwoh *et al.*, 2006).

Not only have these data provided valuable insight into the understanding of some of the mechanisms that lead to NS but they have also highlighted the vital role played by podocytes in the glomerular filtration barrier (Antignac, 2002; Pollak, 2002). Over 50 genes have now been linked to monogenic forms of NS, with autosomal recessive (AR) and autosomal dominant (AD) modes of inheritance, manifesting in childhood and adulthood (Sadowski *et al.*, 2015) (**Table 1.1**).

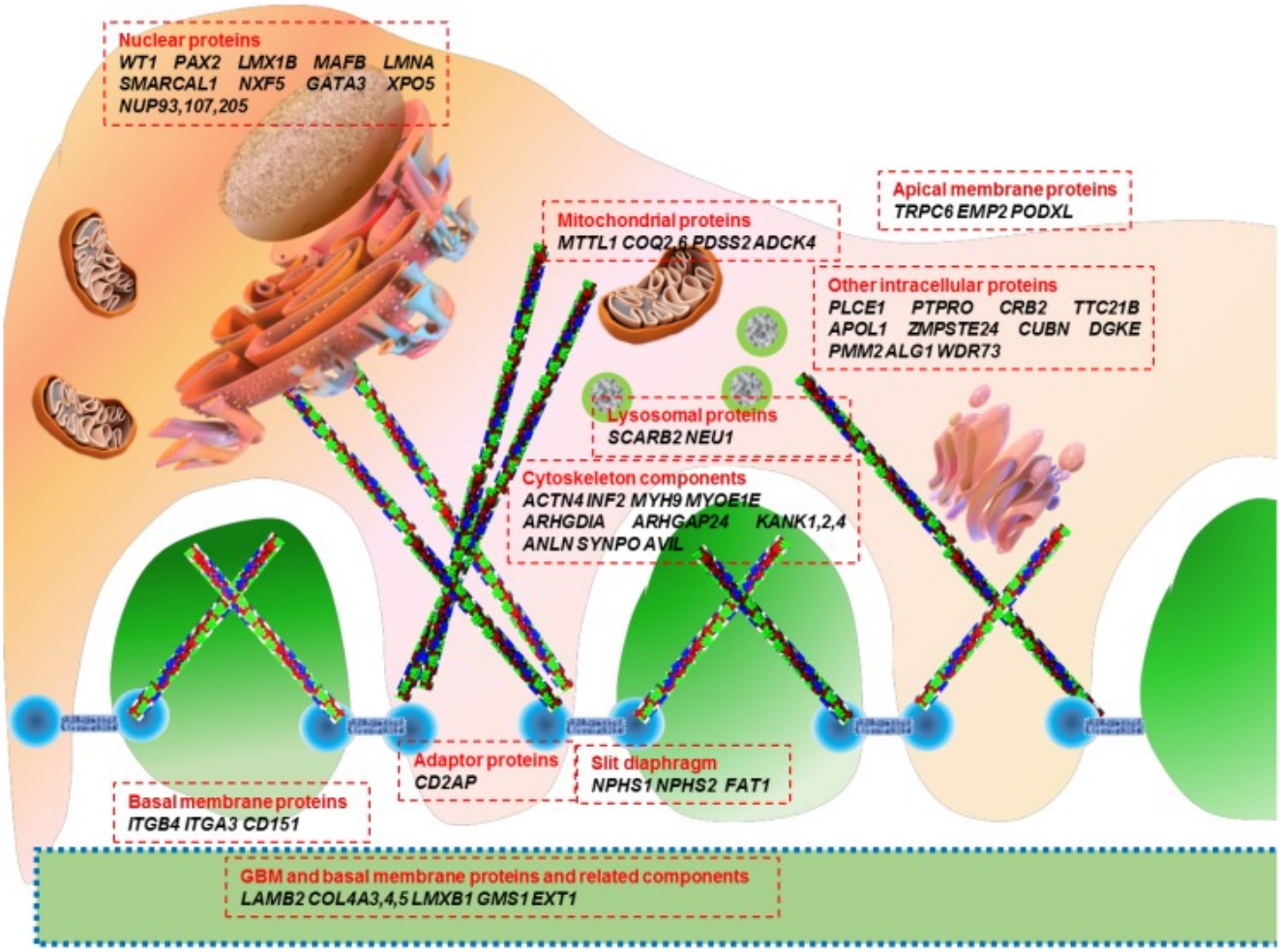


Figure 1.5: Podocyte genes associated with nephrotic syndrome (NS)

Table 1.1: Genes causally associated with monogenic forms of NS and FSGS (Pollak, 2014)

Gene	Locus	Inheritance	Protein	Phenotype
NPHS1	19q13.1	AR	Nephrin	Congenital nephrotic syndrome
NPHS2	1q25.2	AR	Podocin	Congenital, infant, childhood-onset SRNS, FSGS
PLCE1	10q23.33	AR	Phospholipase C epsilon 1	Early-onset DMS, FSGS
CD2AP	6p12	AD	CD2-associated protein	FSGS, CD2AP-deficient mouse model develops severe proteinuria
TRPC6	11q22.1	AD	TRPC6	Adult-onset FSGS
ACTN4	19q13	AD	Alpha-actinin 4	Adult-onset FSGS
INF2	14q32.33	AD	Inverted formin 2	Adult-onset FSGS
MYO1E	15q22.2	AR	Myosin 1E	Childhood-onset SRNS
ARHGAP24	4q22.1	AD	Arhgap24 (RhoGAP)	Adolescent-onset FSGS
WT1	11p13	AD	Wilms tumor 1	Frasier or Denys-Drash syndrome or isolated NS

1.4.1 Monogenic forms of adult-onset NS and FSGS

Point mutations in the *Inverted formin 2* (*INF2*) gene are the most common cause of AD FSGS (Barua *et al.*, 2013; Brown *et al.*, 2010). The *INF2* gene belongs to the formin gene family, a cluster of heterogeneous actin polymerizing molecules that plays a part in regulating a wide range of cytoskeleton-dependent cellular processes (Chhabra *et al.*, 2009; Gupton *et al.*, 2007). Barua *et al.* (2013) identified AD *INF2* mutations in 9% of families with familial FSGS. *INF2* associated adult-onset FSGS has variable penetrance. In contrast to *INF2*, variants in the *alpha actinin 4* (*ACTN4*) and *transient receptor potential 6* (*TRPC6*) ion channel channel genes accounted for 3% and 2% of inherited FSGS, respectively. Although mutations in the *INF2* gene are a minor cause of monogenic FSGS, they are more common than other known AD genes related with FSGS (Barua *et al.*, 2013).

Kaplan *et al.* (2000) and Winn *et al.* (2005) identified FSGS-causing mutations in the *ACTN4* and *TRPC6* cation channel genes, respectively. *ACTN4* and *TRPC6* associated FSGS have similar phenotypes; proteinuria with progressive CKD. The *ACTN4* gene belongs to a group of four actinin genes. These genes are biochemically similar and encode highly homologous proteins.

The *ACTN4* gene is found on chromosome 19q13 and is notably expressed in human glomeruli (**Figure 1.5**). Variants in the *ACTN4* gene increase the affinity of the gene product to filamentous actinin, hence, altering the mechanical properties of the podocyte by affecting the mechanical properties of actin fibres. *ACTN4* associated FSGS mutations are highly, but not completely, penetrant (Weins *et al.*, 2007). The *TRPC6* cation channel gene is a constituent of the glomerular slit diaphragm and is expressed in podocyte cells (**Figure 1.5**). Mutations in *TRPC6* appear to cause an increase in cation channel activity through gain of function mutations (Reiser *et al.*, 2005; Winn *et al.*, 2005). Reiser *et al.* (2005) identified AD *TRPC6* variants in five families with FSGS. Data from the study suggest that the *TRPC6* cation channel plays a vital role in regulating podocyte function and structure.

1.4.2 Monogenic forms of childhood NS and FSGS

Several genes are commonly mutated in childhood NS, however, variants in the *nephrin* (*NPHS1*) and *podocin* (*NPHS2*) genes are the most common cause of NS in children (Sadowski *et al.*, 2015). AR NS occurs when two copies of the mutant allele are inherited, one from each parent. Generally, AR forms of a disease are rarer, more aggressive than AD, and present at an earlier age (Pollak, 2014). The *NPHS1* gene which encodes a transmembrane protein named nephrin is a vital component of the slit diaphragm (Huber and Benzing, 2005). Mutations in the *NPHS1* appear to cause disruption of the glomerular filtration barrier resulting in loss of proteins (Hinkes *et al.*, 2007). Currently, there are approximately 200 disease-causing *NPHS1* mutations that have been described (Pollak, 2014).

The *NPHS2* gene encodes a protein called podocin that belongs to the statin family, and was described as the most common cause of SRNS and SR-FSGS in children (Boute *et al.*, 2000). Podocin is solely expressed in the podocyte cells and interacts with nephrin to initiate nephrin signalling (Huber *et al.*, 2003; Roselli *et al.*, 2002). Podocin also has the ability to stabilize contacts between podocytes by interacting with CD2-associated protein (encoded by *CD2AP*) (**Figure 1.5**) (Schwarz *et al.*, 2001). Hence, podocin plays a vital role in the maintenance of glomerular perm-selectivity by maintaining structural integrity and function of the slit diaphragm (Niaudet, 2004).

Individuals with two pathogenic *NPHS2* variants appear to have an early age of presentation

from 3 months to 6 years (Hinkes *et al.*, 2007). *NPHS2* mutations have been found to be causally linked to 6-17% of sporadic and 28-39% of monogenic SRNS cases (Pollak, 2014). Asharam *et al.* (2018) reported on 33 Indian and 31 black children from Kwa-Zulu Natal with SSNS and SRNS. *APOL1* variants were not associated with NS, but the *NPHS2* V260E allele was strongly associated with SRNS in black children. Out of 30 black children with SRNS, 8 were homozygous for the V2690E variant (OR 21; 95% CI, 2.8 - 960) and no heterozygotes were observed. This variant was absent in all Indian children with SRNS and absent in the one black child with SSNS. One out of 55 black controls was heterozygous for this variant.

1.5 Justification for study

Individuals with the *APOL1* G1 and G2 risk alleles (1 or 2 copies) have been shown to have an elevated risk for NS. In South African adults, there is a strong association between *APOL1* risk variants and HIV-associated nephropathy (HIVAN) with an OR of 89 (95% CI, 17.7 - 912) in the presence of two *APOL1* risk alleles (Kasembeli *et al.*, 2015). However, the *APOL1* risk alleles were not significantly associated with FSGS in black South African adults (OR=6.03; 95% CI, 0.08 - 527) and data on *APOL1* risk variants with FSGS in black South African children is limited. At the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) the most common type of glomerular disease in children is FSGS, with a high proportion of black children progressing to ESKD.

In South Africa, there are limited genetic studies on kidney disease, including FSGS, among children. Studies have shown that variants in the *NPHS2* gene are the most common cause of NS in children and the majority of black children with FSGS do not respond to steroid treatment. An assay that could predict which children are likely to respond to steroid treatment would be of significant clinical value.

1.6 Hypothesis, aim and objectives

1.6.1 Hypothesis

In black South African children with NS, *APOL1* and *NPHS2* variants are significant genetic contributors to the risk of developing FSGS, and could influence the clinical response to corticosteroid therapy and progression to ESKD

1.6.2 Aim

To determine genetic associations with *apolipoprotein L1* (A*POL1*) risk variants and *podocin* (*NPHS2*) variants with FSGS in black South African children.

1.6.3 Objectives

- Recruit paediatric study participants with FSGS based on inclusion/exclusion criteria from two clinics in Johannesburg
- ***APOL1***
    - Sequence three *APOL1* risk variants in all cases and controls
    - Perform case:control comparison with FSGS
- ***NPHS2***
    - Sequence all 8 exons in the *NPHS2* gene in cases and selected variants in controls
    - Perform case:control comparison with SRNS, SSNS and controls
- Assess disease progression from diagnosis to ESKD in the context of genetic variants
- Examine allelic segregation of *APOL1* risk alleles and the *NPHS2 V260E* variant in two familial cases

2. Methods and materials

2.1 Study design

This was a patient cohort study. In the case:control studies, controls were ethnically and geographically equivalent.

2.2 Study participants

The study participants included paediatric patients between the ages 2 and 17 years with biopsy-proven FSGS currently treated by the Division of Paediatric Nephrology of Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and Morningside Mediclinic (MM). Only children with biopsy proven FSGS, younger than 18 years at diagnosis, self-reported black African ethnicity, adequate data in their clinical files and accessible for participation were included. Children with HIV-associated glomerular disease on biopsy and non-black South Africans were excluded. The cases were sporadic, with the exception of two pairs of affected siblings whose nuclear families (parents and children) were included in the study.

The controls included 226 DNA samples from participants in Soweto (appropriately consented), and the samples were stored in the Sydney Brenner Institute for Molecular Bioscience (SBIMB) biobank. Of these, 176 were included in the *APOL1* risk variant association analysis and 50 for comparison with *NPHS2* variants.

2.3 Definition of terms for this study

Onset of Focal Segmental Glomerulosclerosis (FSGS): defined as date of first renal biopsy that confirmed the diagnosis of FSGS

Onset of End Stage Kidney Disease (ESKD): defined as onset of initiation of renal replacement therapy which included: chronic dialysis treatment and/or kidney transplantation; or an estimated glomerular filtration rate (GFR) <= 15 ml/min/1.73m^2

Steroid Sensitive Nephrotic Syndrome (SSNS): remission of nephrotic syndrome is induced within six to eight weeks of using oral corticosteroid therapy. Remission was defined as a spot or random protein:creatinine ratio of <0.2 g/mmol; or a urine dipsticks measurement with a reading that confirms a "trace"; or "no protein" for at least 3 consecutive days.

Steroid Resistant Nephrotic Syndrome (SRNS): Remission of nephrotic syndrome is not induced within six to eight weeks of oral corticosteroid therapy.

2.4 Data capture

The patient files were kept in a secure filing room at both clinics. These files were screened to identify participants based on inclusion and exclusion criteria. Data were extracted and entered onto a REDCap database (**Appendix A**)(Harris *et al.*, 2009). Five data collection instruments were included on REDCap; demographics, patient follow-up details, medical history and baseline information, annual information and genetics. The following data were recorded for demographics; study ID, first name, surname, self-reported ethnicity, sex, date of birth and province of birth. Patient follow-up details included clinic name, clinic file number and contact details for the patient. Medical history and baseline information consisted of anthropometry (height and weight), clinical information (HIV status (positive, negative, unknown), transplant status (yes/no), on dialysis (yes/no), date of diagnosis, and serum creatinine).

Information from annual follow-up visits was used to assess kidney function through quantification of glomerular function rate (GFR) and change in kidney function over time. The files were used to retrieve height, weight and serum creatinine values with corresponding dates, to assess rate of disease progression over the years the patient was being followed-up. This was achieved by using serum creatinine and height to estimate glomerular function rate using the Bedside Schwartz equation. According to Schwartz *et al.* (2009), the Bedside Schwartz formula is the best for estimating GFR in children. GFR was calculated as follows; GFR (ml/min/1.73 m^2) = 0.413 × (Height in cm / serum creatinine in µmol/L).

The following genetic information was recorded; *APOL1* genotypes and haplotypes (G0= ATI, G1= GGI and GTI, G2= ATD) and *NPHS2* sequence variant analysis data. Haplotypes were manually assigned and when they could not be resolved because more than one locus was heterozygous, compatibility with the common mutation-associated haplotype was assessed.

Using REDCap, all identifying data were removed and codes provided prior to exporting the data for analysis to ensure anonymity and maintain confidentiality.

2.5 DNA extraction and quality check

Following assent and parental consent, 5ml blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube from each participant. Ten millilitres of blood were collected from consenting adults in the familial cases. EDTA tubes containing blood samples was inverted 7-10 times immediately after collection to prevent clotting. Blood samples were stored in a -20^{o}C freezer. Genomic DNA was extracted from peripheral blood samples using the salting-out procedure (Miller *et al.*, 1988). The salting out procedure took place over a period of three days. Solutions for the salting-out procedure were made prior to DNA extraction (**Appendix B**).

Whole blood samples in EDTA as anti-coagulant were taken out of the freezer and left to thaw at room temperature. Blood was decanted into 50ml Nunc tubes and cold sucrose-Triton X lysing buffer was added to make up the volume to 40ml. This step was performed to lyse red blood cells and other components The sucrose-Triton X solution was stored at a temperature of 4^{o}C. Tubes were inverted several times to mix both substances and centrifuged at 2400rpm

at 4°C for 10 minutes. The resulting supernatant was discarded into bleach. Precaution was taken when discarding the supernatant, ensuring the pellet was not dislodged. The pellet, containing the nucleated white blood cells, was washed with 25ml sucrose-Triton X solution and put into a freezer at -20°C for 5 minutes. Tubes were inverted and centrifuged at 2400rpm at 4°C for 5 minutes. The pellet was washed a third time with 15ml sucrose-Triton X solution. Washes were carried out 3 times to ensure removal of most of the red cell debris. Three milliliters of T20E5, 200μl of 10% SDS and 500μl of proteinase-K mix were added to the pellet to lyse white blood cells and degrade proteins. Tubes were inverted to mix components and incubated overnight at 42°C.

One milliliter of saturated NaCl was added to the lysate and the solution was agitated vigorously for 15 seconds. Tubes were placed in a -20°C freezer for 10 minutes and centrifuged for 38 minutes at 2400rpm at 4°C. Supernatant containing the DNA was transferred into a new tube. Fifteen milliliters of room temperature absolute ethanol were added to the tubes. DNA was not visible and could not be spooled out, so it had to be precipitated. Tubes were placed in a -20°C freezer overnight.

Samples were taken out from the freezer, inverted and centrifuged at 2400rpm at 4°C for 20 minutes. Precaution was taken when discarding the supernatant, ensuring the DNA was not discarded. Seventy percent ethanol was added to make the volume up to 10ml. Tubes were inverted and centrifuged 2400rpm at 4°C for a further 10 minutes. Supernatant was discarded and tubes were inverted and left to air dry. Four hundred and ten microliters of TE buffer was added to each of the tubes.

The concentration and quality of the DNA samples were examined on the NanoDrop spectrophotometer. The following readings were taken for each sample; concentration (ng/μl), and absorbance at 230, 260 and 280 nm, and ratios A260/280 and A260/230. The A260/280 ratio was used to asses to purity of DNA (possible protein or RNA contamination). A ratio of ~1.8 is expected and indicates the DNA sample is pure. A ratio lower than expected indicates the presence of protein contamination and a ratio higher than expected indicates RNA contamination. The A260/230 ratio was a secondary measure to asses DNA purity. The

expected A260/230 ratio is in the range 2.0-2.2. A ratio lower than the expected range would indicate the presence of chemical contaminants (Mackey and Chomczynski, 1997).

5X TBE buffer was diluted to 1X with deionised water. One hundred milliliters of 1X TBE were added to a cylinder with 0.8g of agarose. The cylinder containing TBE buffer and agarose was heated in a microwave for 2 minutes. The cylinder was placed in cold water to cool down the solution before 3µl of ethidium bromide was added. The solution was poured into a tray and left to set for half an hour. In each well, a mixture of 2µl of loading dye, 1µl of DNA and 3µl of deionised water was added. The voltage for the gel electrophoresis, in a solution of 1X TBE buffer was set to 100V and 96MA for 30 minutes. The gel was viewed under UV light (**Appendix C**).

2.6 Sanger sequencing and sequence analysis

Sanger sequencing was performed by Inqaba Biotec™. All PCR products were Sanger sequenced on the ABI 3500 XL as per manufacturer protocol. The genotypes of the 3 *APOL1* variants were obtained only in the forward direction using a forward primer for Sanger sequencing and the following PCR primers: forward – 5'TCAGCTGAAAGCGGTGAACA3' and reverse - 5'GGCATATCTCTCCTGGTGGC3' (**Figure 2.1**). Exons 1 to 8 of the *NPHS2* gene were Sanger sequenced using primers from Boute *et al.* (2000), for exons 2-7. The exon 1 primer set from Boute *et al.* (2000) failed to amplify the samples in this study. A new PCR primer set was designed for *NPHS2* exon 1 (forward – 5'CCAGAGCTTGCGATGAGCTTCTGTATC3', reverse 5'CCGTTCCTGGGAACCTGAGCATCCAGC3').

ATCTCAGCTGAAAGCGGTGAACAGGTGGAGAGGGTTAATGAACCCAGCATCCTG
GAAATGAGCAGAGGAGTCAAGCTCACGGATGTGGCCCCTGTAAGCTTCTTTCTTG
TGCTGGATGTAGTCTACCTCGTGTACGAATCAAAGCACTTACATGAGGGGGCAA
AGTCAGAGACAGCTGAGGAGCTGAAGAAGGTGGCTCAGGAGCTGGAGGAGAAG
CTAAACATTCTCAACAATAATTATAAGATTCTGCAGGCGGACCAAGAACTGTGA
CCACAGGGCAGGGCAGCCACCAGGAGAGATATGCCTGGCAGGGGCCAGGACAA
AATGCAAACTTTTTTTTTTTCTGA

Figure 2.1 Sequence highlighting forward PCR/sequencing primer (blue), SNP1 rs73885319 (yellow) SNP2 rs60910145 (green), INDEL rs71785313 (red) and reverse PCR primer (blue) for the *APOL1* fragment (262bp)

For the *APOL1* amplicon, sequences were obtained only in the forward direction, because the variants were known. For the *NPHS2* exons, sequences were obtained in the forward and

reverse directions, to confirm accuracy of base calling. The sequences were multiple aligned using the ClustalW method of the Geneious software (http://www.geneious.com/) (Kearse *et al.*, 2012). They were compared with reference sequences obtained from the Ensembl genome browser (https://www.ensembl.org/index.html). All variants from the reference sequence that were detected in the *NPHS2* exons were noted and included in the results. All variants were included in the haplotype analysis to assess the likely single of multiple origin of the mutation-associated haplotype. The controls were not sequenced for all the exons and therefore haplotypes could not be deduced for comparison.

2.7 Data analysis

Line graphs were used to depict the clinical course of eGFR over time for each participant.

The association between *APOL1* risk alleles and FSGS was tested by using Fisher's exact to compare frequencies of *APOL1* risk alleles in SS-FSGS cases, SR-FSGS cases without *NPHS2* V260E, all FSGS cases without *NPHS2* V260E, and controls. Individuals with *NPHS2* E/E and V/E were left out from the analysis since they appear to have a monogenic form of SR-FSGS and would bias the association analyses. Odds ratios (ORs) with 95% confidence intervals (CIs) where calculated for the association between 0 versus "1 or 2" *APOL1* risk alleles and FSGS (dominant model) and "0 or 1" versus 2 *APOL1* risk alleles and FSGS (recessive model).

NPHS2 allele and genotype frequencies were compared in 3 groups; SS-FSGS, SR-FSGS and controls. ORs with 95% CIs were calculated for the association between the V260E variant (0 versus 1 V260E variant and 0 versus 2 V260E variants) and SRNS.

A non-parametric Kaplan-Meier plot was used to assess disease progression in terms of "kidney survival", measured as time from diagnosis to ESKD. The Kaplan-Meier is a statistical method used to analyse the time from a specific point (diagnosis of biopsy-proven FSGS) to the occurrence of an event (ESKD) (Kaplan and Meier, 1958).

2.8 Ethics and permission

Ethical clearance was obtained from the Human Research Ethics Committee (HREC) (Medical) (Approval code: M170657) (**Appendix D**). All participants assented to participate in the study and their parents or caregivers provided informed consent according to the documents approved by the (HREC) (**Appendices E to L**).

Documents:

1. Forms for parent/legal guardians for child with FSGS (**Appendix E**) – study information sheet (**Appendix EI**), consent form (**Appendix EII**) and consent form for storing blood and DNA (**Appendix EIII**)
2. Forms for parent/legal guardians for child pedigree (**Appendix F**) – study information sheet (**Appendix FI**), consent form (**Appendix FII**) and consent form for storing blood and DNA (**Appendix FIII**)
3. Forms for child with FSGS ages 7-12 (**Appendix G**) – study information sheet (**Appendix GI**), assent form (**Appendix GII**) and assent form for storing blood and DNA (**Appendix GIII**)
4. Forms for child pedigree ages 7-12 (**Appendix H**) – study information sheet (**Appendix HI**), assent form (**Appendix HII**) and assent form for storing blood and DNA (**Appendix HIII**)
5. Forms for child with FSGS ages 13-17 (**Appendix I**) – study information sheet (**Appendix II**), assent form (**Appendix III**) and assent form for storing blood and DNA (**Appendix IIII**)
6. Forms for child pedigree ages 13-17 (**Appendix J**) – study information sheet (**Appendix JI**), assent form (**Appendix JII**) and assent form for storing blood and DNA (**Appendix JIII**)
7. Forms for patient with FSGS aged >17 (**Appendix K**) – study information sheet (**Appendix KI**), consent form (**Appendix KII)** and consent form for storing blood and DNA (**Appendix KIII**)
8. Forms for adult pedigree (**Appendix L**) – study information sheet (**Appendix LI**), consent form (**Appendix LII**) and consent form for storing blood and DNA (**Appendix LIII**)

3. Results

3.1 Characteristics of study participants

The files of 48 patients with FSGS were identified from CMJAH and MM. Only 32 of these patients met all the study selection criteria. Sixteen participants were excluded from this study due to incomplete data in files and not being currently followed up. Twelve patients were recruited from CMJAH and 20 patients were recruited from the MM. There was one sibling pair from each site. Two hundred and twenty-six controls from the SBIMB biobank were used in this study. One hundred and seventy-six controls were used for comparison with *APOL1* variants. Fifty controls were used for comparison with *NPHS2* variants. The FSGS cases were grouped into SSNS and SRNS. SRNS was more frequent (69%) compared to SSNS (31%). There were 13 (41%) males and 19 (59%) females altogether. The groups did not differ significantly by sex (p=0.99) (**Table 3.1**).

Overall, twenty-five participants had an age of onset <10 years old, and 7 participants had an age of onset ≥10 years old. When comparing the SSNS and SRNS groups, there was a significant difference in the mean age of onset of FSGS (4.7 and 7.1 years respectively; p= 0.02) (**Table 3.1**). The clinical course of eGFR over time for each participant is shown in **Table 3.2**. Generally, steroid resistant participants had an overall decline in kidney function while steroid sensitive participants maintained kidney function.

For those participants who progressed to ESKD, 4 were on dialysis in the SRNS group and none in the SSNS group. Ten participants had undergone a kidney transplant in the SRNS group and none in the SSNS group. The two groups differed significantly by ESKD (p= 0.002) (**Table 3.1**). There were 2 familial cases of FSGS with affected siblings; monozygotic twins from CMJAH and affected sisters from MM. The monozygotic twins from CMJAH were of Sotho ethnicity, and affected siblings from MM were of Twsana and Zulu ethnic origins. One affected twin is currently on dialysis, and one affected sibling has undergone a kidney transplant. Both pairs of affected siblings were steroid resistant.

Table 3.1: Characteristics of black South African children with FSGS****

Characteristic	**FSGS (SD) n=32***	**SSNS (SD) n=10**	**SRNS (SD) n=22**	**P value****
Sex				
Male	13 (40.6%)	4 (40%)	9 (40.9%)	0.9999
Female	19 (59.4%)	6 (60%)	13 (59.1)	
Mean age of onset (years)	6.34 (3.19)	4.7 (2.00)	7.1 (3.34)	0.023
On dialysis				
Yes	4	0	4	0.283
No	28	10	18	
Transplant				
Yes	10	0	10	0.013
No	22	10	12	
ESKD				
Yes	13***	0	13	0.002
No	19	10	9	

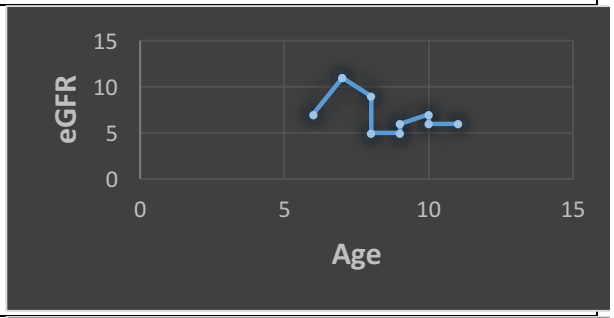
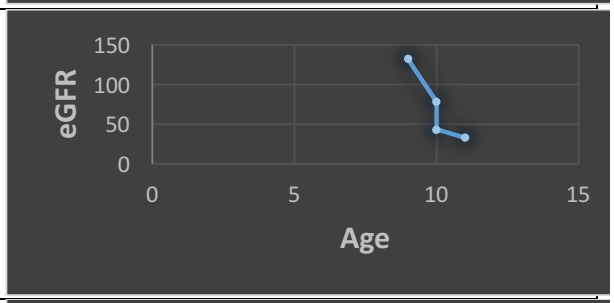
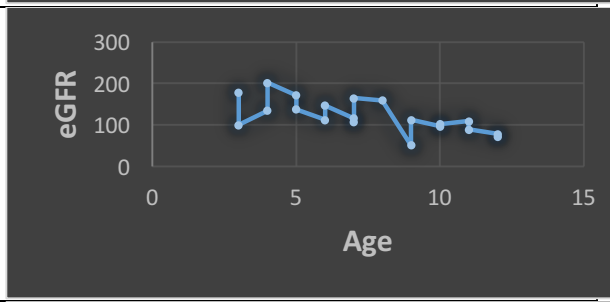
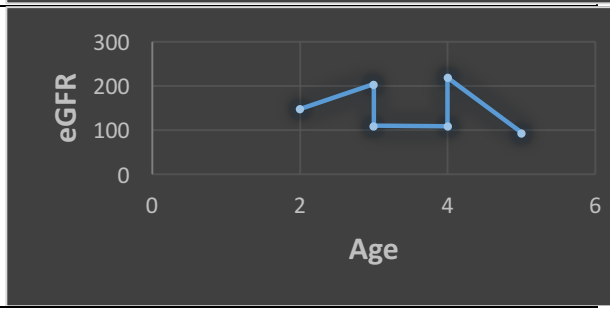
* This includes 2 sets of siblings

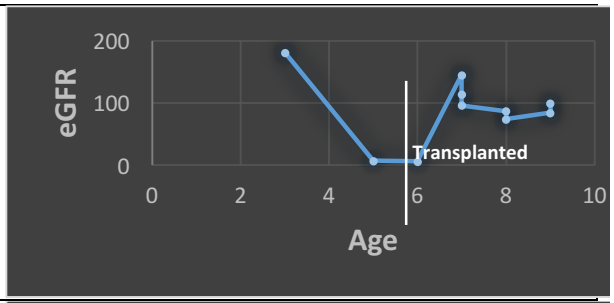
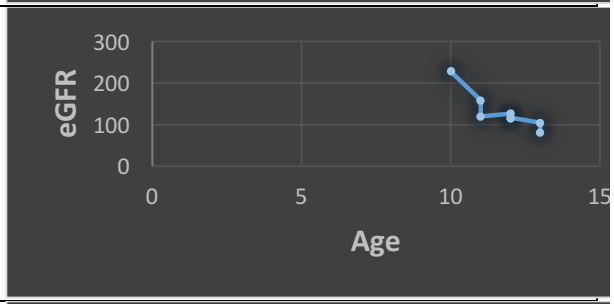
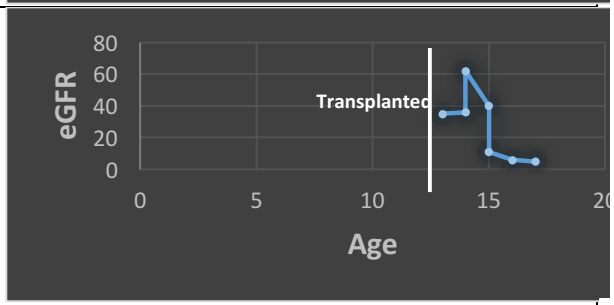
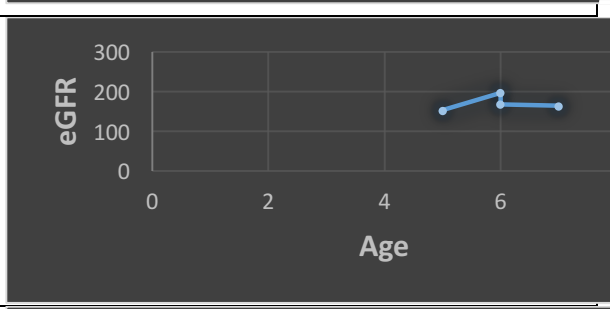
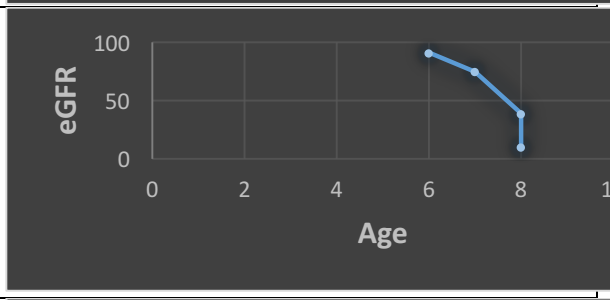
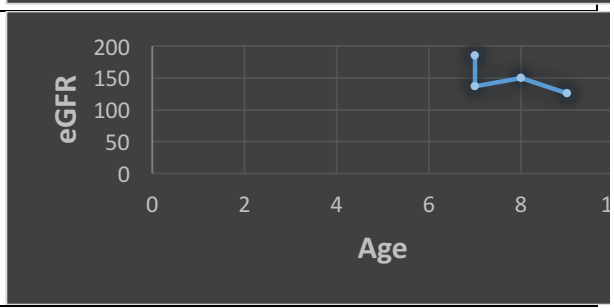
**Comparison between cases with steroid sensitive nephrotic syndrome (SSNS) and steroid resistant nephrotic syndrome (SRNS)

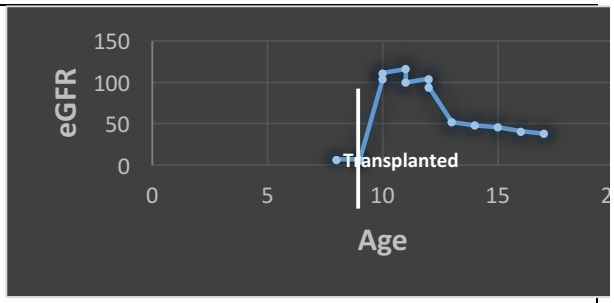
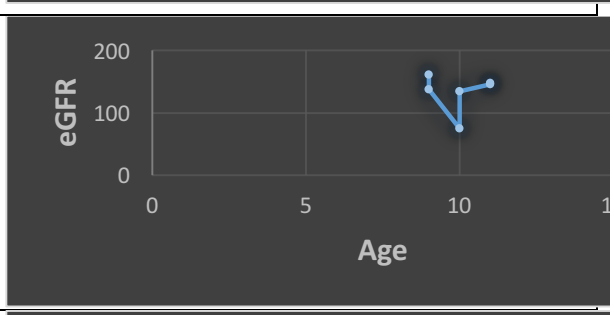
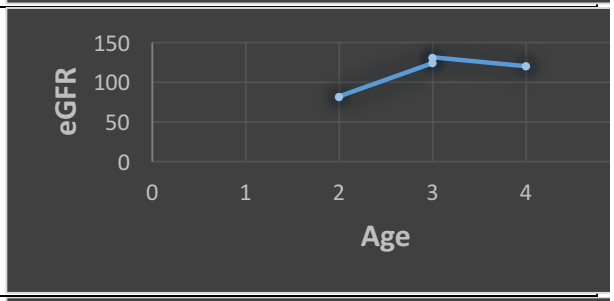
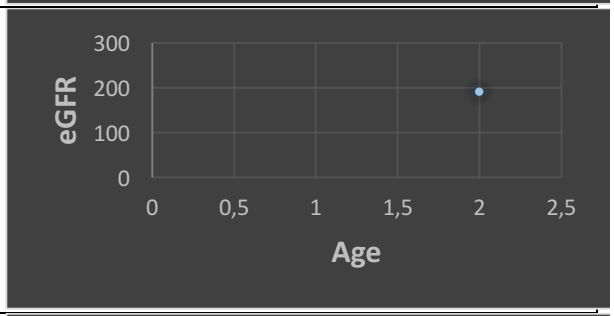
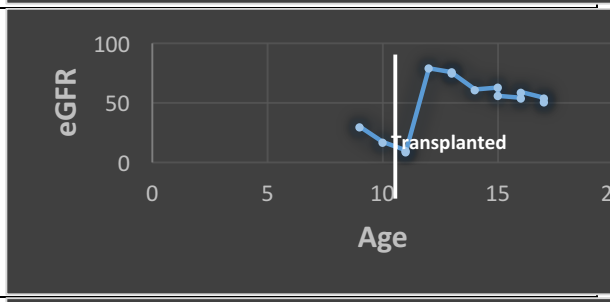
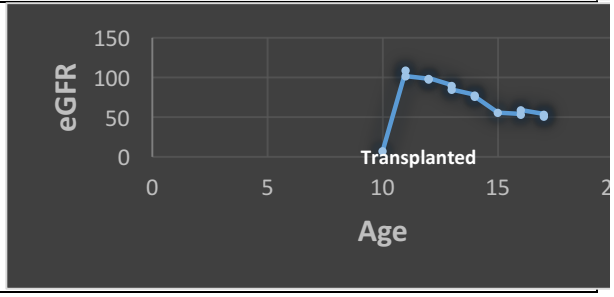
***One patient has dialysis followed by a kidney transplant

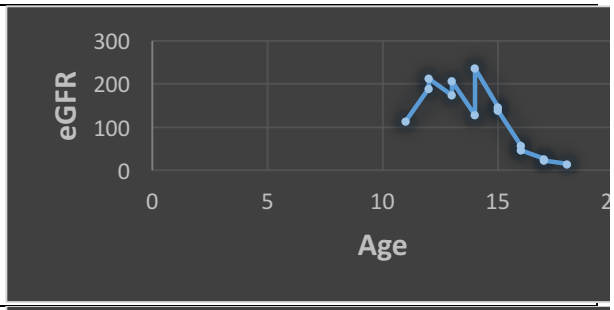
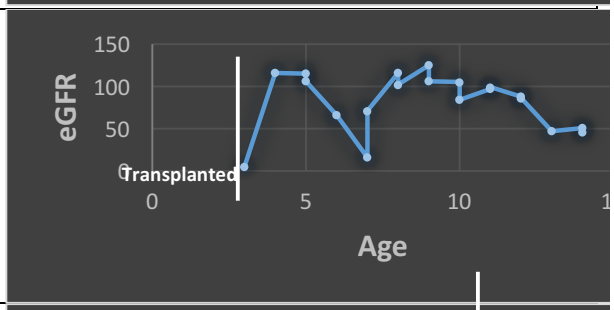
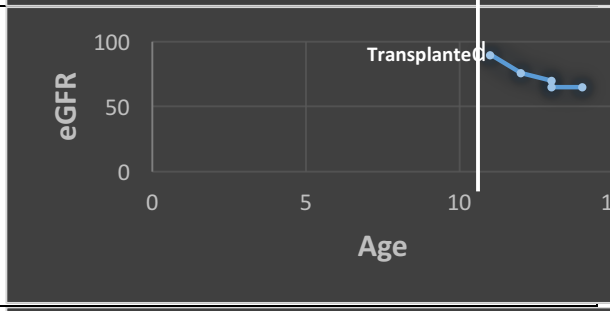
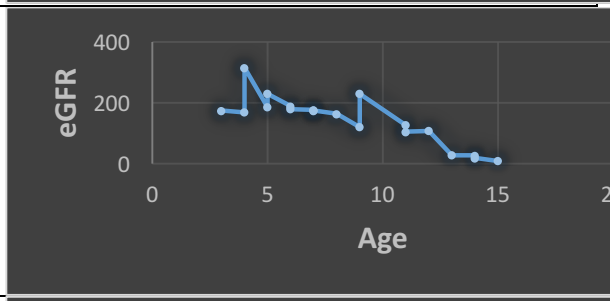
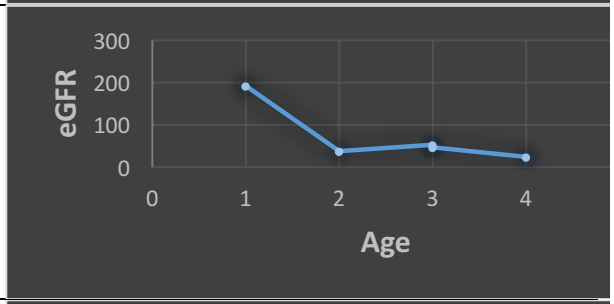
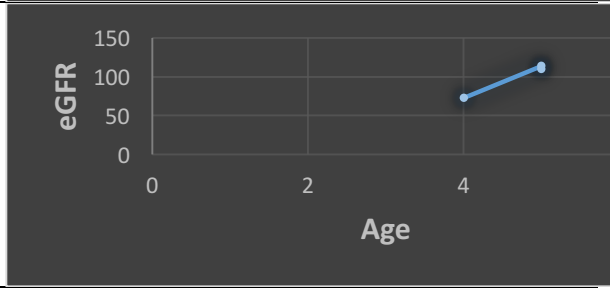
****Fishers exact statistical test used to compare between SSNS and SRNS

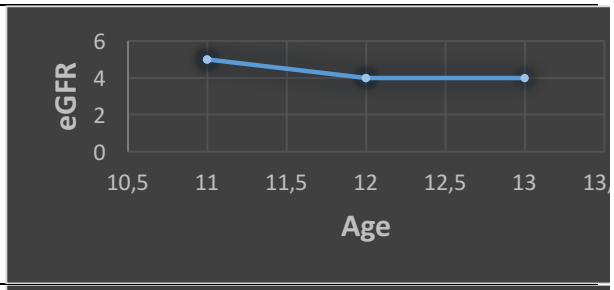
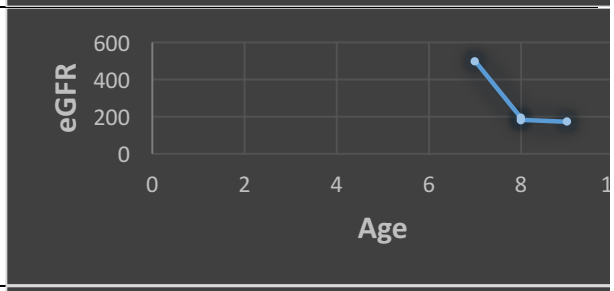
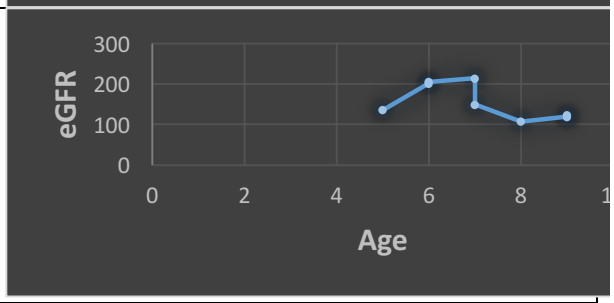
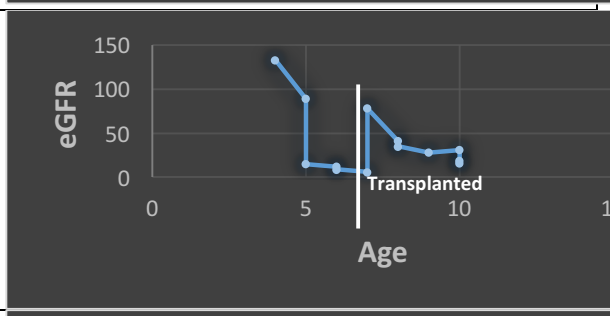
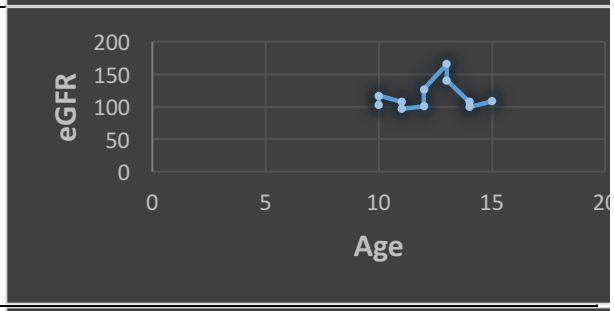
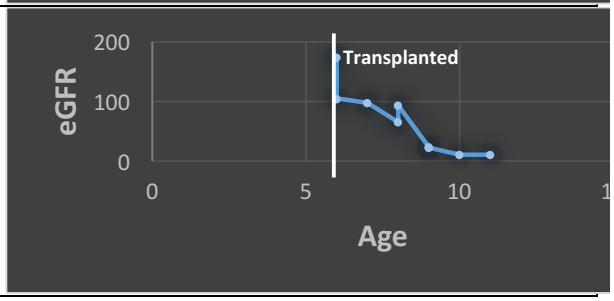
Table 3.2 Clinical course of eGFR over time for each participant

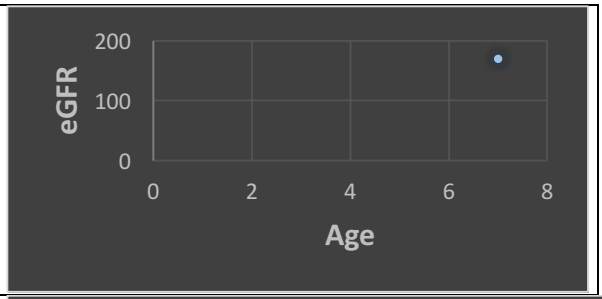
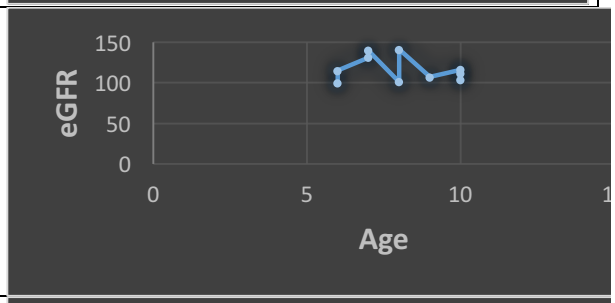
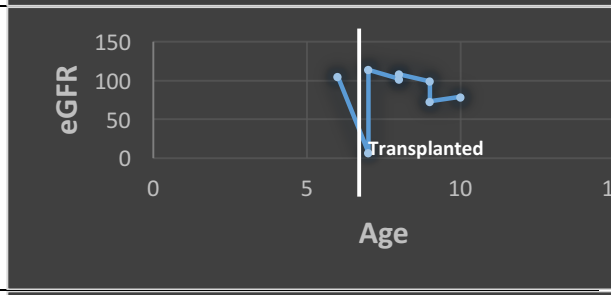
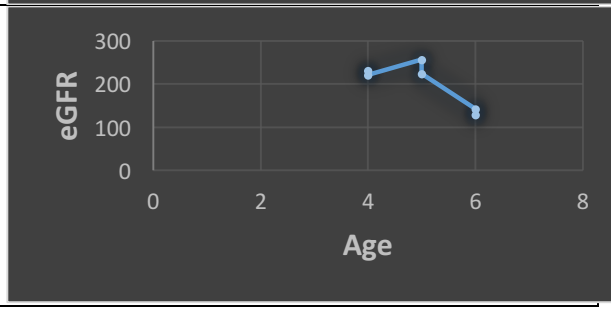
Study ID	SR/ SS	Dialysis	Transplant	Progression graph
KD0013A	SR	YES	NO	
KD0021A	SR	NO	NO	
KD0045A	SR	NO	NO	
KD0059A	SS	NO	NO	

KD0068A	SR	NO	YES	
KD0278A	SR	NO	NO	
KD0076A	SR	YES	YES	
KD0084A	SS	NO	NO	
KD0092A	SR	YES	NO	
KD0101A	SS	NO	NO	

KD0117A	SR	NO	YES	
KD0149A	SR	NO	NO	
KD0158A	SS	NO	NO	
KD0210A *	SS	NO	NO	
KD0241A	SR	NO	YES	
KD0255A	SR	NO	YES	

KD0264A	SR	YES	NO	
KD0308A	SR	NO	YES	
KD0319A	SR	NO	YES	
KD0362A	SR	NO	NO	
KD0370A	SR	NO	NO	
KD0381A	SR	NO	NO	

KD0397A	SR	NO	NO	
KD0416A	SS	NO	NO	
KD0427A	SS	NO	NO	
KD0434A	SR	NO	YES	
KD0442A	SR	NO	NO	
KD0515A	SR	NO	YES	

KD0528A **	SS	NO	NO	
KD0531A	SS	NO	NO	
KD0544A	SR	NO	YES	
KD0553A	SS	NO	NO	

*Study participant KD0210A had 2 eGFR values at age 2. The average of values 165 and 175 are shown in the graph.

** Study participant KD0528A had 2 eGFR values at age 7. The average of values 167 and 174 are shown in the graph.

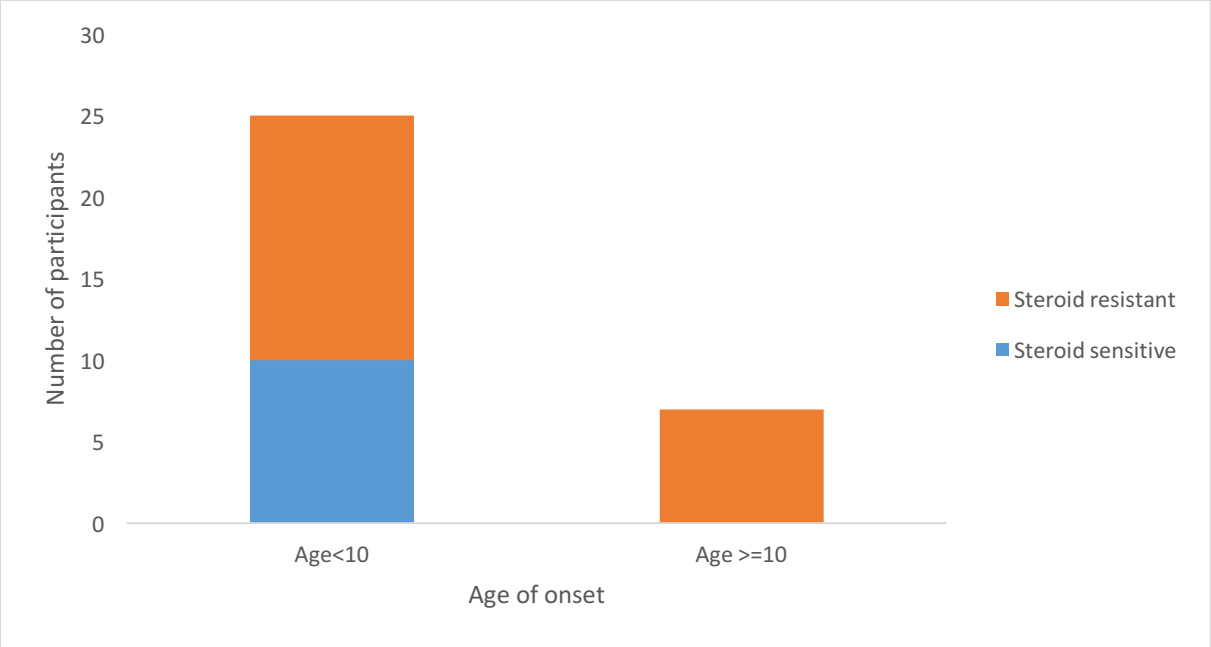


Figure 3.1: Bar graph showing participants grouped into age of onset <10 and ≥10 years old and stratified by steroid resistant and steroid sensitive

Study participants were from different ethnolinguistic origins in South Africa, including Xhosa, Tsonga, Tswana, Swati, Sotho, Pedi and Zulu (**Figure 3.2**). The majority of the study participants were Sotho speakers (Northern and Southern combined).

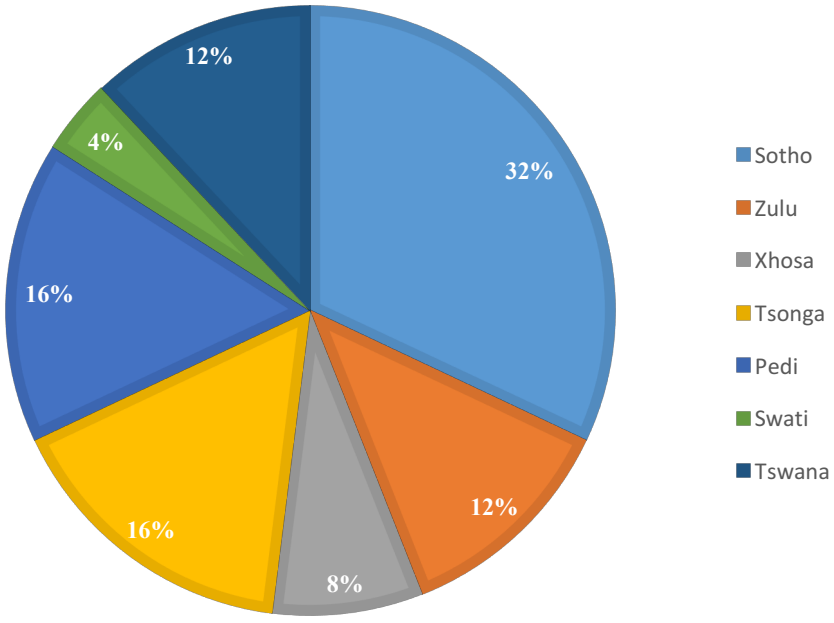


Figure 3.2: Pie chart showing ethnolinguistic origins of study participants

3.2 *APOL1* associations with FSGS

Since the results can be skewed by family relationships, one affected member was removed from each family for *APOL1* association analysis (30 unrelated individuals). *APOL1* genotyping data for all FSGS cases is shown in **Table 3.3**. *APOL1* genotype distribution among FSGS (SRNS and SSNS) unrelated cases and controls is shown in **Table 3.4**. Among the unrelated FSGS cases, allele frequencies for the *APOL1* G1 and G2 risk alleles were 10% and 22%, respectively. Allele frequencies for G1 and G2 alleles were 8% and 13% for the controls, respectively (**Table 3.4**)

Table 3.3: *APOL1* genotyping data for all FSGS cases in this study

Study ID	rs73885319	rs60910145	rs71785313*	Haplotypes**
KD0013A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0021A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0045A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0059A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0068A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0076A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0084A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0092A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0101A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0117A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0149A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0158A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0210A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0241A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0255A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0264A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0278A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0308A	A/A	T/T	D/D	ATD/ATD (G2/G2)
KD0319A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0362A	A/G	T/G	I/D	GGI/ATD (G1/G2)
KD0370A	A/A	T/T	D/D	ATD/ATD (G2/G2)
KD0381A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0397A	A/A	T/T	I/D	ATI/ATD (G0/G2)
KD0416A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0427A	A/A	T/T	I/I	ATI /ATI (G0/G0)
KD0434A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0442A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0515A	A/A	T/T	I/I	ATI/ATI (G0/G0)

KD0528A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0531A	A/G	T/G	I/I	ATI/GGI (G0/G1)
KD0544A	A/A	T/T	I/I	ATI/ATI (G0/G0)
KD0553A	A/A	T/T	I/I	ATI/ATI (G0/G0)

*****I= insertion, D= deletion

**Haplotypes: G0= ATI, G1= GGI and GTI, G2= ATD. Haplotypes were assigned by hand and when more that one locus was heterozygous, compatibility with common haplotypes as the most likely scenario was adopted.

Table 3.4: *APOL1* genotype distribution among FSGS cases and controls

Genotype Frequency	**Allele Frequency**	**FSGS (30)**	**SS-FSGS (10)**	**SR-FSGS (20)***	**Controls (n=176)**
0 risk allele					
G0/G0		14 (46%)	3 (30%)	11 (55%)	117 (66.5%)
1 risk allele					
G0/G1		5 (17%)	3 (30%)	2 (10%)	20 (11.4%)
G0/G2		8 (27%	4 (40%)	4 (20%)	27 (15.3%)
Total		**13 (44%)**	**7 (70%)**	**6 (30%)**	**47 (26.7%)**
2 risk alleles					
G1/G1		0	0	0	2 (1.1%)
G1/G2		1 (3%)	0	1 (5%)	3 (1.7%)
G2/G2		2 (7%)	0	2 (10%)	7 (4%)
Total		**3 (10%)**	**0**	**3 (15%)**	**12 (6.8%)**
	G0 allele	0.683	0.650	0.700	0.798
	G1 allele	0.100	0.150	0.075	0.077
	G2 allele	0.216	0.200	0.225	0.125

* Of the 20 SR-FSGS 20 cases only 5 cases did not have the V260E variant (3 of these individuals had the G0/G0 genotype, 1 individual had G0/G2 genotype and 1 individual had G2/G2 genotype)

The effect size (OR) and statistical significance for SS-FSGS cases vs controls were OR=4.63; 95% CI, 1.15 – 18.5; p=0.03 in the dominant model and OR=0.63; 95% CI, 0.03 – 11.3; p=0.75 in the recessive model (**Table 3.5**). The OR and statistical significance for SR-FSGS cases without V260E (V260E data presented in the next section, but relevant to the *APOL1* analysis and therefore included here) were OR=1.3; 95% CI, 0.21 – 8.13; p=0.76 in the dominant model and OR=3.4; 95% CI, 0.35 – 33.0; p=0.29 in the recessive model (**Table 3.5**). Since there are

only 5 individuals who were SR-FSGS without V260E, these results need to be interpreted with caution. The FSGS cases without V260E were pooled (n=15; 10 SS and 5 SR). The OR and statistical significance for all FSGS cases without V260E were OR=2.97; 95% CI, 1.01 – 8.75; p=0.048 in the dominant model and OR=0.98; 95% CI, 0.12 – 8.07; p=0.98 in the recessive model (**Table 3.5**).

Table 3.5: Association of *APOL1* kidney disease risk variants with FSGS versus controls for dominant and recessive models*

Number risk allele	SS-FSGS N=10	SR-FSGS w/o** NPHS2 V260E N=5	All FSGS w/o** NPHS2 V260E N=15	Controls N=176
0	3	3	6	117
1	7	1	8	47
2	0	1	1	12
Dominant model: 1 or 2 RA vs. 0 RA	4.63, p=0.03 (CI: 1.15, 18.5)	1.3, p=0.76 (CI: 0.21, 8.13)	2.97, p=0.048 (CI:1.01, 8.75)	Reference
Recessive model: 0 or 1 RA vs. 2 RA	0.63, p=0.75 (CI: 0.03, 11.3)	3.4, p=0.29 (CI: 0.35, 33.0)	0.98, p=0.98 (CI:0.12, 8.07)	Reference

*CI = 95% confidence interval
**w/o = without

3.3 *NPHS2* associations with FSGS

3.3.1 Variants in the *NPHS2* gene

NPHS2 exon sequencing was performed for all 32 cases (including sib pairs). Two 5 primer UTR variants (rs78541594 and rs12406197) have been previously reported on short genetic variations database (dbSNP) and are predicted to be likely benign. The COSM900289 variant has been previously reported in Catalogue of Somatic Mutations in Cancer (COSMIC) database and has a FATHMM score of 0.093. FATHMM predicts scores of functionality ranging from 0-1, with scores greater than 0.7 highlighting pathogenicity. Two missense variants (A242V and V260E) and 5 synonymous variants (G34G, S48S, S96S, A318A and L346L) were observed (**Table 3.6**). All synonymous variants have been previously reported on dbSNP and are

predicted to be benign. A242V is a common polymorphism in exon 5 (frequency in cases (5/32) and controls (9/50)). The Minor allele frequency (MAF) for A242V according to the 1000 Genomes project combined data is 7% in Africans, and this variant was not observed in Asians and Europeans. Since the A242V variant has a MAF >0.05 and was only observed in Africans, it is considered to be an African specific variant. *NPHS2* V260E is a known pathogenic variant that results in a nucleotide change from adenine to thymine and an amino acid change from valine to glutamic acid . The ExAC database reports a frequency of 0.002% in Africans, and it is not present in African Americans, Asians and Europeans. Therefore, exon 6 was sequenced in 50 black controls to detect the V260E variant.

Table 3.6: Variants in the *NPHS2* gene in 30 unrelated black South African paediatric FSGS cases

Variant characteristics				SS-FSGS N=10			SR-FSGS N=20			MAF according to the 1000 Genomes Project combined data		
Exon	SNP ID	cDNA	Protein	Het	Hom	Allele Freq	Het	Hom	Allele Freq	Eur	Afr	Asian
1	rs78541594			1	0	0.05	2	0	0.05	0.003	0.230	0
1	rs12406197			3	0	0.15	7	9	0.63	0.239	0.133	0.119
1	rs1079292	c.102A>G	p.Gly34Gly	3	5	0.65	3	16	0.88	0.008	0.177	0.019
1	rs111306764	c.144C>T	p.Ser48Ser	3	0	0.15	2	0	0.05			
2	rs3738423	c.288C>T	p.Ser96Ser	1	0	0.05	1	0	0.03	0.087	0.094	0.095
4	COSM900289			0	0	0	1	0	0.03	-	-	-
5	rs61747727	c.725C>T	p.Ala242Val* A242V	1	0	0.05	4	0	0.10	0	0.073	0
6	rs775006954	c.779T>A	p.Val260Glu** V260E	0	0	0	4	11	0.65	-	-	-
8	rs1410592	c.954C>T	p.Ala318Ala	6	2	0.50	9	0	0.23	0.615	0.595	0.491
8	rs3818587	c.1038A>G	p.Leu346Leu	1	0	0.05	1	0	0.03	0.087	0.095	0.094

* SIFT and PolyPhen predict Ala242Val is deleterious and probably damaging, respectively
** SIFT and PolyPhen predict Val260Glu is damaging and probably damaging, respectively

3.3.2 *NPHS2* associations with FSGS (SRNS & SSNS)

The *NPHS2* V260E variant was present in 15/30 unrelated FSGS cases (11 were homozygous and 4 were heterozygous). The electropherograms for the 3 V260E genotypes (homozygous for wildtype allele (T/T), heterozygous (T/A), homozygous for variant (A/A)) in the forward and reverse directions are shown in **Figure 3.3**. The V260E variant was only observed in individuals with SRNS and not in any of the SSNS cases.

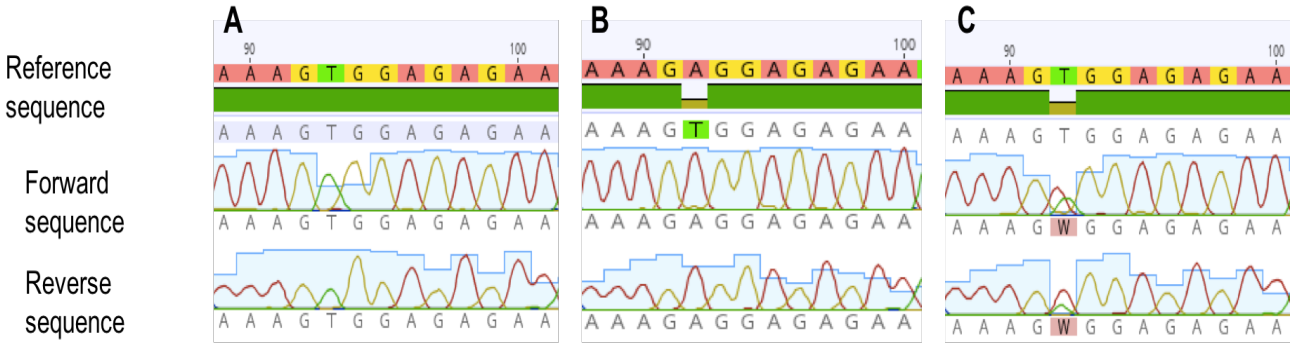


Figure 3.3: Electropherograms showing sequence data from individuals (A) Homozygous for the *NPHS2* V260 allele (T/T) (B) Homozygous for the *NPHS2* 260E allele (A/A) (C) Heterozygous for the *NPHS2* V260E allele (A/T), from the sequencing in the forward and reverse directions.

Allele and genotype frequencies of *NPHS2* V260E were compared in three groups (SS-FSGS, SR-FSGS and controls) (**Table 3.7**). None of the controls were homozygous for *NPHS2* V260E, however one black healthy control was heterozygous for the *NPHS2* V260E variant. The FSGS cases were grouped into SRNS (22/32) (69%) and SSNS (10/32) (31%) (including sib pairs).

Table 3.7 Allele and genotype frequencies for *NPHS2* V260E in SS-FSGS, SR-FSGS and controls

	NPHS2 V260E	**SS-FSGS N=10**	**SR-FSGS N=20**	**Controls N=50**
Genotypes	V/V	10 (100%)	5 (25%)	49 (98%)
	V/E	0	4 (20%)	1 (2%)
	E/E	0	11 (55%)	0
Allele frequency	V	1	0.35	0.99
	E	0	0.65	0.01

One affected sibling was removed from each familial case for *NPHS2* V260E associations (30

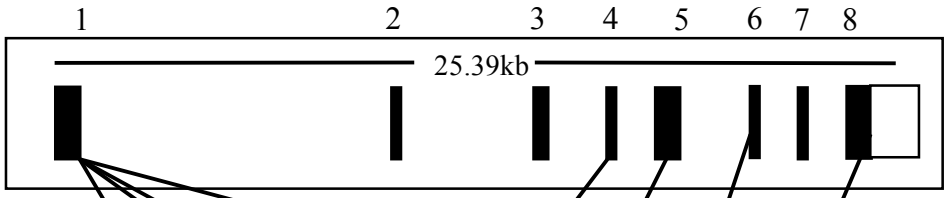
unrelated individuals). There were no significant differences in genotype frequencies between SS cases and controls (p= 0.99). There was a significant difference in genotype frequencies between SR and SS cases (p= 0.00012) and between SR cases and controls (p= 2.07e-10). The OR and statistical significance were assessed for carrying 1 or 2 *NPHS2* V260E variants in SRNS cases (**Table 3.8**). Carrying one copy of *NPHS2* V260E had an OR of 36.8 (95% CI, 3.4 - 396.7; p= 0.002) for SRNS that increased to 101.2 (95% CI, 10.7 - 995.9; p=7.76e-8) when carrying two copies of *NPHS2* V260E.

Table 3.8: *NPHS2* associations with unrelated FSGS cases (n=30) (SS (n=10) and SR (n=20))

Comparison	**Fisher's exact p value**	**OR (95% CI)**
SSNS vs. controls	**0.99**	
SSNS vs. SRNS	**0.00012**	
SRNS vs. controls	**2.07e-10**	
SRNS vs. controls 1 versus 0 2 versus 0	 0.002 7.76e-8	 36.8 (3.4 to 396.7) 101.2 (10.7 to 955.9)

Table 3.9 shows the *NPHS2* haplotypes associated with the V260E variant in the SR-FSGS cases with the V260E variant. Of the 26 independent *NPHS2* 260E-associated alleles (from 11 homozygotes and 4 heterozygotes), 24 were compatible with the TTGCCCAC haplotype, and two had alternate 260E-associated haplotypes (CGGTCCAC and TGGCCCAC). There is a core haplotype around the 260E variant (CCAC).

Table 3.9: Different *NPHS2* haplotypes identified with map showing their physical location in *NPHS2* exons in unrelated SR-FSGS individuals with the V260E variant*



	rs115256710	rs12406197	rs1079292	rs111306764	COSM900289	rs61747727	rs775006954	rs141 0592	
	T/C	**G/T**	**A/G**	**C/T**	**C/A**	**C/T**	**T/A****	**C/T**	**E chromosomes N=26**
Haplotype 1	T	T	G	C	C	C	A	C	24/26
Haplotype 2	C	G	G	T	C	C	A	C	1/26
Haplotype 3	T	G	G	C	C	C	A	C	1/26

***Affected siblings were also E/E

****The *NPHS2* V260E variant that results in an amino acid change from valine to glutamic acid

3.3.3 *NPHS2* and *APOL1* association with age of onset of FSGS

This section reports on the variants in both *NPHS2* and *APOL1* in each of the cases and aims to provide information on their dual role in kidney disease and age of onset of FSGS. The V260E variant was not observed in any of the SSNS cases (**Figure 3.4A**). In the SRNS cases, *NPHS2* V260E was present in the homozygous state in 59% (13/22) and heterozygous state in 18% (4/22) (including sib pairs) (**Figure 3.4B**). Both sibs in the two familial cases were homozygous for V260E. The *APOL1* risk allele status is shown for each individual and none of the SSNS cases had 2 *APOL1* risk alleles. In the SRNS groups, individuals with 0 or 1 *APOL1* risk variants had a spread of ages of onset, but individuals with 2 *APOL1* risk alleles tended to have an earlier age of onset.

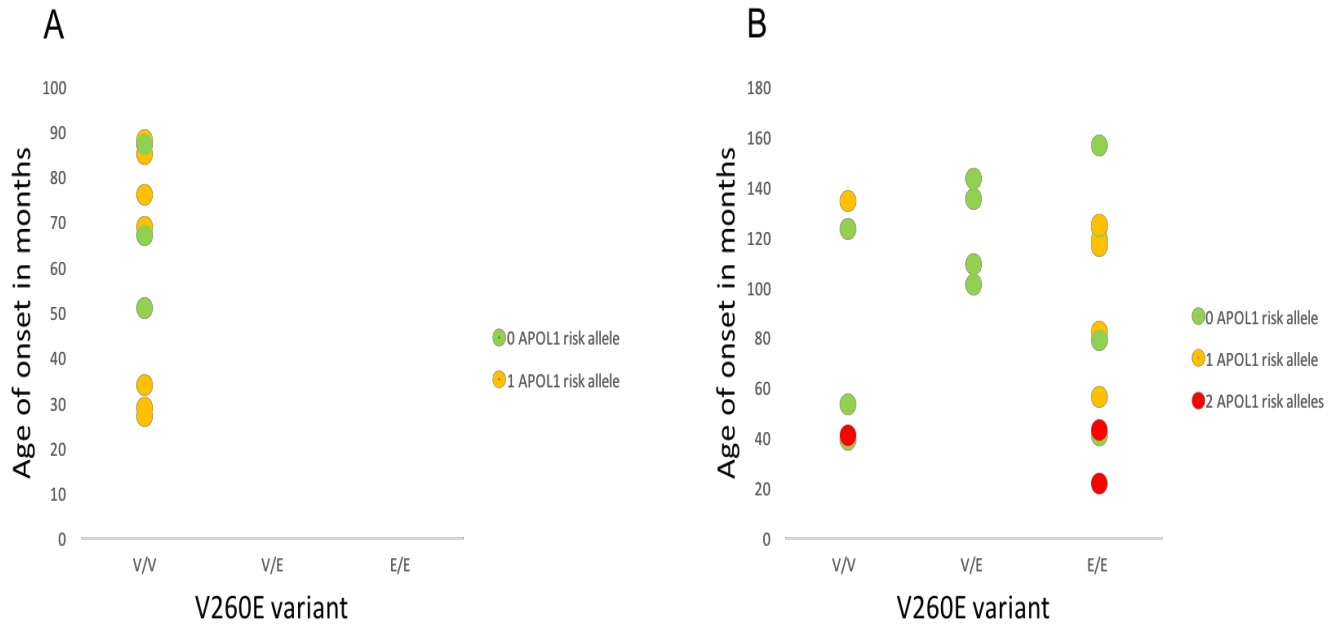


Figure 3.4: A) Comparison of SSNS (n=10) and SRNS (B)(n=22) (including sib pairs) cases showing *NPHS2* V260E genotypes, age of onset (vertical axis) and colour coding according to number of *APOL1* risk alleles.

A time-to-event analysis was performed for V260E by measuring the time from biopsy-proven FSGS to progression to ESKD, and comparing individuals who had SSNS (0 *NPHS2* V260E variant), SRNS (0 *NPHS2* V260E variant) and SRNS (1 or 2 *NPHS2* V260E variants). Survival curves were combined for individuals with 1 and 2 *NPHS2* V260E variants, as there was no significant difference between the two (p= 0.21). The Kaplan-Meier plot depicts the kidney survival among 32 paediatric patients with FSGS. Out of the 32 FSGS cases, 13 progressed to ESKD. Vertical lines indicate duration of observation from the time of diagnosis in individuals

who have not yet reached kidney failure. In terms of the number of individuals with ESKD, the *NPHS2* V260E variant was present in 77% (10/13) of cases. A mean kidney survival of 69.2 and 26.8 months was observed when the V260E variant was absent and present in SRNS cases, respectively. There was a significant difference in kidney survival among the three groups with a log rank p value of 0.026 (**Figure 3.5**). The sample size for individuals with the V260E variant was too small to stratify the sample according to *APOL1* genotypes for the Kaplan-Meier kidney survival plot.

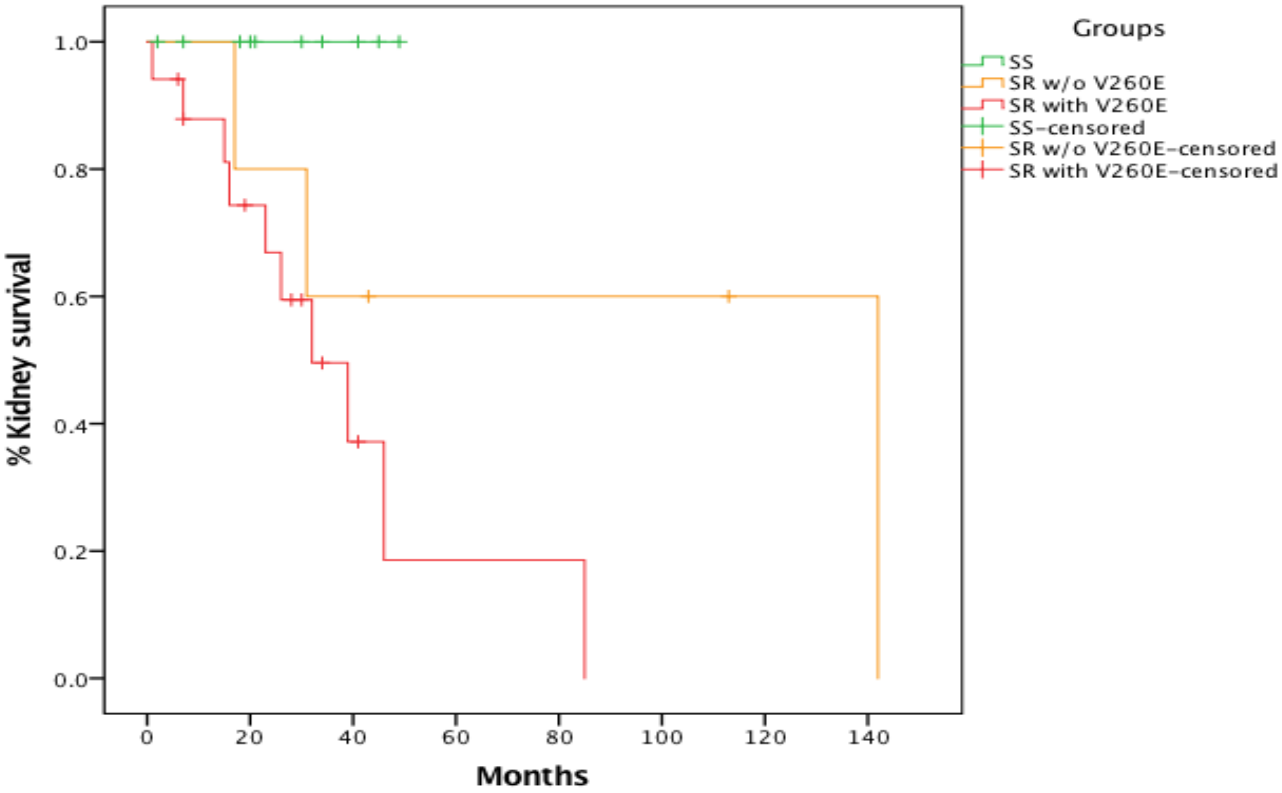


Figure 3.5: Kaplan-Meier plot showing kidney survival from biopsy proven diagnosis to renal failure determined by either the start of dialysis, at the time of kidney transplant or individuals with eGFR <15ml/min/1.73m^2 for individuals with SSNS (0 *NPHS2* V260E variant), SRNS (0 *NPHS2* V260E variant) and SRNS (1 or 2 *NPHS2* V260E variants). Vertical lines indicate duration of observation from the time of diagnosis in individuals who have not yet reached renal failure.

The pedigrees of families A and B are shown in **Figure 3.6** Notably the age of a biopsy-confirmed FSGS differed by 7 years for the two sibling from family A who are homozygous for V260E. Proband II.3 was 3 years old when a renal biopsy confirmed FSGS and a renal transplant was performed at the age of 7 years. Proband II.2 was 10 years old at the time of biopsy-confirmed FSGS. The mother and father each have 1 *APOL1* high risk allele. The mother

passed on 1 high risk allele (G1) to two of her children, one of whom was unaffected (proband II.2). Proband II.3 inherited two low risk alleles, one from each parent. Both parents were heterozygous for V260E mutation and both affected sibs were homozygous for the mutation. In Family B, monozygotic twins were affected with FSGS (SRNS). Proband II.2 was 6 years old when FSGS was confirmed, and is currently on dialysis. Proband II.3 was 9 years old when diagnosed with FSGS and is not on dialysis. The difference in age of onset may be due to environmental modifiers. DNA samples were unavailable for the unaffected children. The mother had 1 high risk *APOL1* allele, which she passed on to both affected children. Both parents were heterozygous for V260E,and each passed on the mutation to their affected children who were homozygous for V260E.

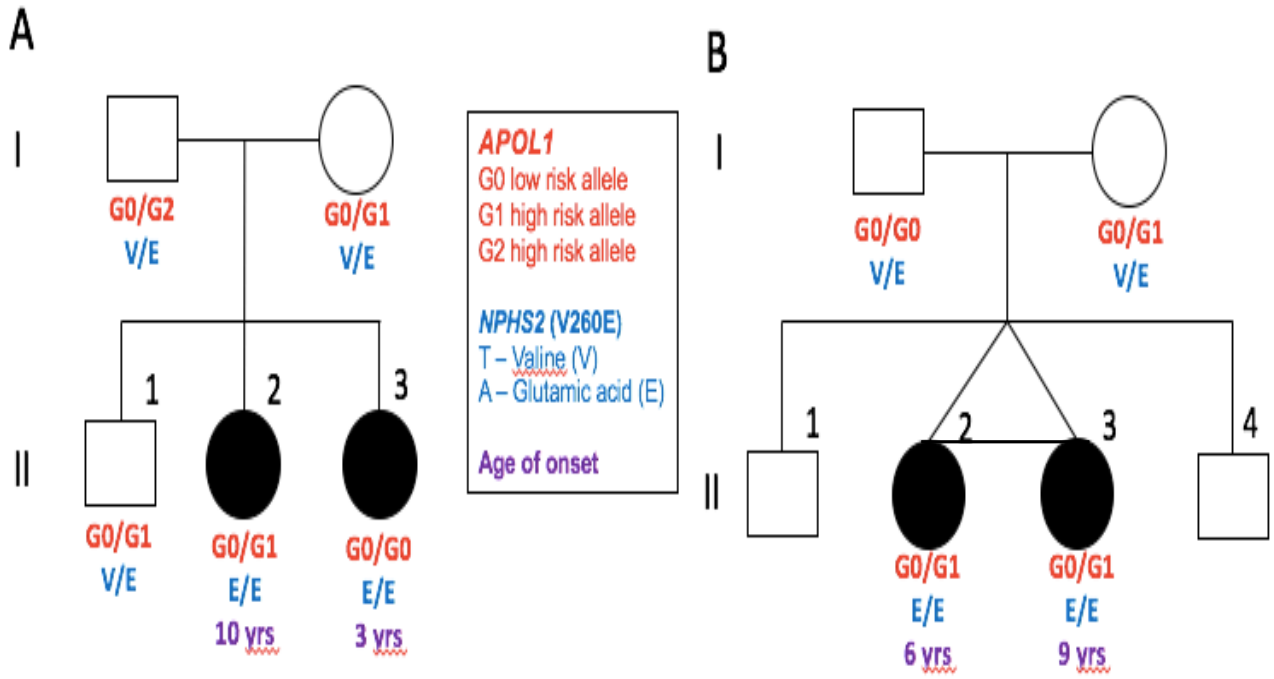


Figure 3.6: Pedigree showing segregation of *APOL1* alleles (G0, G1 and G2) and *NPHS2* V260E variant in the two familial cases. In family B, blood samples were not available for the unaffected siblings.

4. Discussion

Previous studies have found a relatively strong association between *APOL1* risk alleles and genotypes and FSGS in African American adults (OR=17) (Kopp *et al.*, 2011), but no significant association in black South African adults with FSGS (OR=6.03) (Kasembeli *et al.*, 2015). In the

presence of HIV infection, South African adults did however show a very strong association between *APOL1* risk variants and HIVAN (OR=89) (Kasembeli *et al.*, 2015). In African American children with FSGS, 78% were found to carry two *APOL1* risk variants with a median age of onset of 12 years (Woroniecki *et al.*, 2016). The role of *APOL1* risk variants with FSGS in black South African children is unresolved; a previous study did not find significant associations in a group of 31 children with SR-FSGS (Asharam *et al.*, 2018). Monogenic forms of FSGS in children and adults have been described involving over 50 genes and *NPHS2* is often implicated in childhood SRNS. The aim of this study was therefore to determine genetic associations with *apolipoprotein L1* (A*POL1*) risk variants and *podocin* (*NPHS2*) variants in black South African children with FSGS.

4.1 Study participant characteristics

FSGS is the most common histological finding in NS patients with approximately 80% being steroid resistant (Bagga and Mantan, 2005; Han and Kim, 2016), and some differences in the proportions across ethnic groups. The frequency of FSGS is two to three times higher in individuals with African ancestry compared to individuals with European ancestry (Sprangers *et al.*, 2016). In my study, a larger proportion of unrelated black African children with FSGS were steroid resistant (67%) compared to steroid sensitive (33%); a figure much higher than the proportion described by Chanchlani and Parekh (2016). This study showed steroid resistance in 27% of Africans compared to 20% of Europeans (Chanchlani and Parekh, 2016). This is in agreement with several South African studies that found that steroid resistance is more common in black children (Asharam *et al.*, 2018; Bakhiet, 2016; Bhimma *et al.*, 1997), supporting the assertion that ancestry plays a vital role in susceptibility to disease (Chanchlani and Parekh, 2016).

There was a significant difference in the mean age of onset between SSNS (4.7 years) and SRNS (7.1 years). This is somewhat unexpected, as SRNS cases usually have an earlier age of onset (Nourbakhsh and Mak, 2017; Tan *et al.*, 2018). The difference in average age of onset between SSNS and SRNS cases is difficult to explain but may be due to sampling bias - as children may have been referred at different time periods after their diagnosis of FSGS, or the observed effect might be a consequence of the small sample size.

Generally, among steroid resistant participants, there was an overall decline in kidney function (measured with eGFR), while steroid sensitive participants maintained their kidney function (**Table 3.2**). Previous studies by Bakhiet (2016) and Zagury *et al.* (2013) reported reduced eGFR (<90 ml/min/1.73m^2) in individuals with SRNS compared to those with SSNS, which confirms the findings in this study. If there is a continual decline in eGFR, there is an increased risk of progression to ESKD (van Biljon, 2011; Li *et al.*, 2009; Zagury *et al.*, 2013). In several of the participants in my study, the eGFR increased with a kidney transplant but then decreased again.

4.2 *APOL1* associations with FSGS

In my study, the combined G1 and G2 risk allele frequency among paediatric FSGS cases was 32%. This was higher than the ~18.4% observed in adult black South Africans by Kasembeli *et al.* (2015), and lower than the reported allele frequencies in West Africa (Yoruba ~45%) and African Americans in a cohort of patients with renal disease (~34%) (Genovese *et al.*, 2013; Kasembeli *et al.*, 2015; Kopp *et al.*, 2011). It is hypothesized that in the last 10 000 years, *APOL1* variants arose in sub-Saharan Africans, most likely in West Africans, where they were subjected to strong recent positive selection likely due to the presence of trypanosomiasis (Limou *et al.*, 2015; Smith and Malik, 2009). There is evidence of a recent selective sweep, seen as extended haplotype homozygosity in the region harbouring the *APOL1* variants observed only in Yoruba individuals from Nigeria (Genovese *et al.*, 2010; Limou *et al.*, 2015). The lower frequency in my study suggests a possible relaxation of selection pressures once the population migrated out of a trypanosomiasis endemic region, or gene flow from West Africans into the gene pool of different ethnic groups in sub-Saharan Africa with lower allele frequencies, possibly from the Bantu expansion out of West Africa (Kasembeli *et al.*, 2015; Tishkoff *et al.*, 2009). In this study, the combined allele frequency for G1 and G2 was 21% in controls, similar to what was observed in KwaZulu-Natal (19%)(Asharam *et al.*, 2018).

The *APOL1* G1 and G2 risk alleles are found at high frequencies outside Africa only in individuals with recent African origin. During the 16th to 19th century, approximately 11 million Africans were forced to migrate from the Atlantic coastal regions of Africa to the Caribbean and America during the trans-Atlantic slave trade (Postma, 2003). The *APOL1* G1 and G2 frequencies from African individuals along the Atlantic coastal regions are reflected in the

high allele frequencies in African Americans and Hispanic Caribbeans (Tishkoff *et al.*, 2009). The frequency of individuals carrying 2 *APOL1* risk alleles is approximately 10-15% in African Americans and in Hispanic individuals that migrated from the Caribbean to the New York region, the *APOL1* risk alleles occur at much lower frequencies (Behar *et al.*, 2011; Rosset *et al.*, 2011).

Even though some studies state that 2 *APOL1* risk alleles are associated with a later age of onset of FSGS (Kopp *et al.*, 2015), there are others that state that two *APOL1* risk alleles are associated with an earlier age of onset of FSGS (Hoy *et al.*, 2015). In my study, individuals with 2 *APOL1* risk alleles appeared to have an early age of onset (**Figure 3.4b**), however, because the sample size was small, the study was underpowered to reach a definitive conclusion.

It is argued by many studies that *APOL1* risk alleles have a stronger effect in a recessive model (Limou *et al.*, 2015; Tayo *et al.*, 2013; Tzur *et al.*, 2010). However, my study failed to identify a significant association of *APOL1* risk variants with FSGS in unrelated black South African paediatric patients consistent with other studies, in the recessive model (Kasembeli *et al.*, 2015). Even though most studies argue for a recessive model, an additive or dominant model cannot be completely excluded. In my study, a weak association for the dominant model with p values of 0.048 and 0.03 was noted for all FSGS cases without V260E and SS-FSGS cases only, respectively. Along this line, studies by Kanji *et al.* (2011) and Tayo *et al.* (2013) found that carrying one risk allele, either the G1 or G2, is associated with an earlier age of onset in non-diabetic ESKD patients. In my study, *APOL1* risk alleles were found to be significantly associated with SSNS (p=0.03). These results contradict findings by Adeyemo *et al.* (2018), who found no association (p=0.5) between *APOL1* risk alleles and SSNS in African American children.

4.3 *NPHS2* associations with SR-FSGS

Previous reports describe *NPHS2* variants as the most common cause of AR SRNS (Online Mendelian Inheritance in Man [OMIM] #600995) in childhood (Sadowski *et al.*, 2015; Weber *et al.*, 2004). In an international cohort of 1783 SRNS families, Sadowski *et al.* (2015) identified *NPHS2* variants in 13% of SRNS patients that comprised 34% of all genetic variants identified.

In my study, a total of 10 variants were identified in the *NPHS2* gene. Two were 5' UTR variants (rs12406197 and rs78541594), that were previously reported and predicted to be likely benign. These 2 variants have MAFs of 13.3% and 23% in African populations, respectively. A non-coding variant, COSM900289, is a somatic cell mutation that is found in human cancers. It has a FATHMM score of 0.93 and is predicted to be pathogenic, but has not previously been associated with kidney disease. Five synonymous variants were identified (G34G, S48S, S96S, A318A and L346L). All 5 synonymous variants were previously described and predicted to be benign/likely benign. Two missense variants (A242V and V260E) were identified in exon 5 and 6, respectively.

In my study, 4 individuals were heterozygous for the A242V variant, and no homozygotes were observed. Variant A242V found in exon 5 was predicted to be deleterious and probably damaging by SIFT and PolyPhen, respectively. Weber *et al.* (2004) also detected A242V in heterozygous state in a SRNS cohort, with an allele frequency of 4% among patients with African ancestry. This frequency is lower than the 8% observed in my study cohort. A study by Tonna *et al.* (2008) found that A242V was one of the most common variants found in a FSGS patient cohort. However, A242V did not associate with proteinuria, or appear to cause FSGS (Tonna *et al.*, 2008). The ExAC browser reports 29 homozygotes for the A242V variant. However, homozygous events for this variant are rare (Tonna *et al.*, 2008). This variant has a minor allele frequency (MAF) of 7% in African populations and has not been observed in European or Asian populations. Because it is common in African populations, it is unlikely that this variant alone has a severe pathogenic effect, however, it can occur in compound heterozygosity to cause a pathogenic effect.

The *NPHS2* V260E variant was found in exon 6 and predicted to be damaging and probably damaging by SIFT and PolyPhen, respectively. The V260E variant was present in 77% of steroid resistant FSGS (SR- FSGS) (including 2 sib pairs) cases among black children. Out of the 20 unrelated SR-FSGS cases, 4 and 11 individuals were heterozygous and homozygous for the V260E variant, respectively. The V260E variant was absent in 5 SR-FSGS cases. The allele frequency for the *NPHS2* 260E allele was 65% in the cases and 1% in the controls. The *NPHS2* V260E variant has a population frequency of 0.002% in Africans in the ExAC database (http://exac.broadinstitute.org/) and has not been detected in Europeans, Asians, or African Americans (Karczewski *et al.,* 2016). In this study, the V260E variant was found to be highly

associated with SR-FSGS (p=2.07e-10), consistent with findings by Asharam *et al.* (2018).

The maintenance and structural integrity of the podocyte slit diaphragm is dependent on podocin localization in lipid rafts at the insertion of the slit diaphragm. The V260E variant is predicted to act like the European founder variant R138Q, by disrupting the transport of the modified podocin protein to the plasma membrane and retaining it in the endoplasmic reticulum (Asharam *et al.*, 2018; Roselli *et al.*, 2002, 2004). Roselli *et al.* (2004) suggests the development of therapeutic approaches to correct protein processing and permit normal cellular trafficking of podocin in patients with *NPHS2* mutations.

The V260E variant was found in homozygous state in 55% (11/20) and heterozygous state in 20% (4/20) of the unrelated SR-FSGS cases. This variant was previously observed in the homozygous state in 27% (8/30) of unrelated black children with non-familial SR-FSGS from KwaZulu-Natal (Asharam *et al.*, 2018). Children homozygous for the variant had SR-FSGS and did not respond to steroid therapy, consistent with findings of disease severity of V260E (Weber *et al.*, 2004; Asharam *et al.*, 2018). The V260E variant has also been observed in the homozygous state in several consanguineous families with SRNS (Machuca *et al.*, 2010; Santín *et al.*, 2011; Weber *et al.*, 2004). Homozygous V260E variants have also been reported in Spanish consanguineous families and 3 unrelated families (2 from Comoros Island and 1 from Saudi Arabia) with SRNS (Santín *et al.*, 2011; Weber *et al.*, 2004). Twenty-four out of the 26 *NPHS2* 260E-associated alleles were compatible with the TTGCCCAC haplotype, and two had different 260E-associated haplotypes (CGGTCCAC and TGGCCCAC). These results suggest a single origin for the V260E allele with some haplotype decay, likely arising from recombination events, giving rise to the two alternate haplotypes that retain a core haplotype (CCAC) including the V260E mutation.

Asharam *et al.* (2018) identified the V260E variant in black African children of Zulu ethnicity. They hypothesized that the variant may be present in the wider Zulu population and likely in other Bantu speaking populations, due to the estimated age of the mutation. My study strengthens and expand the association of the *NPHS2* V260E variant with SRNS in black African children of different ethnic origins in South Africa, including Tsonga, Tswana, Swati, Sotho, Pedi and Zulu.

Weber *et al.* (2004) and Santín *et al.* (2011) suggests that the majority of patients with 2 pathogenic *NPHS2* variants present with SRNS at an earlier age (mean age of onset less than 6 years), compared to individuals who are heterozygous or compound heterozygous for the pathogenic variant. In my study, participants who were homozygous for the *NPHS2* V260E variant had variable age of onset, while individuals heterozygous for the V260E variant appeared to have a later age of onset, but the sample size is too small to assess statistical significance (**Figure 3.4b**).

The majority of the studies report homozygous genotypes for the V260E variant (Asharam *et al.*, 2018; Santín *et al.*, 2011; Weber *et al.*, 2004) and heterozygous genotypes (possibly compound heterozygotes) for this variant and other variants in *NPHS2* is rare. Hence, the identification of four heterozygotes in my study was unexpected. Out of the 4 individuals who were heterozygous for the V260E variant, 2 were also heterozygous for the A242V variant. It is possible that these 2 individuals present with compound heterozygosity for the V260E variant and the A242V on the other allele and that this may be causal for SRNS. McKenzie *et al.* (2007) suggests compound heterozygous variants are a very rare cause of familial FSGS, but Tonna *et al.* (2008) reported the A242V variant in compound heterozygosity with R229Q in patients with SR-FSGS. Further studies would be required to determine if A242V acts in combination with V260E in a compound heterozygous state. Individuals who were heterozygous for the V260E variant could have a second variant an *NPHS2* intron that was not Sanger sequenced in this study.

Further studies to help address the last point could be carried out to determine if the combination of the A242V and V260E variants in a compound heterozygote state are pathogenic. This would involve studying the parents to confirm that each variant is inherited from a different parent, as well as functional studies examining mRNA transcription and protein abundance and localisation (e.g. using and immunohistopathology approach).

Although the majority of the *NPHS2* mutations segregate as autosomal recessive traits, some have been suggested to segregate as autosomal dominant variants (Pollak, 2014). The V290M mutation was detected in the heterozygous state in late-onset, slowly progressing SRNS in central and eastern Europeans (Kerti *et al.*, 2013). Patients carrying the V290M mutation have

been diagnosed with proteinuria. In the general American population, the allele frequency of V290M was 1/13 000, and it has not yet been reported in Western European and North African patients with SRNS (Boute *et al.*, 2000; Machuca *et al.*, 2009; Weber *et al.*, 2004).

4.4 *NPHS2* associations with SS-FSGS

Over the last decade, genetic analyses of familial SRNS have led to the identification of causal mutations in over 50 genes. In contrast to familial SRNS, familial forms of SSNS are relatively rare, representing less than 10% of cases (Dorval *et al.*, 2018). Many studies have examined genes that cause SRNS (e.g. *NPHS1, NPHS2* and *WT1*) for mutations in SSNS patient cohorts, but found no mutations causing monogenic forms of SSNS (Caridi *et al.*, 2009; Ruf *et al.*, 2004). Similarly, in this study, no pathogenic *NPHS2* mutation was found to cause monogenic SSNS. Even though several studies suggest monogenic forms of SSNS is unlikely, one cannot exclude causal variants in coding sequences or non-coding RNA (Dorval *et al.*, 2018).

Many studies suggest a polygenic and multifactorial disease aetiology for familial SSNS (Dorval *et al.*, 2018; Karp and Gbadegesin, 2017). Data presented in this thesis support this statement. Studies by Adeyemo *et al.* (2018), Dorval *et al.* (2018) and Karp and Gbadegesin (2017), identified specific alleles at the *HLA* loci to be risk factors for SSNS, supporting the assumption of a complex pattern of inheritance. Adeyemo *et al.* (2018) identified 2 *HLA-DQA1* variants significantly associated with SSNS (**C34Y:** OR, 3.53; 95% CI, 2.33-5.42; **F41S:** OR, 4.08; 95% CI, 2.70-6.28), but not with SRNS in African American children. Dorval *et al.* (2018) also described a strong association between 2 *HLA-DQA1* gene variants (rs1129740 and rs1071630) and SSNS, with the cases having higher allele frequencies of these variants than the healthy controls. These studies provide confirmation that SRNS and SSNS are indeed separate conditions.

4.5 Disease progression in FSGS patients

In my study, the V260E variant was not present in any of the SS-FSGS patients (**Figure 3.4A**). All individuals with SS-FSGS maintained good kidney function following treatment and none had progressed to ESKD (**Figure 3.5**). This is in concordance with literature that states there is excellent prognosis in children who are steroid sensitive, as they achieve remission within 6-8 weeks of taking oral corticosteroids (Van Husen and Kemper, 2011; Uwaezuoke, 2015).

The results from my study are in agreement with a study by Fuchshuber *et al.* (2001), who found a favourable disease outcome in terms of kidney function in children with SSNS.

In most cases, the progression to ESKD seems to follow a conventional course. Once a considerable portion of the kidney tissue has been damaged, there is a continual decline in function with time, irrespective of the nature of the initial insult (Kriz *et al.*, 1998). This can be seen in the SR-FSGS patients without the V260E variant (**Figure 3.5**). SR-FSGS patients without the V260E variant have a mean kidney survival of 6 years. The mean kidney survival in this study was much lower than the 11.62 years found by Otukesh *et al.* (2009) in Iranian children. However, the Iranian children were treated with cyclophosphamide and mycophenolate mofetil, which may have prolonged their kidney survival. In my study, in the absence of the V260E variant, SR-FSGS individuals had 50% kidney survival at 12 years (**Figure 3.5**). These findings are similar to previously published data that found SR-FSGS patients to have 50% kidney survival at 13 years (Beins and Dell, 2015).

The Kaplan-Meier plot shows progression to ESKD was significantly faster in individuals who have the *NPHS2* V260E variant (**Figure 3.5).** The censored markings (short vertical lines) on the graph depict the cases in which ESKD had not yet occurred during the time the individual was observed, and therefore we only know the total number of months during which the individual did not develop ESKD. The plot suggests that individuals who did not have the *NPHS2* V260E variant progressed more slowly to ESKD; the plots differed significantly by the log rank test (p=0.026). Asanuma and Mundel (2003) and Kwoh *et al.* (2006) state that podocyte injury can occur through interference in the structure of the slit diaphragm or modification of its components. Since the V260E variant is hypothesized to alter podocin and affect the structural integrity of the slit diaphragm, it provides an explanation for the rapid decline in kidney function in the presence of the V260E variant. The findings from this study is in agreement with literature that states the presence of 2 copies of a *NPHS2* mutation leads to a more rapid progression to ESKD (Weber *et al.*, 2004).

4.6 Familial cases of SR-FSGS

In my study, segregation of the *NPHS2* V260E variant in the 2 familial cases suggests an autosomal recessive pattern of inheritance. The *APOL1* risk variants did not appear to

influence age of onset in these families. The range for age of onset is similar to what has been previously reported in a study of paediatric sporadic SRNS and in family studies (Asharam *et al.*, 2018). There was a 3-year difference in age of onset between the monozygotic twins. Along the same line in a monozygotic triplet of males, Dorval *et al.* (2018) identified that only 2 brothers developed SSNS at the same age, while the third brother was unaffected at the time the family was studied. In my study, one twin had an earlier age of onset compared to the other twin. This was possibly due to an environmental factor, infection or toxins. This was an unexpected finding, since monozygotic twins would be expected to share similar environmental exposures and share the same genetic background. Further investigation would be necessary to understand why the 2 affected sibling pairs, both homozygous for the V260E variant, were diagnosed with FSGS at different ages and progressed at different rates.

The families were from different ethnic origins in South Africa, including Sotho, Zulu and Tswana ancestry. Asharam *et al.* (2018) identified the V260E variant in children from the Zulu ethnic group, which comprises the largest ethnic group in South Africa and this is confirmed in my study. Asharam *et al.* (2018) suggested that this variant may be present in other ethnic groups in South Africa. The V260E variant has been previously observed in consanguineous African families with SRNS from areas in and around the Indian Ocean, particularly in areas linked with the 17th to 19th century Omani Empire. This suggests that travel and migration linked with this empire resulted in the wide distribution of this variant across Africa (Bouchireb *et al.*, 2014).

4.7 Recommendations

The findings of this study not only confirm the aetiology of AR SRNS disease among selected paediatric SR-FSGS cases, but since the V260E mutation is common among South African children with SRNS, it could be used as a diagnostic marker strongly predicting a steroid resistant therapeutic response. V260E is a founder mutation in the populations since it is predominantly present on a single haplotype. It's autosomal recessive inheritance means that both parents are expected to be carriers and therefore have a 1 in 4 risk of a homozygote child. It is unclear if this variant is fully penetrant in the presence of kidney dysfuction and a larger sample is needed to understand this more fully. It would be a useful predictive test in children with either familial FSGS or sporadic FSGS and could be used to avoid the administration of immunosuppressant therapy that is likely to result in adverse effects, with

no benefit to the patient (Benoit *et al.*, 2010; Santín *et al.*, 2011). For the children who do not respond to steroid therapy, alternative treatment options can be used such as non-immunosuppressant therapy or renin-angiotensin-aldosterone inhibition monotheraphy (RIM), as suggested by Kangovi *et al.* (2012). The use of RIM has been shown to decrease proteinuria and preserve renal function in paediatric patients with FSGS (Fitzwater *et al.*, 1990).

A higher incidence of SR-FSGS is reported among children with African ancestry. Another implication of testing for the *NPHS2* V260E variant is that it provides an opportunity for genetic counselling in affected families. Identifying the V260E variant in a child with SR-FSGS may help the parents in decision making when planning future pregnancies. If both parents are carriers of the V260E variant, there is a 25% chance with every pregnancy that their child could be homozygous for the variant. The results could also be used for prenatal genetic screening and selective termination of affected foetuses. Lastly, if a sibling of a SRNS patient with the V260E variant develops NS, immunosuppressant therapy could be avoided (Rood *et al.*, 2012).

4.8 Strengths and limitations of the study

The results of my study strengthen the association between the V260E variant and SRNS in black South African children. The study provides a possible precision medicine approach by genotyping the V260E variant in black children with FSGS and identifying which children are not likely to respond to oral corticosteroid therapy. The limitations of the study include its modest size and the fact that the age of onset is reflected as the age at biopsy confirmed diagnosis and may, in fact, have been considerably earlier. The study was underpowered to detect definitive associations with *APOL1* risk variants, hence, we cannot confirm the apparent association of two *APOL1* risk alleles in SRNS children with a younger age of disease onset.

4.9 Future research

There are several questions that remain unanswered. How well does this small study reflect the distribution of *NPHS2* mutations in all black South African children with SRNS? What is the clinical utility of the test and are clinicians likely to request the test and to use it for making decisions about therapy? Why is there a variable age of onset among homozygotes for V260E,

even among monozygotic twins? What is the causal genotype in heterozygotes for the V260E mutation (i.e. is there another mutation in *NPHS2* that was not detected as it is not in a coding region of the gene that was sequenced in my study)? Further studies to help address the last point could be carried out to determine if the combination of the A242V and V260E variants in a compound heterozygote state are pathogenic. This would involve studying the parents to confirm that each variant is inherited from a different parent, as well as functional studies examining mRNA transcription and protein abundance and localisation (e.g. using and immunohistopathology approach). The question of age of onset could be addressed with further studies to determine why monozygotic twins develop FSGS at different ages, and have different rates of progression. It is also important to study a larger cohort to confirm the findings from this study.

4.10 Conclusion

There was a weak association between *APOL1* risk alleles and FSGS in patients without the V260E variant in the dominant model, but not in the recessive model. Individuals with 2 *APOL1* risk alleles appeared to have an earlier age of presentation in SRNS cases but the sample size was too small to make a definitive conclusion. No association was identified with any of the *NPHS2* variants in the SSNS cases. The *NPHS2* V260E variant was found to be strongly associated with SRNS in black South African children and has not been observed in European and Asian populations. This study identified a monogenic form of SRNS with an AR pattern of inheritance in black children. Out of 22 SR-FSGS individuals, 17 carried either 1 or 2 copies of the V260E variant. Two individuals heterozygous for the V260E variant were also heterozygous for the A242V variant. However, further studies would be needed to determine whether the combination of the 2 variants together is pathogenic. Progression to ESKD was significantly faster in black South African children with SRNS in the presence of the V260E variant.

The high frequency of the V260E variant in black African children of different ethnic origins in South Africa, including Tsonga, Tswana, Swati, Sotho, Pedi and Zulu, presents a potential opportunity for a precision medicine application for NS in Africans. Genotyping the V260E variant in black children with FSGS could provide useful information in a clinical setting to guide treatment, may prevent the need for renal biopsy and avoid side effects of

glucocorticoid and other immunosuppressive therapies, and facilitate genetic counselling in affected families.

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6. Appendix

Appendix A – Redcap instruments

Confidential

Demographics

record id	_____
study number	_____
this is a patient who is	◯ affected but has no family history ◯ affected with a family history ◯ a family member of index case
number of affected family members	_____
surname	_____
first name/s	_____
date of birth	_____
gender	◯ male ◯ female
ethnicity	◯ asian ◯ black ◯ indian ◯ mixed race ◯ white
province of birth	◯ free state ◯ gauteng ◯ kwazulu-natal ◯ limpopo ◯ mpumalanga ◯ northwest ◯ eastern cape ◯ northern cape ◯ western cape

Patient follow-up details

record id	_____
is this patient from	◯ dr göttlich's practice ◯ cmjah
patient's practice file number	_____
patient's hospital number	_____
Patients contact details	
patient contact number (cell)	_____
patient contact number (landline)	_____
patient email address	_____
Next-of-kin's contact details	
next-of-kin cell number	_____
next-of-kin's name	_____ (use format: smith, john)
next-of-kin's, relationship to patient	_____
Alternate next of kin details	
next-of-kin's cell number	_____
next-of-kin's name	_____ (use format: smith, john)
next-of-kin's relationship to patient	_____

Medical history and baseline information

record id	_____
does this patient have biopsy proven FSGS	◯ yes ◯ no
date of FSGS diagnosis (confirmed on biopsy)	_____
age at diagnosis	_____
at diagnosis is this patient	◯ transplanted ◯ on dialysis ◯ no intervention as yet (status recorded should be the date closest to the diagnostic year end)
date of transplant	_____
date dialysis commenced	_____
relationship	◯ index case ◯ mother ◯ father ◯ brother ◯ sister
name of index case	_____ (use lower case and the format: smith, john)
hiv status at the time of FSGS diagnosis	◯ hiv positive ◯ hiv negative ◯ unknown not recorded (this patient must be HIV negative to be included in this study)
height at time of diagnosis (cms)	_____ (this would be the date closest to diagnostic biopsy)
weight at time of diagnosis (kgs)	_____ (this would be the date closest to diagnostic biopsy)
serum creatinine at time of diagnosis (umol/l)	_____ (this would be the date closest to diagnostic biopsy)

Annual follow-up

record id	_____
Follow-up at 6 months	
visit date (6 months)	_____
age at visit (6 months)	_____
height (6months)	_____ (1 decimal place)
weight (6 months)	_____ (1 decimal place)
serum creatinine (6 months) (umol/l)	_____
eGFR (6months) (ml/min/1.73 m2)	_____
eGFR (6months) if documented in patient records (ml/min/1.73 m2)	_____
urine protein creatinine ratio (upcr) (6 months)	_____ (3 decimal places)
Annual follow up	
visit date (12 months)	_____
age at visit (12 months)	_____
annual follow-up - is this patient	◯ transplanted ◯ on dialysis ◯ no intervention as yet
date dialysis commenced	_____
date of transplant	_____

Genetics

record id	
APOL1 SNP 1 rs73885319	
APOL1 SNP 2 rs60910145	
APOL1 INDEL rs71785313	
APOL1 haplotype	
NPHS2 Exon 1	
NPHS2 Exon 2	
NPHS2 Exon 3	
NPHS2 Exon 4	
NPHS2 Exon 5	
NPHS2 Exon 6	
NPHS2 Exon 7	
NPHS2 Exon 8	

Appendix B – Recipes for DNA extraction

SUCROSE-TRITON-X-LYSING BUFFER

1. Add 10ml 1M Tris-HCL pH8, 5ml 1M $MgCl_2$ and 10ml Triton-X 100 and make up to 1L with dH_2O.
2. Autoclave.
3. Keep solution chilled at 4^oC.
4. Add 109.5g sucrose on the day it is meant to be used. **Do not store solution for more than a day.**

20mM TRIS 5mM EDTA (T20E5)

1. Add 20ml 1M Tris-HCL (pH8.0), 10ml 0.5M EDTA (pH 8.0), and make up to 1L with dH_2O.
2. Autoclave.

1X TRIS EDTA (TE) BUFFER

1. Add 10ml 1M Tris-HCL (pH 8), 2ml 0.5M EDTA, and make up to 1L with dH_2O.
2. Autoclave.

PROTEINASE-K MIX

1. For 8 extractions; add 400µl 10% SDS, 16µl 0.5M EDTA, 2.8ml autoclaved dH_2O.
2. Add 800µl Proteinase-K (10mg/ml stock) just before use.

10% SDS

1. Add 10g SDS to 100ml autoclaved dH_2O.

SATURATED SALT SOLUTION

1. Autoclave 100ml dH_2O.
2. Slowly add 40g NaCl until saturated (some may precipitate out).
3. Before use, agitate and let NaCl settle. Use the clear supernatant.

0.5M EDTA

1. Add 93.06g EDTA and make up to 500ml with dH_2O.
2. Use NaOH to get pH to 8.0. **EDTA will only dissolve once the correct pH is reached.**
3. Autoclave.

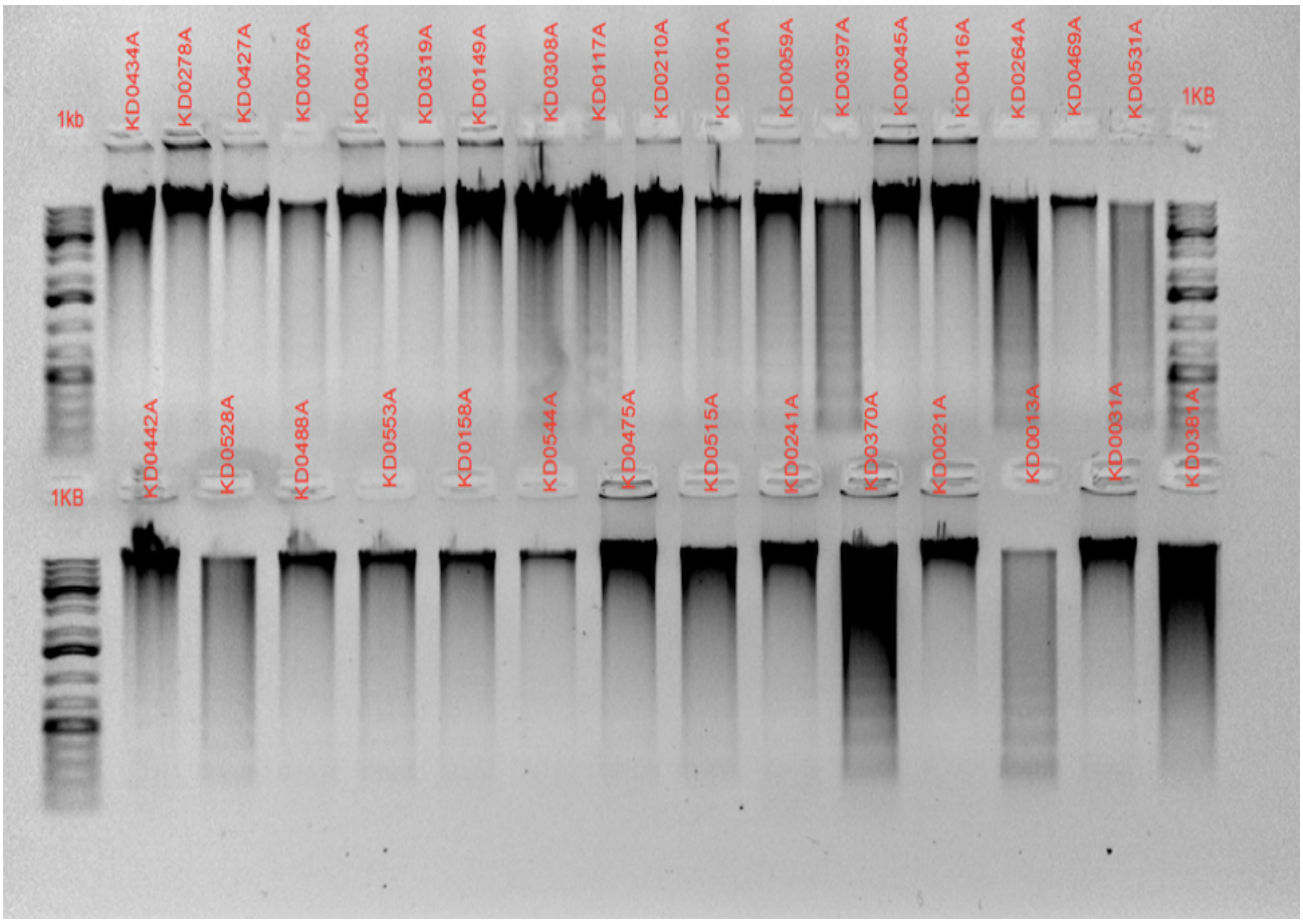
1M $MgCl_2$

1. Add 101.66g $MgCl_2$ and make up to 500mlwith dH_2O.
2. Autoclave.

1M TRIS-HCl pH 8.0

1 Add 121.1g Tris and make up to 1L with dH_2O. Adjust pH with NaOH.
2 Autoclave.

Appendix C – Quality of DNA samples after gel electrophoresis



Appendix D – Ethics certificate

R14/49 Miss Melanie Ann Govender et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170657

NAME: (Principal Investigator)	Miss Melanie Ann Govender et al
DEPARTMENT:	Human Genetics, School of Pathology Sydney Brenner Institute of Molecular Bioscience
PROJECT TITLE:	Role of Apolipoprotein L1 (APOL1) Risk Variants in Onset and Progression of Focal Segmental Glomerulosclerosis (FSGS) in Black South African Paediatric Patients
DATE CONSIDERED:	30/06/2017
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof M. Ramsay and Dr J. Fabian
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	04/08/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in June and will therefore be due in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix E - Forms for parents/legal guardians for child with FSGS

Appendix EI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for parents / legal guardians

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease called focal segmental glomerulosclerois (FSGS). Because your child has FSGS, I would like to invite your child to participate in this project. Before you decide whether or not I can approach your child and invite them to participate in this study, let me give you a bit more information about this research.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is identified by damaged or scarred glomeruli. Glomeruli are tiny vessels that filter the blood, keeping in protein and removing waste which becomes urine. Scarring or damage to the glomeruli prevents the removal of waste products and results in excess protein in the urine (proteinuria). If scarring continues, there is risk of progression to end stage renal disease (ESRD). ESRD is when the kidneys are no longer able to function at a capacity needed for day to day life. ESRD is irreversible and support in the form of dialysis or kidney transplantation is required to sustain life.

Sporadic forms of FSGS can affect children of any age and may lead to ESRD. Risk variants in the *APOL1* (apolipoprotein L1) gene has been identified in individuals with African ancestry and known to affect kidneys. Various studies state that risk variants in the *APOL1* gene increase the progression of FSGS to ESRD. No specific study has been done in black South African children. The purpose of this research is to screen risk variants in the *APOL1* gene (as well as other genes) that is associated with FSGS. We hope that the results will help us to better understand FSGS.

What is involved in the study?

We would like to invite your child to participate in this study in order for us to see how variants in the *APOL1* gene (as well as variants in other genes) are associated with FSGS. If you agree to let your child take part in this research, a nurse will take a sample of blood (5ml) from a vein in your child's arm. The procedure will take approximately 30 minutes. We would also like to ask your permission to access your child's medical records. Here we can get some information that we need like the age of your child, date of diagnosis and how long your child has been receiving treatment

Blood sampling

Blood samples will be taken by a nurse and she will collect 5ml of blood (about one teaspoon) from a vein in your child's arm. Sometimes when blood is taken your child may feel a prick at the place where the needle enters his/her body. Afterwards there may be some slight bruising. Sterile, disposable syringes will be used once only so there is no chance of infection. This procedure is safe and there is only a slight prick as the needle is placed through the skin.

There will be no charge for these blood tests. The results from this blood test will be absolutely confidential, this means a code will be used instead of your child's name and my supervisors and I will be the only people to know the code. Your child's blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. We will ask you to sign a consent form to give us permission to use your child's blood sample for no other purposes other than that described to you.

DNA testing

All the cells in our bodies contain a chemical called DNA that we inherit from our parents. It carries the instructions for making us living organisms. It contains all our genes. We can take the DNA out of the cells that are in the sample of blood that we take from your child's arm. Once we have removed the DNA from the blood we can screen for risk variants within the *APOL1* gene. Please note that all DNA samples will be safely stored in a biobank at SBIMB and my supervisors and I will be the only people who will have access to them. We will ask you to sign a consent form to give us permission to use your child's DNA for no other purposes other than those described to you. However, if at some future date we realize there are other studies that we would like to carry out on your child's DNA samples, such tests would only be performed if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your child's identity will be anonymous as your child's sample will be identified by a code.

Possible risks

Your child may experience slight discomfort, bleeding, and/or bruising during blood sampling.

Possible benefits

This study is not likely to directly benefit your child.

Costs to you

Your child will not be paid for participating in this study. The costs for testing any of your child's samples collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research

Whether or not you agree that I can approach your child and invite him/her to participate in the study is entirely up to you, your child is under no pressure to participate. If you decide that your child can participate, and later on change your mind, your child is free to stop participating at any time. If you decide you would rather your child does not participate this will not affect the treatment that your child's receives at this hospital. The law says that we need to ask you if you are willing for us to approach your child and invite them to participate. We also need to ask your child to agree to take part in the study. If your child does not want to take part, then we may not be able to include him / her in the study.

Records of your child's participation in this research

If your child chooses to participate in this project, nobody will know his/her identity and we will keep your child's personal information safe. Only my supervisors and I will have access to

your child's data. Your child will receive a special study code, so your child's name will not be used. Your child's study information will be stored in a password protected, secure database.

Publication of the results of the research

The results of this research may appear in scientific publications without identifying your child in any way. The results will be given to your child's doctor who will give you and your child the information.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -

Prof Michele Ramsay – 011 717 6635

Dr June Fabian – 011 356 6395

If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider letting your child participate in the study, please don't hesitate to contact me should you have any questions.

Melanie Govender – 061 476 6702

Appendix EII – Consent form

Study code: _____

Parent / legal guardian consent form

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The purpose of this study, procedures to be followed, risks and benefits, have been explained to me.
- I understand that a sample of blood (5ml) will be taken from a vein my child's arm.
- I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research.
- I have read this consent form and agree to let my child participate in this research study with the understanding that I may withdraw my child at any time.
- I am aware that my child will only participate in the study if he or she is happy to do so.
- I have been told that I will be given a signed and dated copy of this consent form if I would like one.

_____ _____

Name of Child Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix EIII – Consent form for storing blood and DNA

Study code:_____

Parent / legal guardian consent form for blood and DNA storage and future testing

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of consent is for me to inform the researcher what they can or cannot do with my child's samples.
I understand that all procedure/tests on my child's stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I am in agreement that my child's DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I am in agreement that the data generated from my child's DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I am in agreement that the information from my child's DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my child's DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that my child will not benefit directly from the research done on his/her DNA.

YES ☐ NO ☐

I understand that I may withdraw my child from the study at any time.

YES ☐ NO ☐

_____	_____
Name of child	Date
_____	_____
Signature of Parent or Legal Guardian	Date
_____	_____
Signature of Person Obtaining Consent	Date

Appendix F - Forms for parents/legal guardians for child pedigree

Appendix FI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for parents / legal guardians of child pedigrees

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease called focal segmental glomerulosclerois (FSGS). Because there is a history of FSGS in your family, I would like to invite your child to participate in this project. Before you decide whether or not I can approach your child and invite them to participate in this study, let me give you a bit more information about this research.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is identified by damaged or scarred glomeruli. Glomeruli are tiny vessels that filter the blood, keeping in protein and removing waste which becomes urine. Scarring or damage to the glomeruli prevents the removal of waste products and results in excess protein in the urine (proteinuria). If scarring continues, there is risk of progression to end stage renal disease (ESRD). ESRD is when the kidneys are no longer able to function at a capacity needed for day to day life. ESRD is irreversible and support in the form of dialysis or kidney transplantation is required to sustain life.

Familial cases can be divided into two groups according to their mode of inheritance; recessive disease with an early onset and autosomal dominant disease with a later onset. Generally, FSGS with a recessive mode of inheritance present in early childhood and by contrast, FSGS with a dominant mode of inheritance present at a later stage. Risk variants in the *APOL1* (apolipoprotein L1) gene has been identified in individuals with African ancestry and known to affect kidneys. Various studies state that risk variants in the *APOL1* gene (as well as other genes) increase the progression of FSGS to ESRD. No specific study has been done in black South Africans. The purpose of this research is to screen risk variants in the *APOL1* gene that is associated with FSGS and by looking at familial cases, association between *APOL1* variants and progression of disease can be determined. We hope that the results will help us to better understand FSGS.

What is involved in the study?

We would like to invite your child to participate in this study in order for us to see the association between *APOL1* variants (as well as variants in other genes) and progression of FSGS. If you agree to let your child take part in this research, a nurse will take a sample of blood (5ml) from a vein in your child's arm. The procedure will take approximately 30 minutes.

Blood sampling

Blood samples will be taken by a nurse and she will collect 5ml of blood (about one teaspoon) from a vein in your child's arm. Sometimes when blood is taken your child may feel a prick at the place where the needle enters his/her body. Afterwards there may be some slight

bruising. Sterile, disposable syringes will be used once only so there is no chance of infection. This procedure is safe and there is only a slight prick as the needle is placed through the skin. There will be no charge for these blood tests. The results from this blood test will be absolutely confidential, this means a code will be used instead of your child's name and my supervisors and I will be the only people to know the code. Your child's blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. We will ask you to sign a consent form to give us permission to use your child's blood sample for no other purposes other than that described to you.

DNA testing

All the cells in our bodies contain a chemical called DNA that we inherit from our parents. It carries the instructions for making us living organisms. It contains all our genes. We can take the DNA out of the cells that are in the sample of blood that we take from your child's arm. Once we have removed the DNA from the blood we can screen for risk variants within the *APOL1* gene. Please note that all DNA samples will be safely stored in a biobank at SBIMB and my supervisors and I will be the only people who will have access to them. We will ask you to sign a consent form to give us permission to use your child's DNA for no other purposes other than those described to you. However, if at some future date we realize there are other studies that we would like to carry out on your child's DNA samples, such tests would only be performed if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your child's identity will be anonymous as your child's sample will be identified by a code.

Possible risks

Your child may experience slight discomfort, bleeding, and/or bruising during blood sampling.

Possible benefits

This study is not likely to directly benefit your child.

Costs to you

Your child will not be paid for participating in this study. The costs for testing any of your child's samples collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research

Whether or not you agree that I can approach your child and invite him/her to participate in the study is entirely up to you, your child is under no pressure to participate. If you decide that your child can participate, and later on change your mind, your child is free to stop participating at any time. If you decide you would rather your child does not participate this will not affect your child's in any way. The law says that we need to ask you if you are willing for us to approach your child and invite them to participate. We also need to ask your child to agree to take part in the study. If your child does not want to take part, then we may not be able to include him / her in the study.

Records of your child's participation in this research

If your child chooses to participate in this project, nobody will know his/her identity and we will keep your child's personal information safe. Only my supervisors and I will have access to your child's data. Your child will receive a special study code, so your child's name will not be used. Your child's study information will be stored in a password protected, secure database.

Publication of the results of the research

The results of this research may appear in scientific publications without identifying your child in any way. The results will be given to an attending clinician who will give you and your child the information.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -

Prof Michele Ramsay – 011 717 6635

Dr June Fabian – 011 356 6395

If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider letting your child participate in the study, please don't hesitate to contact me should you have any questions.

Melanie Govender – 061 476 6702

Appendix FII – Consent form

Study code: _____

Parent / legal guardian consent form for child pedigrees participation

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The purpose of this study, procedures to be followed, risks and benefits, have been explained to me.
- I understand that a sample of blood (5ml) will be taken from a vein my child's arm.
- I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research.
- I have read this consent form and agree to let my child participate in this research study with the understanding that I may withdraw my child at any time.
- I am aware that my child will only participate in the study if he or she is happy to do so.
- I have been told that I will be given a signed and dated copy of this consent form if I would like one.

_____ _____

Name of Child Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix FIII – Consent form for storing blood and DNA

Study code:_____

Parent / legal guardian consent form for blood and DNA storage and future testing in child pedigrees

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of consent is for me to inform the researcher what they can or cannot do with my child's samples.
I understand that all procedure/tests on my child's stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I am in agreement that my child's DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I am in agreement that the data generated from my child's DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I am in agreement that the information from my child's DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my child's DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that my child will not benefit directly from the research done on his/her DNA.

YES ☐ NO ☐

I understand that I may withdraw my child from the study at any time.

YES ☐ NO ☐

_____ _____

Name of child Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix G - Forms for child with FSGS ages 7-12

Appendix GI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for children aged 7 – 12

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease which you have. I would like to ask you to take part in this study. Before you decide whether or not you would like to take part in this study, let me tell you more about this study.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is a disease that results in damage to the tiny tube like parts of the kidney. These tiny tubes help the kidney to remove harmful waste from your body. When these tiny tubes are damaged, kidneys are no longer able to remove these harmful wastes. If damage continues, special treatment may be required.

DNA is made up of something called nucleotides. There are four different types of nucleotides: adenine, thymine, cytosine, and guanine. Our body is made up of many cells. Inside all the cells in our body is the chemical DNA. Cells get their instructions on what do to from DNA. DNA code is held by the different letters of the nucleotides (for example ACT GTC ATT). The different letters can make up a gene. Each gene has a special job to do. A mistake happens when one of the letters are replaced with another or removed. This can result in the gene not been able to do its job. This study will examine if the mistakes in the nucleotides in the gene of interest (as well as mistakes in other genes) in this study is related to FSGS (the disease that you have).

What is involved in the study?

I would like to invite you to take part in this study in order for us to see if these mistakes in the gene of interest (as well as mistakes in other genes) is related to FSGS. If you agree to take part in this research, a nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. The will take about 30 minutes.

Blood sampling

A nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. Sometimes when blood is taken you may feel a tiny prick at the place where the needle enters your body. Afterwards there may be slight bruising where the needle was. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. My supervisors and I will be the only people who will have access to it. We will ask you to sign an assent form to give us permission to use your blood sample for this study.

DNA testing

We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood, we will look for the changes or mistakes in the gene of interest in this study. We will ask you to sign an assent form to give us permission to use your DNA. However, if at some future date we realize there are other

studies that we would like to carry out on your DNA sample, such tests will only be carried out if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your sample will be identified by a code, so your name will not be made known.

Possible risks
You may experience slight pain, bleeding, and/or bruising during blood sampling.

Benefits
This study will not benefit you directly.

Costs to you
You will not be paid for taking part in this study. The costs for testing your sample collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research
You have the right to agree or refuse to take part in this study. If you decide to take part and later change your mind, you are free to stop at any time, and it will not affect you in any way.

Records of your participation in this research
Nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used.

Publication of the results of the research
The results of this research may appear in scientific publications without using your name.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -
Prof Michele Ramsay – 011 717 6635
Dr June Fabian – 011 356 6395
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider taking part in this study, please don't hesitate to contact me should you have any questions.
Melanie Govender – 061 476 6702

Appendix GII – Assent form

Study code:_____

Assent form for children aged 7 - 12

Project title: Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The study has been explained to me.
- I understand that a sample of blood that's about one teaspoon in volume will be taken from my arm.
- I have been told who to contact if anything worries me or if I have any problems about this study.
- I have asked questions and had them answered.
- I have read this form (or had it read to me).
- I agree to take part in the study.
- I understand that I can leave the study at any time.
- I have been told that I will get a copy of this form.

_____ _____

Signature of Child/ thumb print Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix GIII – Assent form for storing blood and DNA

Study code:_____

Assent form for blood and DNA storage and future testing – children aged 7 - 12

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

I understand how blood will be collected and what it will be used for. I understand that the reason for assent is for me to let the researcher know what they can or cannot do with my samples.
I understand that all tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I understand that my DNA may be stored and used for the purpose of this study.

YES ☐ NO ☐

I understand that the data generated from my DNA may be made available to me by my doctor if I want it.

YES ☐ NO ☐

I understand that every time a new study is done on DNA, permission will be obtained.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may leave the study at any time.

YES ☐ NO ☐

_____ _____
Signature of Child/ thumb print Date

_____ _____
Signature of Parent or Legal Guardian Date

_____ _____
Signature of Person Obtaining Consent Date

Appendix H - Forms for child pedigree ages 7-12

Appendix HI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for child pedigrees aged 7 – 12

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease. Because two or more people in your family have this disease, I would like to ask you to take part in this study. Before you decide whether or not you would like to take part in this study, let me tell you more about this study.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is a disease that results in damage to the tiny tube like parts of the kidney. These tiny tubes help the kidney to remove harmful waste from your body. When these tiny tubes are damaged, kidneys are no longer able to remove these harmful wastes. If damage continues, special treatment may be required.

DNA is made up of something called nucleotides. There are four different types of nucleotides: adenine, thymine, cytosine, and guanine. Our body is made up of many cells. Inside all the cells in our body is the chemical DNA. Cells get their instructions on what do to from DNA. DNA code is held by the different letters of the nucleotides (for example ACT GTC ATT). The different letters can make up a gene. Each gene has a special job to do. A mistake happens when one of the letters are replaced with another or removed. This can result in the gene not been able to do its job. This study will see if the mistakes in the nucleotides in the gene of interest (as well as mistakes in other genes) in this study is related to FSGS. This study will also see if different members of your family has different disease phenotypes (this mean that in one member of your family the disease is worse than in other/s member/s.

What is involved in the study?

I would like to invite you to take part in this study in order for us to see if these mistakes in the gene of interest (as well as mistakes in other genes) has a different effect in different members of your family. If you agree to take part in this research, a nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. The will take about 30 minutes.

Blood sampling

A nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. Sometimes when blood is taken you may feel a tiny prick at the place where the needle enters your body. Afterwards there may be slight bruising where the needle was. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. My supervisors and I will be the only people who will have access to it. We will ask you to sign an assent form to give us permission to use your blood sample for this study.

DNA testing

We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood, we will look for the changes or mistakes

in the gene of interest in this study. We will ask you to sign an assent form to give us permission to use your DNA. However, if at some future date we realize there are other studies that we would like to carry out on your DNA sample, such tests will only be carried out if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your sample will be identified by a code, so your name will not be made known.

Possible risks
You may experience slight pain, bleeding, and/or bruising during blood sampling.

Benefits
This study will not benefit you directly.

Costs to you
You will not be paid for taking part in this study. The costs for testing your sample collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research
You have the right to agree or refuse to take part in this study. If you decide to take part and later change your mind, you are free to stop at any time, and it will not affect you in any way.

Records of your participation in this research
Nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used.

Publication of the results of the research
The results of this research may appear in scientific publications without using your name.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -
Prof Michele Ramsay – 011 717 6635
Dr June Fabian – 011 356 6395
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider taking part in this study, please don't hesitate to contact me should you have any questions.
Melanie Govender – 061 476 6702

Appendix HII – Assent form

Study code:_____

Assent form for child pedigrees aged 7 - 12

Project title: Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The study has been explained to me.
- I understand that a sample of blood that's about one teaspoon in volume will be taken from my arm.
- I have been told who to contact if anything worries me or if I have any problems about this study.
- I have asked questions and had them answered.
- I have read this form (or had it read to me).
- I agree to take part in the study.
- I understand that I can leave the study at any time.
- I have been told that I will get a copy of this form.

_____ _____

Signature of Child/ thumb print Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix HIII – Assent form for storing blood and DNA

Study code:_____

Assent form for blood and DNA storage and future testing – child pedigrees aged 7 - 12

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

I understand how blood will be collected and what it will be used for. I understand that the reason for assent is for me to let the researcher know what they can or cannot do with my samples.
I understand that all tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I understand that my DNA may be stored and used for the purpose of this study.

YES ☐ NO ☐

I understand that the data generated from my DNA may be made available to me by a doctor if I want it.

YES ☐ NO ☐

I understand that every time a new study is done on DNA, permission will be obtained.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may leave the study at any time.

YES ☐ NO ☐

_____ _____
Signature of Child/ thumb print Date

_____ _____
Signature of Parent or Legal Guardian Date

_____ _____
Signature of Person Obtaining Consent Date

Appendix I - Forms for child with FSGS ages 13-17

Appendix **II** – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for children aged 13 – 17

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease which you have. I would like to ask you to take part in this study. Before you decide whether or not you would like to take part in this study, let me tell you more about this study.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is a disease that results in damage to the tiny vessels in the kidney. These tiny vessels help the kidney to remove harmful waste from your body. When these tiny vessels are damaged, kidneys are no longer able to remove these harmful wastes. If damage continues, special treatment may be required (for example, you may require a new kidney).

DNA is made up of something called nucleotides. There are four different types of nucleotides: adenine, thymine, cytosine, and guanine. Our body is made up of many cells. Inside all the cells in our body is the chemical DNA. Cells get their instructions on what do to from DNA. DNA code is held by the different letters of the nucleotides (for example ACT GTC ATT). The different letters represent different instructions. Each gene has a special job to do. A mistake happens when one of the letters are replaced with another or removed. This can result in the gene not been able to do its job. This study is going to look for mistakes in the nucleotides in the *APOL1 gene* (as well as mistakes in other genes) that is believed to be associated with FSGS (the disease that you have).

What is involved in the study?

I would like to invite you to take part in this study in order for us to see if these mistakes in the *APOL1* gene (as well as mistakes in other genes) is associated with FSGS. If you agree to take part in this research, a nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. The will take about 30 minutes.

Blood sampling

A nurse will take a sample of blood that is about a teaspoon (5 ml) from your arm. Sometimes when blood is taken you may feel a tiny prick at the place where the needle enters your body. Afterwards there may be slight bruising where the needle was. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. My supervisors and I will be the only people who will have access to it. We will ask you to sign an assent form to give us permission to use your blood sample for this study.

DNA testing
We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood, we can look for the changes or mistakes in *APOL1* gene as mentioned above. We will ask you to sign an assent form to give us permission to use your DNA. However, if at some future date we realize there are other studies that we would like to carry out on your DNA sample, such tests will only be carried out if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your sample will be identified by a code, so your identity will be anonymous.

Possible risks
You may experience slight pain, bleeding, and/or bruising during blood sampling.

Benefits
This study will not benefit you directly.

Costs to you
You will not be paid for taking part in this study. The costs for testing your sample collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research
You have the right to agree or refuse to take part in this study. If you decide to take part and later change your mind, you are free to stop at any time, and it will not affect you in any way.
Records of your participation in this research
Nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used.

Publication of the results of the research
The results of this research may appear in scientific publications without using your name.

CONTACT DETAILS
If you have any problems or any questions at any time you can contact these telephone numbers: -
Prof Michele Ramsay – 011 717 6635
Dr June Fabian – 011 356 6395
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider taking part in this study, please don't hesitate to contact me should you have any questions.
Melanie Govender – 061 476 6702

Appendix **I**II – Assent form

Study code:_____

Assent form for children age 13 - 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The procedures to be followed, risks and benefits of the study has been explained to me.
- I understand that a sample of blood (5ml) that's about one teaspoon in volume will be taken from a vein in my arm.
- I have been told who to contact if anything concerns me about the study.
- I have asked questions that concerns me and had them answered.
- I have read this form.
- I agree to take part in the study.
- I understand that I can leave the study at any time.
- I have been told that I will get a copy of this form.

_____ _____

Signature of Child/ thumb print Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix **I**IIII – Assent form for storing blood and DNA

Study code:_____

Assent form for blood and DNA storage and future testing – children aged 13 - 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of assent is for me to inform the researcher what they can or cannot do with my samples.
I understand that all procedure/tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I understand that my DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I understand that the data generated from my DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I understand that the information from my DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may withdraw from the study at any time.

YES ☐ NO ☐

_____ _____

Signature/thumb print of child Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix J - Forms for child pedigree ages 13-17

Appendix JI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for child pedigrees aged 13 – 17

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease. Because two or more people in your family has this disease, I would like to ask you to take part in this study. Before you decide whether or not you would like to take part in this study, let me tell you more about this study.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is a disease that results in damage to the tiny vessels of the kidney. These vessels help the kidney to remove harmful waste from your body. When these tiny vessels are damaged, kidneys are no longer able to remove these harmful wastes. If damage continues, special treatment may be required (such as requiring a new kidney).

DNA is made up of something called nucleotides. There are four different types of nucleotides: adenine, thymine, cytosine, and guanine. Our body is made up of many cells. Inside all the cells in our body is the chemical DNA. Cells get their instructions on what do to from DNA. DNA code is held by the different letters of the nucleotides (for example ACT GTC ATT). The different letters can make up a gene. Each gene has a special job to do. A mistake happens when one of the letters are replaced with another or removed. This can result in the gene not been able to do its job. This study will see if the mistakes in the nucleotides in the *APOL1* gene (as well as mistakes in other genes) is related to FSGS. This study will also see if different members of your family has different disease phenotypes (this mean that in one member of your family the disease is worse than in other/s member/s.

What is involved in the study?

I would like to invite you to take part in this study in order for us to see if these mistakes in the *APOL1* gene (as well as mistakes in other genes) has a different effect in different members of your family. If you agree to take part in this research, a nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. The will take about 30 minutes.

Blood sampling

A nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. Sometimes when blood is taken you may feel a tiny prick at the place where the needle enters your body. Afterwards there may be slight bruising where the needle was. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. My supervisors and I will be the only people who will have access to it. We will ask you to sign an assent form to give us permission to use your blood sample for this study.

DNA testing
We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood, we will look for the mistakes in the *APOL1* gene. We will ask you to sign an assent form to give us permission to use your DNA. However, if at some future date we realize there are other studies that we would like to carry out on your DNA sample, such tests will only be carried out if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your sample will be identified by a code, so your name will not be made known.

Possible risks
You may experience slight pain, bleeding, and/or bruising during blood sampling.

Benefits
This study will not benefit you directly.

Costs to you
You will not be paid for taking part in this study. The costs for testing your sample collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research
You have the right to agree or refuse to take part in this study. If you decide to take part and later change your mind, you are free to stop at any time, and it will not affect you in any way.

Records of your participation in this research
Nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used.

Publication of the results of the research
The results of this research may appear in scientific publications without using your name.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -
Prof Michele Ramsay – 011 717 6635
Dr June Fabian – 011 356 6395
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider taking part in this study, please don't hesitate to contact me should you have any questions.
Melanie Govender – 061 476 6702

Appendix JII – Assent form

Study code:_____

Assent form for child pedigrees aged 13 - 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The procedures to be followed, risks and benefits of the study has been explained to me.
- I understand that a sample of blood (5ml) that's about one teaspoon in volume will be taken from a vein in my arm.
- I have been told who to contact if anything concerns me about the study.
- I have asked questions that concerns me and had them answered.
- I have read this form.
- I agree to take part in the study.
- I understand that I can leave the study at any time.
- I have been told that I will get a copy of this form.

_____ _____

Signature of Child/ thumb print Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix JIII – Assent form for storing blood and DNA

Study code:_____

Assent form for blood and DNA storage and future testing – child pedigrees aged 13 - 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of assent is for me to inform the researcher what they can or cannot do with my samples.
I understand that all procedure/tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I understand that my DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I understand that the data generated from my DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I understand that the information from my DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may withdraw from the study at any time.

YES ☐ NO ☐

_____	_____
Signature/thumb print of child	Date
_____	_____
Signature of Parent or Legal Guardian	Date
_____	_____
Signature of Person Obtaining Consent	Date

Appendix K - Forms for patient with FSGS aged >17

Appendix KI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for patient aged > 17

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease which you have. I would like to ask you to take part in this study. Before you decide whether or not you would like to take part in this study, let me tell you more about this study.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is a disease that results in damage to the tiny vessels in the kidney. These tiny vessels help the kidney to remove harmful waste from your body. When these tiny vessels are damaged, kidneys are no longer able to remove these harmful wastes. If damage continues, special treatment may be required (for example, you may require a new kidney).

DNA is made up of something called nucleotides. There are four different types of nucleotides: adenine, thymine, cytosine, and guanine. Our body is made up of many cells. Inside all the cells in our body is the chemical DNA. Cells get their instructions on what do to from DNA. DNA code is held by the different letters of the nucleotides (for example ACT GTC ATT). The different letters represent different instructions. Each gene has a special job to do. A mistake happens when one of the letters are replaced with another or removed. This can result in the gene not been able to do its job. This study is going to look for mistakes in the nucleotides in the *APOL1 gene* (as well as mistakes in other genes) that is believed to be associated with FSGS (the disease that you have).

What is involved in the study?

I would like to invite you to take part in this study in order for us to see if these mistakes in the *APOL1* gene (as well as mistakes in other genes) is associated with FSGS. If you agree to take part in this research, a nurse will take a sample of blood that is about one teaspoon (5 ml) from your arm. The will take about 30 minutes.

Blood sampling

A nurse will take a sample of blood that is about a teaspoon (5 ml) from your arm. Sometimes when blood is taken you may feel a tiny prick at the place where the needle enters your body. Afterwards there may be slight bruising where the needle was. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. My supervisors and I will be the only people who will have access to it. We will ask you to sign an assent form to give us permission to use your blood sample for this study.

DNA testing
We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood, we can look for the changes or mistakes in *APOL1* gene as mentioned above. We will ask you to sign an assent form to give us permission to use your DNA. However, if at some future date we realize there are other studies that we would like to carry out on your DNA sample, such tests will only be carried out if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your sample will be identified by a code, so your identity will be anonymous.

Possible risks
You may experience slight pain, bleeding, and/or bruising during blood sampling.

Benefits
This study will not benefit you directly.

Costs to you
You will not be paid for taking part in this study. The costs for testing your sample collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research
You have the right to agree or refuse to take part in this study. If you decide to take part and later change your mind, you are free to stop at any time, and it will not affect you in any way.
Records of your participation in this research
Nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used.

Publication of the results of the research
The results of this research may appear in scientific publications without using your name.

CONTACT DETAILS
If you have any problems or any questions at any time you can contact these telephone numbers: -
Prof Michele Ramsay – 011 717 6635
Dr June Fabian – 011 356 6395
If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider taking part in this study, please don't hesitate to contact me should you have any questions.
Melanie Govender – 061 476 6702

Appendix KII – Consent form

Study code:_____

Consent form for patient > age 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The procedures to be followed, risks and benefits of the study has been explained to me.
- I understand that a sample of blood (5ml) that's about one teaspoon in volume will be taken from a vein in my arm.
- I have been told who to contact if anything concerns me about the study.
- I have asked questions that concerns me and had them answered.
- I have read this form.
- I agree to take part in the study.
- I understand that I can leave the study at any time.
- I have been told that I will get a copy of this form.

_____ _____

Signature of Child/ thumb print Date

_____ _____

Signature of Parent or Legal Guardian Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix KIII – Consent form for storing blood and DNA

Study code:_____

Consent form for blood and DNA storage and future testing – patient aged > 17

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of assent is for me to inform the researcher what they can or cannot do with my samples.
I understand that all procedure/tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I understand that my DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I understand that the data generated from my DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I understand that the information from my DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may withdraw from the study at any time.

YES ☐ NO ☐

_____	_____
Signature/thumb print of child	Date
_____	_____
Signature of Parent or Legal Guardian	Date
_____	_____
Signature of Person Obtaining Consent	Date

Appendix L - Forms for adult pedigree

Appendix LI – Study information sheet

Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

Study information sheet for adult pedigrees

Hello, my name is Melanie Ann Govender and I am a student at the University of the Witwatersrand. I am doing a study on a type of kidney disease called focal segmental glomerulosclerois (FSGS). Because there is a history of FSGS within your family, I would like to invite you to participate in this project. Before you decide whether or not you want to participate in this study, let me give you a bit more information about this research.

What is the research about?

Focal segmental glomerulosclerosis (FSGS) is identified by damaged or scarred glomeruli. Glomeruli are tiny vessels that filter the blood, keeping in protein and removing waste which becomes urine. Scarring or damage to the glomeruli prevents the removal of waste products and results in excess protein in the urine (proteinuria). If scarring continues, there is risk of progression to end stage renal disease (ESRD). ESRD is when the kidneys are no longer able to function at a capacity needed for day to day life. ESRD is irreversible and support in the form of dialysis or kidney transplantation is required to sustain life.

Familial cases can be divided into two groups according to their mode of inheritance; recessive disease with an early onset and autosomal dominant disease with a later onset. Generally, FSGS with a recessive mode of inheritance present in early childhood and by contrast, FSGS with a dominant mode of inheritance present at a later stage. Risk variants in the *APOL1* (apolipoprotein L1) gene has been identified in individuals with African ancestry and known to affect kidneys. Various studies state that risk variants in the *APOL1* gene increase the progression of FSGS to ESRD. No specific study has been done in black South Africans. The purpose of this research is to screen risk variants in the *APOL1* gene that is associated with FSGS and by looking at familial cases, association between *APOL1* variants and progression of disease can be determined. We would also like to test additional compelling kidney associated genetic variants in the FSGS cases and families. We hope that the results will help us to better understand FSGS.

What is involved in the study?

We would like to invite you to participate in this study in order for us to see the association between *APOL1* variants (and variants in other genes) and progression of FSGS. If you agree to participate in this research, a nurse will take a sample of blood (10ml) from a vein in your arm. The procedure will take approximately 30 minutes.

Blood sampling

Blood sample will be taken by a nurse and she will collect 10ml of blood from a vein in your arm. Sometimes when blood is taken you may feel a prick at the place where the needle enters your body. Afterwards there may be some slight bruising. Sterile, disposable syringes will be used once only so there is no chance of infection. This procedure is safe and there is

only a slight prick as the needle is placed through the skin. There will be no charge for these blood tests. The results from this blood test will be absolutely confidential, this means a code will be used instead of your name and my supervisors and I will be the only people to know the code. Your blood sample will be stored in the Sydney Brenner Institute for Molecular Biosciences (SBIMB) biobank. We will ask you to sign a consent form to give us permission to use your blood sample for no other purposes other than that described to you.

DNA testing

All the cells in our bodies contain a chemical called DNA that we inherit from our parents. It carries the instructions for making us living organisms. It contains all our genes. We can take the DNA out of the cells that are in the sample of blood that we take from your arm. Once we have removed the DNA from the blood we can screen for risk variants within the *APOL1* gene. Please note that all DNA samples will be safely stored in a biobank at SBIMB and my supervisors and I will be the only people who will have access to them. We will ask you to sign a consent form to give us permission to use your DNA for no other purposes other than those described to you. However, if at some future date we realize there are other studies that we would like to carry out on your DNA samples, such tests would only be performed if permission is given to us by the Human Research Ethics Committee of the University of the Witwatersrand. Your identity will be anonymous as your sample will be identified by a code.

Possible risks

Your may experience slight discomfort, bleeding, and/or bruising during blood sampling.

Possible benefits

This study is not likely to directly benefit you in any way.

Costs to you

You will not be paid for participating in this study. The costs for testing any of your samples collected during the study will be paid by the study budget, which means it will not cost you anything.

Voluntary participation in research

Whether or not you agree to participate in the study is entirely up to you, you are under no pressure to participate.

Records of your participation in this research

If you chooses to participate in this project, nobody will know your identity and we will keep your personal information safe. Only my supervisors and I will have access to your data. You will receive a special study code, so your name will not be used. Your study information will be stored in a password protected, secure database.

Publication of the results of the research

The results of this research may appear in scientific publications without identifying you in any way. The results will be given to the attending clinician who will give you the information.

CONTACT DETAILS

If you have any problems or any questions at any time you can contact these telephone numbers: -

Prof Michele Ramsay – 011 717 6635

Dr June Fabian – 011 356 6395

If you have any complaints or problems with this research you may report them to the Chair of the Human Research Ethics Committee at the University of the Witwatersrand on this telephone number – (011) 717 1234.

Thank you very much for taking the time to consider participating in the study, please don't hesitate to contact me should you have any questions.

Melanie Govender – 061 476 6702

Appendix LII – Consent form

Study code: _____

Consent form for adult pedigrees

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

- The purpose of this study, procedures to be followed, risks and benefits, have been explained to me.
- I understand that a sample of blood (10ml) will be taken from a vein my arm.
- I have been told whom to contact if I have questions, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research.
- I have read this consent form and agree to participate in this research study with the understanding that I may withdraw at any time.
- I have been told that I will be given a signed and dated copy of this consent form if I would like one.

_____ _____

Signature of Participant Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix LIII – Consent form for storing blood and DNA

Study code:_____

Consent form for blood and DNA storage and future testing – adult pedigrees

Project title : Role of *APOL1* risk variants in onset and progression of FSGS in black South African paediatric patients

The information around the blood sample and DNA that will be extracted from the blood is clear and the purpose of consent is for me to inform the researcher what they can or cannot do with my samples.
I understand that all procedure/tests on my stored blood and DNA samples will be approved by the Human Research Ethics Committee of the University of the Witwatersrand.

YES ☐ NO ☐

I am in agreement that my DNA may be stored and used for the purposes described in the information sheet.

YES ☐ NO ☐

I am in agreement that the data generated from my DNA may be made available as stated in the information sheet.

YES ☐ NO ☐

I am in agreement that the information from my DNA may be used as stated in the information sheet.

YES ☐ NO ☐

I understand that every time a new study is done on my DNA, permission will be obtained from the ethics committee for the study to make sure that it is used only for the purposes stated.

YES ☐ NO ☐

I understand that I will not benefit directly from the research done on my DNA.

YES ☐ NO ☐

I understand that I may withdraw from the study at any time.

YES ☐ NO ☐

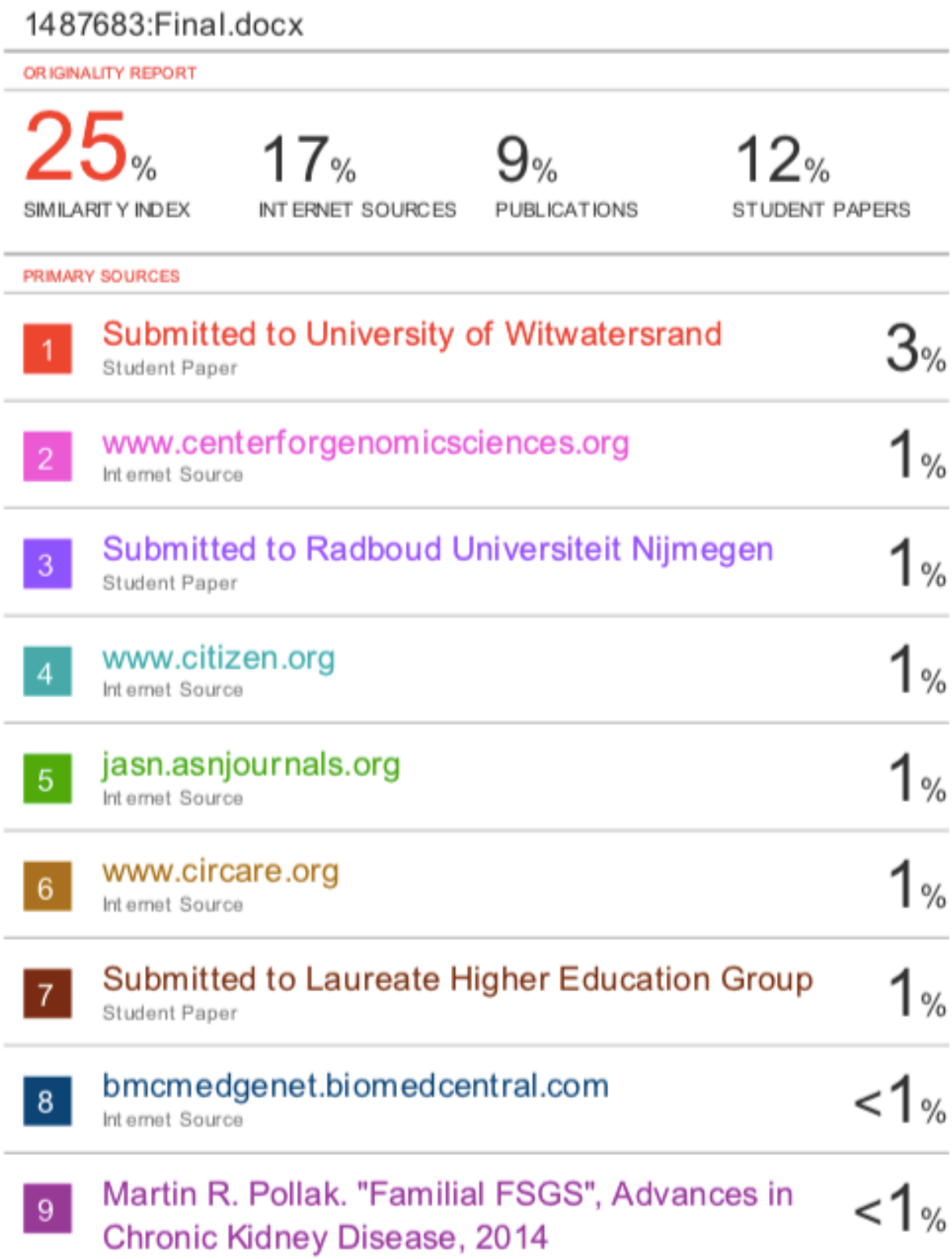
_____ _____

Signature of Participant Date

_____ _____

Signature of Person Obtaining Consent Date

Appendix M– Turnitin originality report



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