

**Genetic Basis of Steroid
Resistant Nephrotic
Syndrome in Indian and
Black South African
Children**

by

Kareshma Asharam

Submitted in partial fulfilment of the academic requirements for the degree of Masters in

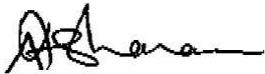
Medical Science in the Department of Paediatrics & Child Health, Nelson R Mandela

School of Medicine, University of KwaZulu-Natal, Durban

2017

Preface

This study represents original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text. The experimental work described in this thesis was carried out in the following labs: Paediatrics and Child Health Laboratory, located in the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, 4013, South Africa and at the Frederick National Laboratory, Frederick, MD, USA; Kidney Disease This research was carried out from September 2010 to December 2014, under the supervision of Professor Rajendra Bhimma (Durban) and Dr Cheryl A Winkler (USA) .



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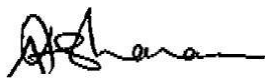


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Declaration

I, **Kareshma Asharam** declare that:

- (i) The research reported in this dissertation, except where otherwise indicated is my Original work.
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Statement

The following publication, fully referenced has been reproduced in part non-continuously throughout the thesis:

A Single *NPHS2* Mutation Explains 27% of Steroid Resistant Nephrotic Syndrome in a Black South African Children

¹Kareshma Asharam BMed Sci Hon, ¹Rajendra Bhimma MD, ^{1,2}Hoosen M Coovadia MD, ¹Wenkosi P Qulu BA, ¹Thajasvarie Naicker PhD, ³Victor A David MS, ⁴Randall Johnson PhD, ⁵Sophie Limou PhD, ⁶Jeffrey B Kopp MD, ⁴George W Nelson PhD, ^{5*}Cheryl A Winkler PhD.

The candidate performed the experiments described in this paper, and where others made contributions it has been duly acknowledged in the text. The candidate drafted this publication in full and they were reviewed by co-authors.

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Professor R Bhimma: Date: 01 September 2017

Dr CA Winkler: Date: 01 September 2017

PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications:

1. R Bhimma, M Adhikari, K Asharam (2006). Management of Steroid Resistant Focal Segmental Glomerulosclerosis in Children using Tacrolimus. *Am J Nephrol.* 26(6) :544-51
2. Bhimma R, Adhikari M, Asharam K (2006). [Steroid-resistant nephrotic syndrome: the influence of race on cyclophosphamide sensitivity.](#) *Pediatr Nephrol:* 21(12):1847-1853
3. Bhimma R, Adhikari M, Asharam K, C Connolly (2008) The spectrum of chronic kidney disease (stages 2–5) in KwaZulu-Natal, South Africa *Pediatr Nephrol.* 23:1841-1846

Journal article submitted (Awaiting response)

Kareshma Asharam BMed Sci Hon, ¹Rajendra Bhimma MD, ^{1,2}Hoosen M Coovadia MD,¹Wenkosi P Qulu BA, ¹Thajasvarie Naicker PhD, ³Victor A David MS, ⁴Randall Johnson PhD, ⁵Sophie Limou PhD, ⁶Jeffrey B Kopp MD, ⁴George W Nelson PhD, ^{5*}Cheryl A Winkler PhD. A Single *NPHS2* Mutation Explains 27% of Steroid Resistant Nephrotic Syndrome in a Black South African Children. *JASN*

International Conference presentations:

1. K Asharam, R Bhimma, M Adhikari, HM Coovadia. Microsatellite analysis of Steroid-resistant Nephrotic Syndrome in Indian and Black Children in South Africa. The 26th International Pediatric Association Congress of Pediatrics 2010-JHB-SA, August 04-August 08, 2010. (abstract no. 407).
2. K Asharam, R Bhimma, M Adhikari, HM Coovadia. Fifteenth Congress of the International Microsatellite analysis of Steroid-resistant Nephrotic Syndrome in Indian and Black Children in South Africa. Pediatric Nephrology Association-New York, August 29 – September 2, 2010. (abstract no. O-42).

National Conference Presentations

1. Asharam K, Bhimma R, Adhikari M. Idiopathic Steroid Resistant Nephrotic syndrome in South African Children. African Paediatric Nephrology Congress 2006. Cape Town March 16-18. Poster presentation.
2. Asharam K, Bhimma R, Adhikari M. Steroid resistant nephrotic syndrome in South African children: The influence of race on cyclophosphamide. South African Paediatric Congress 2006. Sun City Sep 7-10. Poster presentation.
3. Asharam K, Bhimma R, Adhikari M. Steroid resistant nephrotic syndrome in South African children: The influence of race on cyclophosphamide. Oral presentation at the University Research Day 2008.
4. R Bhimma, K Asharam, M Adhikari, HM Coovadia. Microsatellite analysis of Steroid-resistant Nephrotic Syndrome in Indian and Black Children in South Africa. South African Congress of Nephrology CT-SA, 16-19 April 2010.

5. K Asharam, R Bhimma, M Adhikari, HM Coovadia. Microsatellite analysis of Steroid-resistant Nephrotic Syndrome in Indian and Black Children in South Africa. College of Health Sciences: UKZN Research Symposium, September 14 – September 15, 2010.
6. K Asharam, R Bhimma, WP Qulu, T Naicker, V David, S Limou, EB Roemer, JB Kopp, GW Nelson, CA Winkler. *NPHS2* V260E homozygosity is a frequent cause of sporadic steroid resistant nephrotic syndrome in Black South African children. UKZN Research Symposium, 10-11 September, 2015.

Dedication

To my husband, Vikash, my son Yadav and my daughter Tuhina

'Your support, patience and understanding is a true inspiration'

Acknowledgments

To my husband who taught me never to give up hope, who always stood by me and encouraged me through my darkest days. Your ever so gentle back and foot massages is something I will never trade for the world. Thank you for your everlasting support today, tomorrow and always. To my son Yadav and daughter Tuhina, your innocent smiles and gentle kisses makes any day worth living for. Mummy will be forever grateful for your understanding and patience when she has to leave you and attend conferences and training away from home especially during long periods of time. You both are my shining stars and remember nothing is 'impossible', dream, live and love, always reach for the stars my sweethearts.

I wish to express my sincere gratitude to:

Professor R Bhimma, for his invaluable supervision, encouragement, understanding and kindness always. You have seen me through this from day one and I have finally achieved my goal through many challenges but without your support this would not been possible. It has been a pleasure being your student.

Professor M Adhikari, who took me under her wing in 2002, who nurtured and encouraged me, keeping me in focus and teaching me never to lose track of my family. For always having my best interest at heart, no words can express my gratitude.

Professor Cheryl Winkler, for her unconditional support through all these years. Your expert and meticulous assistance with laboratory analysis and great assistance with publications will always be treasured.

Professor Hoosen Coovadia for your encouragement and mentorship.

Elizebeth Binns-Roemer, for your kindness, support and understanding when I was miles always from my family. Thank you, you will never be forgotten.

To laboratory staff and academic staff V David, S Limou, EB Roemer, JB Kopp and GW Nelson at Frederick National Laboratory, Frederick, MD, USA; Kidney Disease Branch, National Institute of Diabetes and Digestive Diseases, Bethesda, MD, USA; I am forever grateful for your constant support.

To all the members in the Department of Paediatrics and Child Health, thank you for all the support and help throughout my degree, with a special thanks to Mrs. D Pillay, Mrs P Narayansamy for your administrative support and to Prof Anna Coutsoudis thank you for your encouragement and support through all these years.

To Dr Kavidha Reddy, my dear friend and confidant, thank you for your undying support for the many years that we are friends, for listening to me and always being there for me and my family. I will forever be indebted to you.

To all my friends that have stood by me over some of my most challenging years at Paediatrics, thank you, you will always be remembered.

To my parents Mr and Mrs Ramcharan, my pillars of strength, my comfort, thank you mum and dad your for unconditional love and support.

To my Brothers and their families thank you for always looking out for me and helping with kids on all my travels, I will forever be grateful.

My Mother in-law, thank you for being mum to the kids when I am away from home, your support never goes unnoticed, Viky, I and the kids greatly appreciate your warmth.

Finally to his divine blessing Shree Hanuman, 'nothing is impossible'

ABSTRACT

Background

NPHS2 mutations are a common cause of childhood steroid resistant nephrotic syndrome (SRNS). *APOL1* variants are strongly associated with focal segmental glomerulosclerosis (FSGS) in African Americans. In South Africa, SRNS is more frequent among Black children compared to Indian children. We investigated whether *NPHS2* and *APOL1* variants contributed to this disparity.

Methods

Indian and Black children with Nephrotic Syndrome (NS) from KwaZulu-Natal Province, South Africa were enrolled; only SRNS children underwent kidney biopsy. The 64 NS cases and 104 controls were sequenced for *NPHS2* and genotyped for *APOL1* risk alleles. A replication group comprised 20 Black SRNS cases with FSGS and 18 controls. Haplotype and coalescence analyses was used to determine relatedness among the children and to age the mutation.

Findings

58% (19/33) of Indian and 97% (30/31) of Black children with NS were steroid resistant (SR) ($p = 0.0002$). 8/30 (26.7%) of all SRNS Black children were homozygous for *NPHS2* V260E and had FSGS. V260E homozygosity was replicated in 6/20 (30%) of Black children with SR-FSGS. V260E was not observed in Indian cases or controls; however, one Black control was heterozygous for V260E (minor allele freq. $\sim 0.07\%$). Children homozygous for V260E developed SRNS at an earlier age than non-carriers (median 34 vs. 78 months, $p=0.009$), and none achieved either partial or complete remission (0% vs. 47%, $p=0.002$) even after intensive treatment. *APOL1* variants did not associate with NS.

Interpretation

Sporadic SRNS among unrelated Black children is partially explained by homozygosity for the V260E pathogenic mutation, present in 26.7% of those with SRNS and 32% with SR-FSGS histology, which is not due to cryptic consanguinity among affected children. Genotyping a single mutation in children of Zulu ancestry with NS will identify those with SR-FSGS making it possible to avoid kidney biopsy and ineffective steroid or additional intensive immunosuppressive treatment in many Black children with NS.

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LIST OF ABBREVIATIONS

SRNS	:	Steroid Resistant Nephrotic Syndrome
NS	:	Nephrotic Syndrome
SR	:	Steroid Resistant
SSNS	:	Steroid Sensitive Nephrotic Syndrome
FSGS	:	Focal Segmental Glomerulosclerosis
MCD	:	Minimal Change Disease
MN	:	Membranous Nephropathy
ESKD	:	End Stage Kidney Disease
CNS	:	Congenital Nephrotic Syndrome
CNF	:	Congenital Nephrotic Finnish-type
GFB	:	Glomerular Filtration Barrier
GBM	:	Glomerular Basement Membrane
GEC	:	Glomerular Endothelial Cells
NPHS1	:	Nephrin
NPHS2	:	Podocin
PLCE1	:	Phospholipase C Epsilon 1
WT1	:	Wilm's Tumor 1
LAMB2	:	Laminin- β 2
CD2AP	:	CD2-Associated Protein
ACTN1	:	Alpha-Actinin 4
TRPC6	:	Transient Receptor Potential Cation Channel, Homolog of 6
INF2	:	Inverted Formin 2
Th1	:	T Helper 1
Th2	:	T Helper 2
IL-13	:	Interleukin 13

suPAR : Soluble Urokinase Plasminogen Activator Receptor
VEGF : Vascular Endothelial Growth Factor
NF- κ B : Nuclear Factor Kappa B
ANGPTL4 : Angiopoietin-like-4
TLRs : Toll Like Receptors
G : Glucocorticoids
C : Calcineurin Inhibitors
S : Synaptopodin
R : Rituximab

Chapter One - Introduction

1.1 The Human Kidney

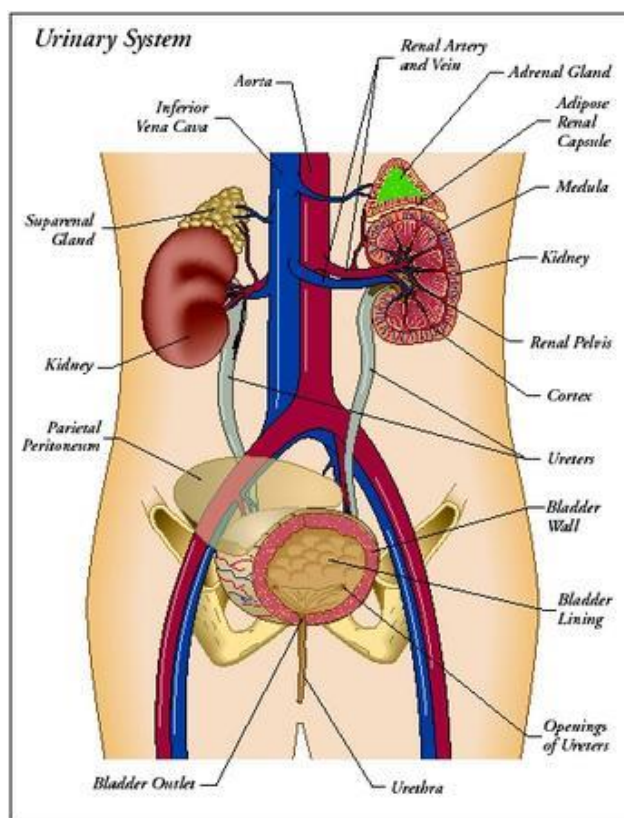


Figure 1.1 The Urinary System (Adapted from <http://www.fags.org/health/Body-by-Design-V2/The-Urinary-System-Design-parts-of-the-urinary-system.html> 26/10/2016 at 12.33pm).

The kidney is situated below the diaphragm and posterior to the liver and the spleen. It is located at the rear wall of the abdominal cavity, just above the waistline (Figure 1.1). Grossly, the kidneys are bean-shaped structures and weigh about 150 grams in the male and about 135 grams in the female. In an adult they are typically 10-12 cm in length, 5-7 cm in width, and 2-3 cm in thickness (Messing EM. (2007) In: Wein AJ (editor-in-chief) and LR 2007). In a term newborn the kidney measures 4–5cm and it is smaller in preterm babies.

The kidneys serve important functions, including filtration and excretion of metabolic waste products (urea and ammonium); regulation of necessary electrolytes, fluid, and acid-base balance; and stimulation of red blood cell production. As the kidneys filter blood, they create urine, which collects in the kidneys' pelvis - funnel-shaped structures that drain down tubes

called ureters to the bladder. They also serve to regulate blood pressure via the renin-angiotensin-aldosterone system, controlling reabsorption of water and maintaining intravascular volume. The kidneys also reabsorb glucose and amino acids and have hormonal functions via erythropoietin, calcitriol, and vitamin D activation.

1.1.1 Structure of the Kidney

1.1.1.1 *Cortex and the Medulla*

The kidney is divided into the cortex and medulla. Two layers form them internally. The outer layer is the **Cortex** that contains:

- Glomeruli
- Proximal Tubules
- Cortical Portions of Loop of Henle
- Distal Tubules
- Cortical Collecting Ducts

The inner layer or **Medulla** is comprised of **Renal Pyramids**. The pyramids contain:

- Medullary portions of the Loops of Henle
- Medullary Portions of the Collecting Ducts

1.2 Functional renal unit

The multiple pyramids taper and join forming a **minor calyx**. Several combined make a **major calyx**. The major calyces join and enter a funnel shaped **renal pelvis** that directs urine into the ureter.

The functional renal unit, the nephron has five parts namely:

- The glomerulus, which is the blood kidney interface, plasma is filtered from capillaries into the Bowman's capsule.
- The proximal convoluted tubule, which reabsorbs most of the filtered load, including nutrients and electrolytes.
- The loop of Henle, which, depending on its length, concentrates urine by increasing the osmolality of surrounding tissue and filtrate.
- The distal convoluted tubule, which reabsorbs water and sodium depending on needs.
- The collecting system, which collects urine for excretion. There are two types of nephrons, those localized to the cortex, and those extending into the medulla. The latter are characterized by long loops of Henle, and are more metabolically active.

1.2.1 Nephrons

Each kidney contains around a million units called nephrons, each of which is a microscopic filter for blood. The nephron, the functional unit of the kidney selectively filters molecules from blood plasma to form a filtrate (Figure 1.2); (Zuo 2006). The nephron has two main parts: the **renal corpuscle** (the mouth and the jawbreaker) and the **renal tubule** (the long and winding neck). The "mouth" of the renal corpuscle is actually called **Bowman's capsule**, which is a double-walled, cup-shaped structure around the glomerulus of each nephron of the vertebrate kidney. It serves as a filter to remove organic wastes, excess inorganic salts, and water and the "jawbreaker" is actually called the **glomerulus**. The glomerulus consists of a tuft of capillaries situated within a Bowman's capsule at the end of a renal tubule in the vertebrate kidney that filters waste products from the blood and thus initiates urine formation. The renal tubule

consists of the **proximal tubule** called the **loop of Henle**. The loop of Henle is a long U-shaped portion that recovers water and sodium chloride from the urine.

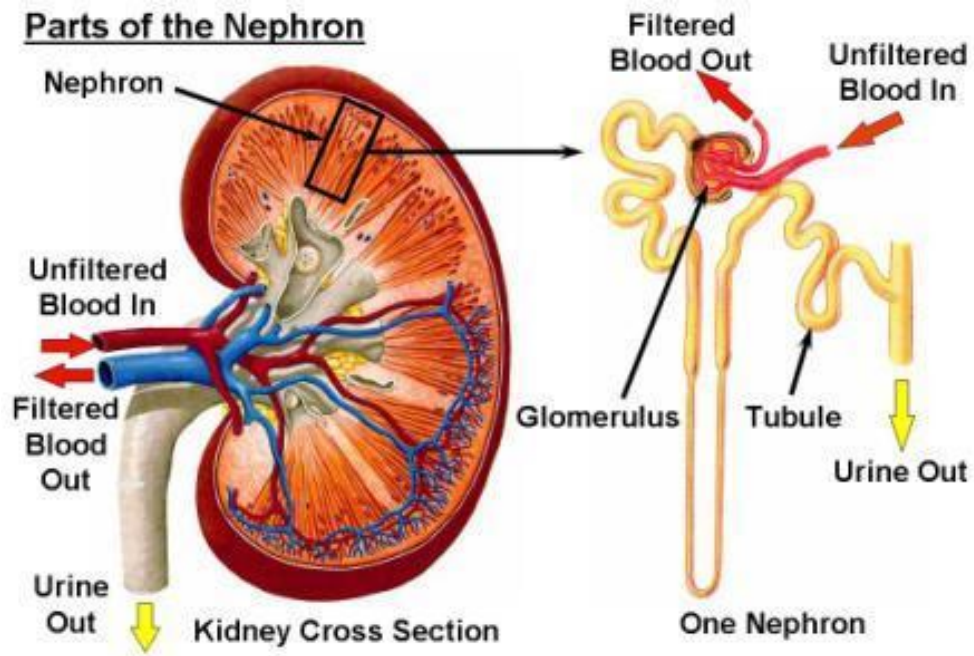


Figure 1.2 Schematic diagram of cross section of kidney linking structure to function. Direction of blood flow and filtration in the nephron is amplified (Adapted from (Falk 2008)).

1.2.2 Glomerulus

The glomerulus is capillary blood vessels actively involved in the filtration of the blood to form urine. The glomerulus is one of the key structures that make up the nephron, the functional unit of the kidney.

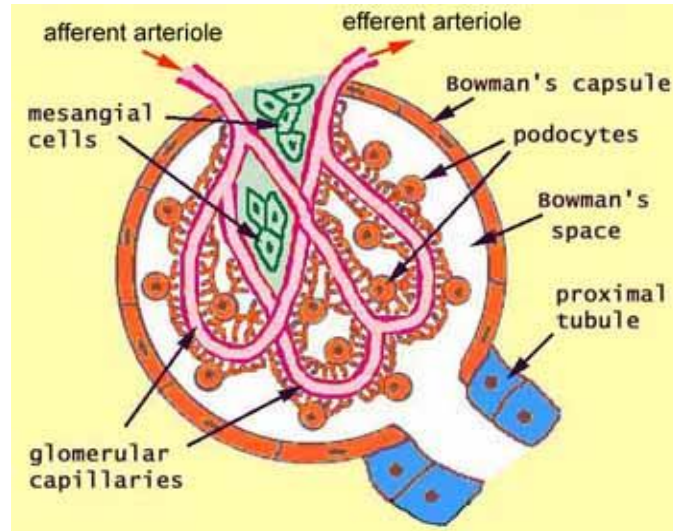


Figure 1.3 Schematic Cross section of glomerulus, showing the flow of blood and filtrate.

(Adapted from Google images on 26/10/2016 at 2.45pm).

https://www.google.co.za/search?q=urinary+system&espv=2&biw=1600&bih=770&site=webhp&source=lnms&tbn=isch&sa=X&sqi=2&ved=0ahUKEwiikprIn_jPAhWhKMAKHTWgDxoQ_AUIBigB#tbm=isch&q=schematic+glomerulus&imgrc=re-4xCa0d-9FcM%3A

The kidney contains approximately one million individual filtering units called glomeruli composed of specialized capillaries. The ultrafiltration of plasma in the kidney occurs through the capillary wall of the glomerulus (Figure 1.3). The glomerular filtration barrier (GFB) is made up of glomerular endothelial cells (GEC's), glomerular basement membrane (GBM) and the podocyte cell layer with their 'slit diaphragms' (Patrakka and Tryggvason 2010). Present data suggest that all three layers of the glomerular capillary wall need to be intact to maintain normal filtration function. However, the studies performed during the last decade have underlined the

role of the podocyte in the filtering process (Patrakka and Tryggvason 2009). Injury to the filtration barrier in renal disorders leads to protein leakage to urine (proteinuria) and progressive renal disease.

1.2.3 Glomerular endothelial cells (GECs)

The endothelial cells of the glomerulus are highly fenestrated. The fenestrae are 70–100 nm in diameter and constitute 20–50% of the glomerular capillary surface area. As the fenestrations are huge compared to the size of albumin, the glomerular epithelial cells (GECs) have not been believed to play an important role in the barrier function for protein filtration. The GECs are, however, covered by an endothelial cell surface layer (mainly glycocalyx) that may hinder the passage of albumin and other plasma proteins into the GBM (Figure 1.4). GECs are likely to be critically involved in the formation and maintenance of the filtration barrier, and probably also act as a true barrier for the traversal of plasma macromolecules.

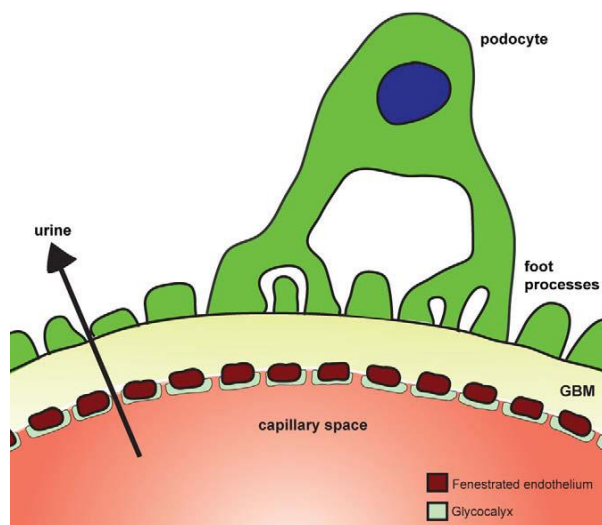


Figure 1.4. Glomerular filtration occurs through the capillary wall into the urinary space. The capillary wall contains an innermost fenestrated endothelium with glycocalyx, the glomerular basement membrane (GBM), and a layer of podocytes with interdigitating foot processes connected by slit diaphragms (Adapted from (Patrakka and Tryggvason 2010)).

1.2.4 Glomerular basement membrane (GBM)

The GBM forms a structural foundation of the glomerular capillary to which endothelial and podocyte cells are anchored on both sides (Figure. 1.5). The main components of the GBM include type IV collagen, laminin, proteoglycans and nidogen. The triple-helical type IV collagen molecules of mature GBM are composed of $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains. The podocyte is a highly specialised epithelial cell covering the outside of the glomerular capillary (Faul, Asanuma et al. 2007). It has large cytoplasmic projections (major process) that divide into long thin processes termed foot process, close to the glomerular capillary (Figure 1.5). The slit diaphragm divides the plasma membrane of the foot processes into basal, apical and lateral surfaces (Patrakka and Tryggvason 2010). Defects of either the GBM or in the slit diaphragm can cause leakage of albumin in the urine (albuminuria) ((Miner 2012)..

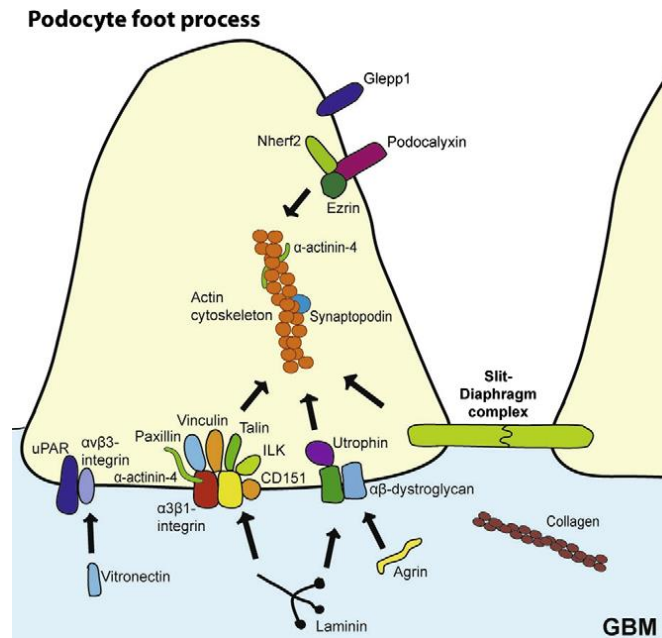


Figure. 1.5. Schematic picture of podocyte foot processes. Foot processes are basally anchored to the components of the GBM via $\alpha 3\beta 1$ integrin, tetraspanin CD151 and $\alpha \beta$ dystroglycan. These transmembrane proteins are linked to the actin cytoskeleton via several adaptor proteins. uPAR receptor is also found at the basal surface of foot processes in where it probably mediates its actions through $\alpha \beta 3$ -integrin. Vitronectin, the extracellular ligand of $\alpha \beta 3$ -integrin, is induced during proteinuria and activate uPAR signaling in podocytes. The slit diaphragm protein complex is linked to actin cytoskeleton. Apical surface of podocytes contain podocalyxin and Glepp1. Podocalyxin is connected to actin via adapter proteins. The actin cytoskeleton of foot processes contains actin-associated proteins α -actinin-4 and synaptopodin, and interconnects three plasma membrane domains of foot processes together (Adapted from (Patrakka and Tryggvason 2010)).

1.2.5 Podocyte

The podocyte is a highly specialized epithelial cell covering the outside of the glomerular capillary (Faul, Asanuma et al. 2007). The podocyte has a prominent cell body and large cytoplasmic projections (major processes) that divide into long thin processes, termed foot

processes, close to the glomerular capillary (Figure. 1.5). The foot processes are attached firmly to the underlying GBM and arranged in a highly organized way so that they envelope the capillary wall in an interdigitating “comb-like” fashion. Foot processes from adjacent podocytes are interconnected by a specialized cell–cell junction of the podocyte, termed the slit diaphragm. The slit diaphragm divides the plasma membrane of foot processes to the basal, the apical, and the lateral surfaces. In the cytoplasm, these three surfaces are interconnected via the actin-based cytoskeleton. Podocyte foot processes with different plasma membrane domains have essential roles in the maintenance of the glomerular filtration barrier.

1.2.6 The slit diaphragm

The slit diaphragm is a highly specialized cell–cell junction of podocytes that interconnects adjacent foot processes. Rodewald and Karnovsky in 1974 proposed that the slit diaphragm has a structured, zipper-like architecture with pores that are smaller than albumin, and therefore could serve as a barrier for protein filtration (Rodewald and Karnovsky 1974). Although this classic model was proposed over 35 years ago, it was only during the last decade that the molecular architecture of the slit diaphragm started to unravel. The slit diaphragm protein complex is composed of proteins common for most cell–cell junctions, including cadherins and catenins, but importantly, also of proteins that are not generally found elsewhere in the body (Figure. 1.6) (Patrakka and Tryggvason 2009); (Lehtonen, Ryan et al. 2005).

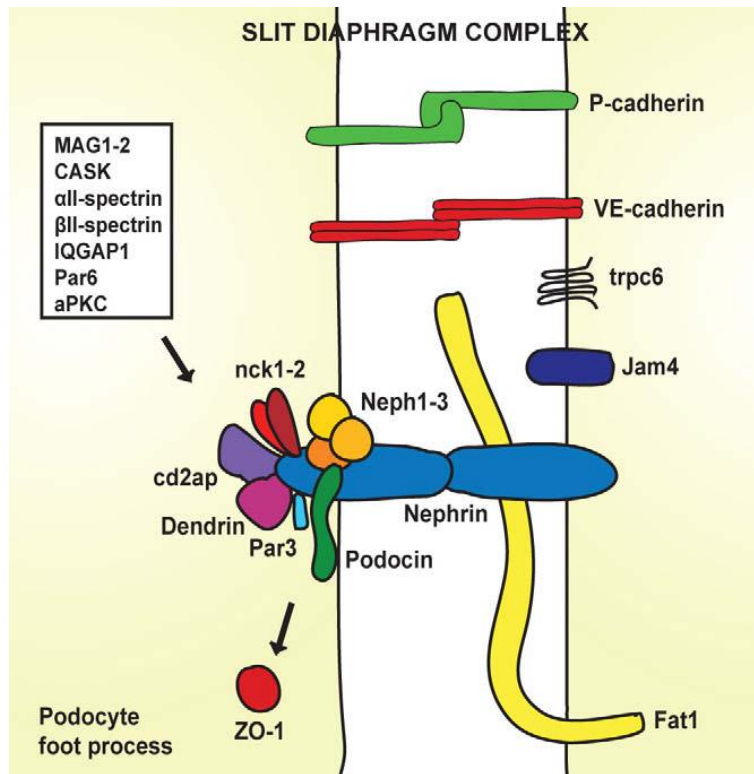


Figure 1.6: Schematic picture of the slit diaphragm components. The slit diaphragm complex is extracellularly formed of at least nephrin, Neph1–3, P-cadherin, VEcadherin, Fat1, and Jam4. Nephrin and cadherin proteins probably interact in a homophilic fashion in the center of the slit diaphragm. Intracellularly, a number of adapter proteins are involved in linking the slit diaphragm to actin (Adapted from (Patrakka and Tryggvason 2010)).

The filtration barrier has a net negative electrical charge therefore the movement of large negatively charged molecules is restricted more than molecules with a positive or neutral charge. Proteins are negatively charged and are not freely filtered by the glomerulus.

Podocin is a hairpin-shaped integral membrane protein with both ends directed into the intracellular space. Podocin interacts directly with nephrin, Neph1 and Cd2ad (Sellin, Huber et al. 2003); (Schwarz, Simons et al. 2001), and seems to be essential for the recruitment of nephrin to the slit diaphragm (Schwarz, Simons et al. 2001). The essential role of podocin in

the glomerular barrier has been shown in podocin deficient mice which lack slit diaphragms and die perinatally due to massive proteinuria (Roselli, Heidet et al. 2004). Trpc6 is found in the slit diaphragm and some Trpc6 mutations found in patients result in increased amplitude and duration of calcium influx after stimulation. Together, these results suggest that impaired slit diaphragm signalling is involved in the development of “Trpc6 nephropathy”. Three cadherin proteins, a large protocadherin Fat1, P-cadherin, and vascular endothelial cadherin (VE-cadherin) have been localized to the slit diaphragm (Patrakka and Tryggvason 2009). Fat1 is a huge protein with 34 tandem cadherin-like repeats, and it is essential for normal filtration barrier as Fat1 knockout mice lack slit diaphragms and exhibit proteinuria (Ciani, Patel et al. 2003). P-cadherin, on the other hand, does not seem to be essential for the functional renal filtration barrier, whereas the role of VE-cadherin in the podocyte is so far unknown (Patrakka and Tryggvason 2009).

Podocytes contribute to the specific size and charge characteristics of the glomerular filtration barrier, and their damage leads to a retractions of their foot processes and proteinuria (Laurens, Battaglia et al. 1995); (Pavenstadt 1998). Podocyte injury leads to many glomerular diseases, including minimal-change nephropathy, focal segmental glomerulosclerosis (FSGS), membranous glomerulopathy, diabetes mellitus and lupus nephritis (Somlo and Mundel 2000); (Kerjaschki 2001). Currently the pathogenic role of foot process effacement in proteinuria is not well understood.

In human genetics much progress has been made toward a molecular understanding of the slit diaphragm and the modulators of foot process architecture. In the past 15 years, human genetic studies revealed that mutations in the genes encoding nephrin (Kestila, Lenkkeri et al. 1998) podocin (Boute, Gribouval et al. 2000), phospholipase C ϵ (Hinkes, Wiggins et al. 2006), and coenzyme Q10 biosynthesis mono-oxygenase 6 (Heeringa, Chernin et al. 2011) give rise to

early-onset proteinuria. Additionally, adult onset proteinuria such as FSGS is associated with mutations in the genes encoding α -actinin 4 (Kaplan, Kim et al. 2000), *CD2AP* (Kim, Wu et al. 2003), *INF2* (Brown, Schlondorff et al. 2010), *TRPC6* (Reiser, Polu et al. 2005); (Winn, Conlon et al. 2005); (Heeringa, Moller et al. 2009) and synaptopodin (Dai, Wang et al. 2010). Mutations in *LMX1B*, which encodes a transcription factor for collagen, result in podocyte abnormalities due to impaired cell adhesion to the abnormal GBM (Morello, Zhou et al. 2001). Similarly, mutations in the gene encoding laminin β 2, another component of the GBM, lead to podocyte injury and proteinuria (Zenker, Aigner et al. 2004). Finally, recent exome sequencing as well as a whole-genome linkage analysis revealed *MYO1E* mutations in childhood proteinuric disease and FSGS; *MYO1E* encodes a mutant form of nonmuscle class I myosin (Mele, Iatropoulos et al. 2011); (Sanna-Cherchi, Burgess et al. 2011).

These gene polymorphisms are not directly linked to podocyte-specific defects, but this is an area of active research at this time. A large locus containing numerous genes was recently identified in African-American populations (Kao, Klag et al. 2008); (Kopp, Smith et al. 2008). Initial studies revealed *MYH9* as a likely gene candidate in this locus. This was an attractive hypothesis, given previous work showing that *MYH9* is responsible for two genetic causes of proteinuria: Epstein and Fechtner syndromes **Table 1.1** (Arrondel, Vodovar et al. 2002). Interestingly, further work revealed that the likely candidate gene conferring risk for kidney disease is apolipoprotein L1 (*APOL1*), a molecule that is known for its trypanolytic properties and that confers an evolutionary advantage in African-Americans (Genovese, Friedman et al. 2010). Furthermore, common variations in *GPC5* (which encodes glypican 5) are also associated with acquired nephrotic syndrome (Okamoto, Tokunaga et al. 2011).

Table 1.1: Candidate genes implicated in various forms of Nephrotic Syndrome

Gene	Location	Protein	Inheritance	Gene	Usual onset
<i>NPHS1</i>	19q13.1	Nephrin	Slit diaphragm	AR	Congenital nephrotic syndrome, characteristic changes
<i>NPHS2</i>	1q25-31	Podocin	Slit diaphragm	AR	Congenital nephrotic syndrome, or early onsets SRNS
<i>PLCE1</i>	10q23-24	Phospholipase C epsilon 1	Intracellular	AR	Early onset SRNS: Diffuse mesangial sclerosis (DMS); FSGS
<i>WT1</i>	11p13	Wilm's tumor 1	Intracellular	AD	Early onset SRNS, Denys-Drash or Frasier syndrome; DMS (Deny's Drash Syndrome); FSGS (Frasier syndrome)
<i>LAMB2</i>	3p21	Laminin-β2	Glomerular basement membrane	AR	Pierson syndrome, early onset SRNS: DMS (Syndromic); FSGS (Isolated)
<i>CD2AP</i>	6p12.3	CD2-associated protein	Slit Diaphragm	SD	Adult onset SRNS (heterozygous), early onset FSGS (homozygous), FSGS
<i>ACTN1</i>	19q13	Alpha-Actinin 4	Intracellular	AD	Adult onset SRNS (incomplete penetrance, slow progression); FSGS
<i>TRPC6</i>	11q21-22	Transient receptor potential cation channel, homolog of 6	Cell surface	AD	Adult onset SRNS; FSGS
<i>INF2</i>	14q32	Inverted formin 2	Intracellular	AD	Adult onset SRNS; FSGS
<i>LMX1B</i>	9q34.1	LIM-homeodomain transcription factor 1β	Intracellular	AD	Nail-patella syndrome; SRNS
<i>APOL1</i>	22p	Apolipoprotein L1	Intracellular	Complex	Adult onset SRNS (incomplete penetrance); FSGS

1.3 History of Nephrotic Syndrome

The term nephrotic syndrome (NS) was first described by Leiter in 1931 “to differentiate the association of massive proteinuria, oedema and hypercholesterolemia “due to what maybe glomerulonephritis” (Leiter 1931). NS is a clinical syndrome characterised by heavy proteinuria ($>40\text{mg/m}^2$ per hour or 50mg/kg per day), hypoalbuminemia (albumin $\leq 2.5\text{mg/dL}$) and hyperlipidemia (serum cholesterol $>200\text{mg/dL}$ or 6.5mmol/L) with or without oedema (Gipson, Massengill et al. 2009). It is also recommended that in children where a 24 hour urine collection is difficult, then the use of the protein: creatinine ratio on a spot early morning sample of urine with a urine protein: creatinine (Up/Ucr) ratio ≥ 2.0 , be used to quantify massive proteinuria (Hogg, Furth et al. 2003). Proteinuria results when there is a weakness in the filter vessel wall that allows protein to leak into the urine. An early event is the loss of integrity of foot processes and the slit diaphragm, leading to foot-process fusion, altering the filtration barrier with resultant protein in the urine (proteinuria) and blood in the urine (haematuria) (Figure 1.7) (Workshop by the British Association for Paediatric Nephrology and Research Unit, Royal College of Physicians (1994) (Hogg, Portman et al. 2000).

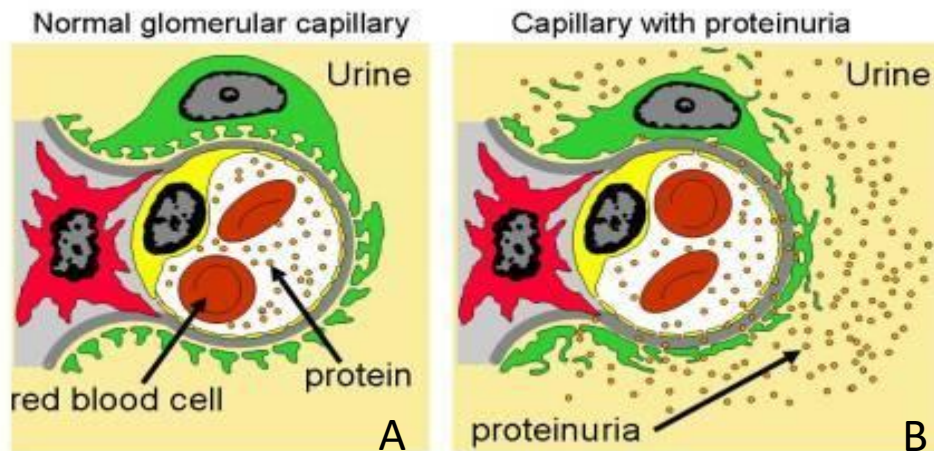


Figure 1.7. A schematic representation of a typical functioning glomeruli versus a diseased glomerulus presenting with proteinuria and hematuria (Adapted from (Falk 2008)).

NS can be classified according to age of onset, as congenital nephrotic syndrome (CNS) that presents from birth to three months, infantile NS presenting after three months to one year of age, and childhood onset NS presenting after one year of age, with those children presenting after eight years of age being classified as having late onset NS (Bhimma 2006).

NS has been separated into 2 broad categories on the basis of patients response to standard steroid therapy, i.e., steroid sensitive nephrotic syndrome (SSNS) and steroid resistant NS (SRNS) (Ruf, Lichtenberger et al. 2004). Most children respond to steroid therapy and show a favourable long-term outcome. However, 10–20% of the patients show resistance to steroid therapy and are classified as SRNS. These patients tend to progress to end stage kidney disease (ESKD) due to progressive damage of the glomerular filtration barrier and will eventually need renal replacement therapy (Yu, Ding et al. 2005).

NS is further categorised into secondary, primary, idiopathic, or familial NS. There are different histological variants of primary idiopathic nephrotic syndrome: minimal-change disease (MCD),

Focal segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN). The latter is rare in children (Bhimma, Naicker et al. 2013)

Minimal change disease and FSGS may represent opposite ends of one pathophysiological process or distinct disease entities. By contrast, membranous nephropathy is a distinct disease associated with prominent immune complex diseases located between glomerular podocytes and the GBM (Eddy and Symons 2003). MCD is characterised by foot process effacement (Figure 1.8) and loss of the normal charge barrier such that albumin leaks out and proteinuria ensues (Han 2006b).

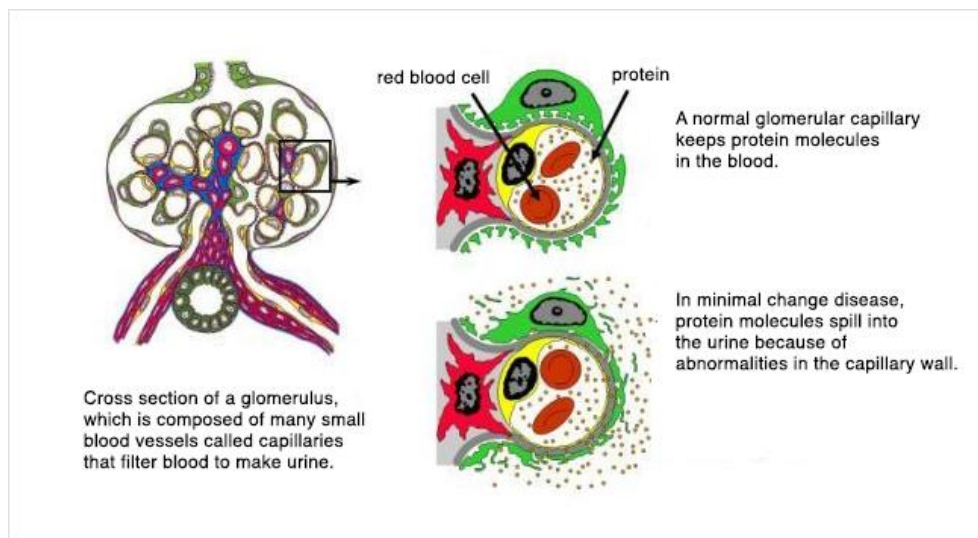


Figure 1.8. A schematic representation of a proteinuria leakage demonstrating Minimal Change disease (Adapted from (Falk 2008)).

Each kidney is made up of approximately one million tiny filters called “glomeruli”. FSGS indicates that some segments of the kidney filters are scarred thus leading to the release of proteins into the urine. (Figure 1.9); (Kriz 2003).

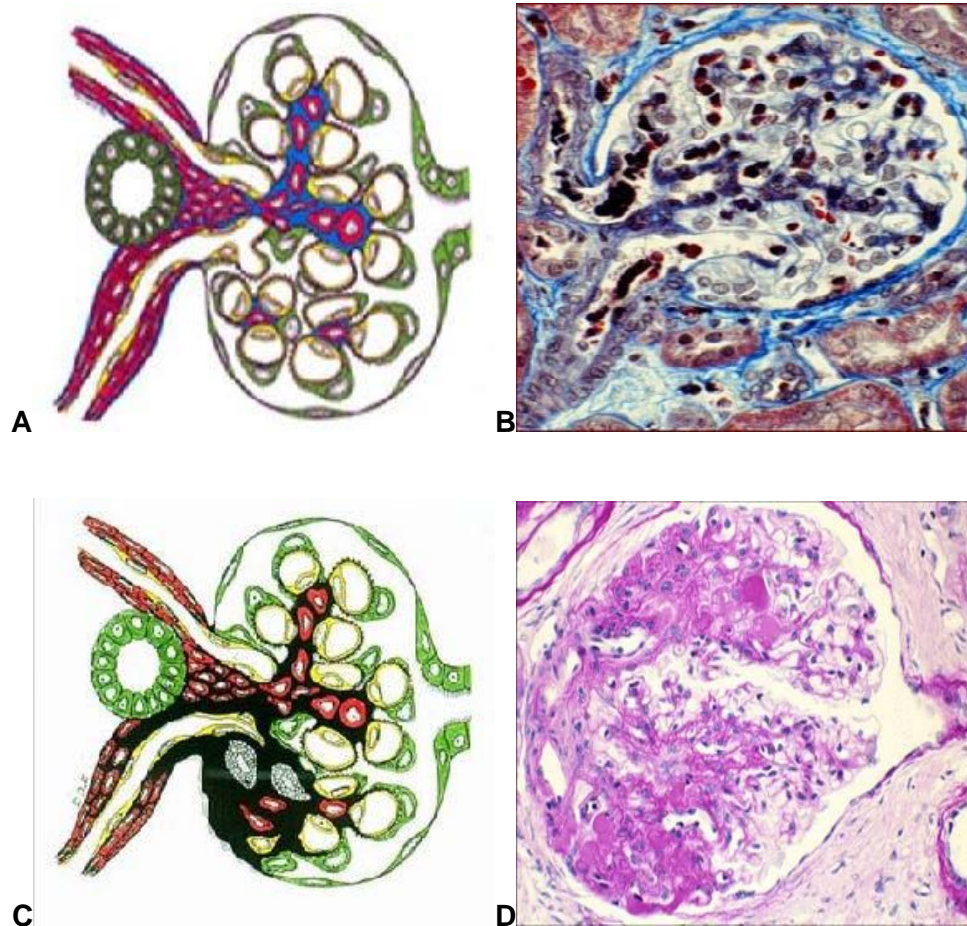


Figure 1.9. Schematic and light micrograph of cross-section of A & B: normal glomeruli; C & D glomeruli with focal segmental glomerulosclerosis (Adapted from (Falk 2008)).

In SRNS, approximately 75% of patients exhibit renal histologic features of FSGS and 20% demonstrate minimal-change disease. Conversely, in SSNS, renal histologic features indicate minimal change disease in 80% of cases and FSGS in 20% (Fuchshuber, Jean et al. 1995). Approximately 10% of children and 50% of adults with idiopathic NS have SRNS, fail to respond

to immunosuppressive treatment and progress to ESKD) within seven years of diagnosis (Korbet 2002).

The worldwide incidence of NS in children in developed countries ranges from 2 to 7 per 100 000 and the prevalence from 12 to 16 per 100 000 children, with approximately a six fold higher rate reported among those with African and South Asian ancestry (Eddy and Symons 2003); (Bhimma, Adhikari et al. 2006); (Copelovitch, Sam OI et al. 2010); (Ingulli and Tejani 1991); (Mubarak, Lanewala et al. 2009).

There have been a few longitudinal studies of children with NS (Table 1.2). Most cohort studies have been retrospective chart reviews with limited access to clinical data. The few prospective studies on childhood NS are registries of short follow-up time, limited clinical data, in ethnically homogenous populations (Hussain, Zello et al. 2013).

Previously it has been reported by Srivastava (Srivastava 1987) that the overall incidence of childhood idiopathic NS has been generally stable, however there has been several reports over the years that the histological pattern is changing. There incidence of FSGS seems to be increasing and this may be due to ethnic origins that may affect histological variants and the response to immunosuppressive treatment (Bhimma, Adhikari et al. 2006); (Bhimma, Adhikari et al. 2008); (Ingulli and Tejani 1991).

Several reasons have been suggested to explain ethnic differences in the incidence of the disease. These include high prevalence of infections, lower socio-economic status with inequalities in access to health care resources, genetics, and environmental factors. The onset of NS has been linked to environmental exposures such as mercury exposure (in adults), history of atopy, and immune response (George 2011); (Meadow and Sarsfield 1981); (Jahan, Hanif et al. 2011); (Cheung, Wei et al. 2004). There have been renal effects found in children as a result of low-level of exposure to cadmium, lead, mercury, and arsenic, however, the role of these

exposures in NS has not been explicitly identified (de Burbure, Buchet et al. 2006). There is some literature describing the associations between childhood NS and socio-demographic factors, but they are mostly cross-sectional studies or prospective studies with limited follow-up, thus restricting our understanding of the determinants of health for children with NS (Vance, Fazan et al. 1980, Vance and Pless 1983); (Naidoo, Moodley et al. 1987); (Soliday and Lande 2002, Soliday, Moore et al. 2002); (Hall, Thorley et al. 2003); (Ruth, Landolt et al. 2004); (Guha, De et al. 2009); (Mitra and Banerjee 2011). As a result, there is a significant gap in the literature on the role of environmental and socio-demographic modifiers of NS in children in the long-term. Socio-demographic factors such as economic status, child quality of life and parental well-being, environmental factors such as exposures to lead or heavy metals, and serological modifiers, clinical factors such as hypertension or body mass index, or genetic factors may account for the variability in incidence and progression rates of the disease among various ethnic groups.

Ethnic origin may affect the histological expression of the diseases in children with NS (Eddy and Symons 2003). Chanchlani et al recently reported that incidence and response to treatment of NS varies by ethnicity (Chanchlani R 2016). Bhimma et al reported that there are distinct differences in the distribution of the various forms of NS within the various population groups in Durban, KwaZulu-Natal (Bhimma, Coovadia et al. 1997). Whilst the disease in the Indian children was shown to parallel that seen in developed countries, with minimal-change disease predominating, the pattern of disease was very different in the black children. Black children had a paucity of minimal-change NS with only 6.8% being considered to have typical steroid-sensitive minimal-change disease. The commonest form of NS seen in black children until a few years after the introduction of the hepatitis B virus vaccine into the Expanded Programme for Immunization in Childhood was Hepatitis B virus-associated membranous nephropathy, which accounted for 86% of cases of NS in black children in Durban, South Africa (Bhimma, Coovadia et al. 1997).

Table 1.2 Observational studies in Children with Nephrotic Syndrome

Author	Year	N	Ethnicity	Mean age at Onset± SD, yrs (Range)	Male (%)	Mean follow-up time± SD, yrs (Range)	Steroid Resistant (%)
Inguli	1991	177	Black and Hispanic	7.3 ± 4.6 (1.0-16.75)	No data	8.25 ± 4.3 (1-15)	15.3%
		65	Caucasian	7.8 ± 4.8 (2-14.8)		8.8 ± 4.1 (2-14.8)	6.2%
Bircan	2002	132	Turkish	4.9 ± 3.56 (1-15)	61.2%	3.4 ± 2.31 (1-6)	13.2%
Ozkaya	2002	392	Turkish	4.6 ± 3.4	59.2%	2 (0.9-16) ^a	23% ^b
Kim	2005	103	Caucasian	4.3 ± 3.5	51%	No data	3.6%
		96	African American	8.2 ± 5.2		No data	11%
Bhimma	2008	816	Black and Indian	4.8 (1.2-16)	60.4%	2.5 (0.1-16.5)	27.3%
Chang	2009	99	Chinese	8.35 ± 4.61 (2-18)	73.7%	5.06 ± 4.35	N/A ^e
Mubarak	2009	538	Pakistani	9.79 ± 4.59 (0.8-18) ^a	64.4%	No data	31.1%
Otukesh	2009	73	Iranian	5.9	52.0%	6.0 ± 4.2 (0.5-16)	100%
Copelovitch	2010	112	Cambodian	8.95 (0.6-15.75)	63.4%	15.1 (3.7-2.73)	16.25%

Banaszak	2012	76	Caucasian (Poland) ^c	2.7 (median)	54.5%	No data	15.8%
		102	Caucasian (Poland) ^d	3.3 (median)	68%	No data	31.3%

Prospective studies

Kumar	2003	290	Northern and Eastern Indian	7.9 ± 5.1	73.4%	No data	38% ^b
Wong (Registry)	2007	49	New Zealand European	6.1 ± 3.8	71.4%	1	19.6%
			Maori, Pacific Islander, Asian				
			Other				
Bakkali (Registry)	2011	231	No data (Netherlands)	5.08	67.1%	4	N/A ^e

^a indicates age of study group, where age at onset not specified

^b estimated based on participants diagnosed with FSGS

^c indicated patients treated with NS between 1986-1995

^d indicated patients treated with NS between 1996-2005

^e no steroid resistance reported

1.4 Pathogenesis

The pathogenesis of NS, in the context of observed immunological dysfunction, and its relationship with proteins of the slit diaphragm and the podocyte cytoskeleton, is unclear. A unifying hypothesis that combines the immunological perturbations with abnormal function at the level of the slit diaphragm is lacking. Figure. 1.10 summarizes the current view on pathogenesis (Sinha and Bagga 2012).

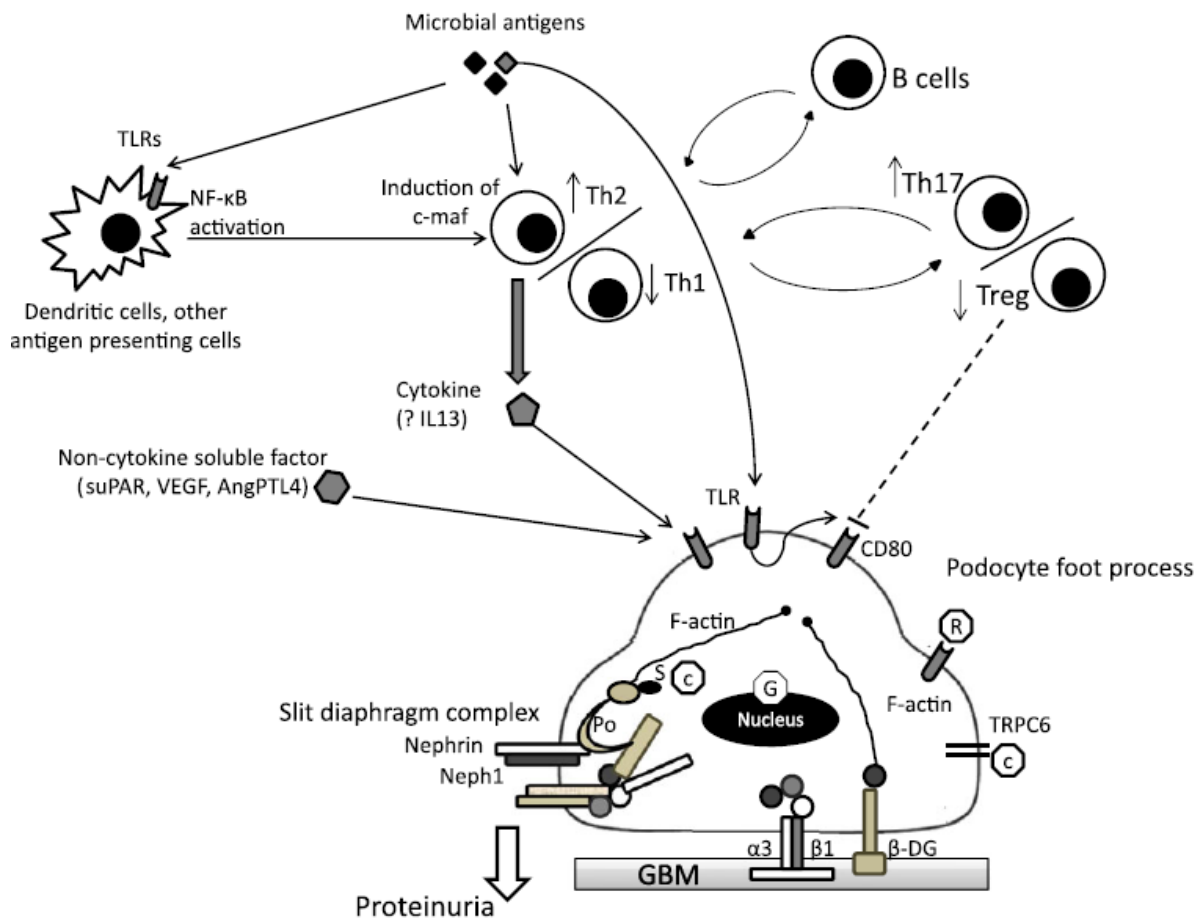


Figure. 1.10 Pathogenesis of idiopathic nephrotic syndrome. Schematic view of podocyte foot process demonstrates its components of the slit diaphragm complex, formed by nephrin, Neph1 and podocin (Po), and the actin cytoskeleton, which also receives inputs from the basolateral domain of the foot process, containing the $\alpha3\beta1$ integrins and α and β dystroglycans (Adapted from (Sinha 2012)).

The dominant paradigm in NS is an imbalance between T helper 1 (Th1) and T helper 2 (Th2) cytokines, and that a soluble mediator, presumably IL-13, increases glomerular permeability. Other molecules suggested as mediators include soluble urokinase plasminogen activator receptor (suPAR), vascular endothelial growth factor (VEGF), and angiotensin-like-4 (ANGPTL4). Signaling through nuclear factor kappa B (NF- κ B) and toll like receptors (TLRs) mediated pathways may polarize adaptive immune responses towards Th2 cells, or directly increase CD80 expression in podocytes.

An imbalance of Th17 and T regulatory responses allows persistent CD80 activation on podocytes, and/or helper responses. Finally, therapeutic agents have direct effects on podocytes; glucocorticoids (G) on gene expression, calcineurin inhibitors (C) on stabilization of synaptopodin (S) or inhibition of TRPC6 channel, and binding of rituximab (R) to the SMPDL-3b protein. These findings demonstrated the importance of the podocyte in the pathogenesis of NS, which led to the proposal that most cases of NS are caused by podocyte defects. Therefore this defect was termed 'podocytopathies' (Wiggins 2007).

More recently single gene mutations affecting podocyte differentiation and function have been described, particularly in steroid resistant disease, predicting unresponsiveness to immunosuppressive therapy. SRNS may be caused by mutations in genes that encode proteins that play key roles in maintaining podocyte ultrastructure (Niaudet 2013).

1.5 Genetics of Nephrotic Syndrome

Genetic mutations are present in 10-20% of patients with SRNS, and in a higher proportion of patients with familial NS (13%) for autosomal recessive and 30% in autosomal dominant NS (Gigante, Piemontese et al. 2011); (Lipska, Balasz-Chmielewska et al. 2013). The age of

disease onset is an important predictor of the odds of findings an abnormality in a particular gene linked to SRNS. To date there are more than 30 genes that have been associated with hereditary SRNS (McCarthy, Bierzynska et al. 2013). In 1998, Kestila *et al.* (Kestila, Lenkkeri et al. 1998) discovered that the *NPHS1* gene, encoding the podocytic protein nephrin, is mutated in the Finnish type of congenital NS (CNS). This finding was the first proof of concept for the heredity of childhood NS. In 2007, Hinkes *et al.* (Hinkes, Mucha et al. 2007) found that mutations in *NPHS1*, *NPHS2* (encoding podocin), *WT1* (exons 8 and 9) and *LAMB1* (encoding laminin- β 2) are causes of two thirds of cases of NS with onset in the first year of life. Mutational analysis of seven podocyte genes (*NPHS1*, *NPHS2*, *WT1*, *CD2AP*, *ACTN4*, *TRPC6* and *PLCE1*) in 19 non-familial childhood-onset, steroid resistant, biopsy-proven FSGS patients revealed variants of *NPHS1*, *NPHS2*, *WT1* and *CD2AP* that could be the cause of the disease in four subjects (21%). In addition, these results have suggested the role of combinations of genetic variants (biogenic heterozygosity) in the pathogenesis of steroid-resistant FSGS (Lowik, Levtchenko et al. 2008);(Santin, Bullich et al. 2011) proved the clinical utility of a genetic testing algorithm, for SRNS occurring before adolescence, based on an analysis of *NPHS1*, *NPHS2*, *WT1* and *PLCE1* genes. On the other hand, Hasselbacher *et al.* (Hasselbacher, Wiggins et al. 2006) and Matejas *et al.* (Matejas, Hinkes et al. 2010) indicated that analysis of the *LAMB1* gene, which is mutated in Pierson syndrome (OMIM #609049), could be included in the diagnostics of early onset NS with absence of extra renal abnormalities (Table 1.2).

The *NPHS1* gene that encodes for nephrin was identified as the causative gene in the most common type of CNS viz. CNS of the Finnish-type (CNF) (Kestila, Lenkkeri et al. 1998). CNS of the Finnish type is an autosomal recessive disorder and is the most common type of CNS found in Finland and other parts of the world (Bolk, Puffenberger et al. 1999). The renal histology of CNS ranges from microcystic dilatation of tubules to minimal change disease, FSGS and diffuse mesangial sclerosis (Hinkes, Mucha et al. 2007); (Santin, Garcia-Maset et al. 2009). *NPHS1* mutations has also been reported in childhood (Philippe, Nevo et al. 2008) and adult-onset SRNS (Santin, Garcia-Maset et al. 2009).

The *NPHS2* gene encodes for podocin, an integral membrane protein found exclusively in glomerular podocytes. Podocin is a 383-amino acid protein of approximately 42kD a lipid-raft-associated protein localized at the slit diaphragm, where it is required for the structural organization and regulation of the glomerular filtration barrier. *NPHS2* encodes an integral membrane protein with one transmembrane domain (105–121 aa) and a 262-amino-acid carboxy-terminal cytoplasmic tail. The transmembrane domain and most of the cytoplasmic tail are homologous to the corresponding regions of the stomatin family proteins. Podocin, however, extends beyond the stomatin-like sequences at both the N and C termini (97 aa and 36 aa, respectively), which show no homology with any known protein sequence (Boute, Gribouval et al. 2000). It is a membrane-anchored protein of the stomatin family with a predicted hairpin-like structure, such that both ends of the protein are located in the cytoplasm (Roselli, Gribouval et al. 2002). Podocin was shown to accumulate in an oligomeric form in lipid raft microdomains and to localize to the slit diaphragm area (Roselli, Gribouval et al. 2002), where it interacts with other slit diaphragm components, such as nephrin and *CD2AP* (Schwarz, Simons et al. 2001); (Huber, Kottgen et al. 2001). Homozygous and compound heterozygous mutations of this gene are most frequently found in children with familial SRNS and less commonly with sporadic SRNS. To date, more than 50 *NPHS2* mutations have been reported in both familial and

sporadic cases of SRNS (Roselli, Moutkine et al. 2004); (Caridi, Bertelli et al. 2003); (Cybulsky, Takano et al. 2009); (Ohashi, Uchida et al. 2003); (Ye, Dhillon et al. 2001); (You, Huo et al. 2008). Half are missense mutations spread over the entire length of the coding sequence of the gene, and the rest are nonsense, protein truncating mutations, insertions, and deletions. Mutations in genes that encode key podocyte and slit diaphragm proteins are implicated in the pathogenesis of SRNS (Hinkes, Mucha et al. 2007). *NPHS2* mutations are present in 10–55% of patients with sporadic and familial SRNS of European ancestry (Hinkes, Mucha et al. 2007); (Karle, Uetz et al. 2002); (Weber, Gribouval et al. 2004); (Ruf, Lichtenberger et al. 2004); (Caridi, Bertelli et al. 2001); (Berdeli, Mir et al. 2007); (Frishberg, Rinat et al. 2002); (Yu, Ding et al. 2005). There is a strong regional bias with 10 to 30 percent of cases of sporadic SRNS in children in the middle east and Europe having this mutation (Weber, Gribouval et al. 2004); (Caridi, Bertelli et al. 2001); (Karle, Uetz et al. 2002); (Frishberg, Rinat et al. 2002); (Caridi, Bertelli et al. 2003); (Berdeli, Mir et al. 2007), whereas the frequency of mutations is low in African-Americans (Chernin, Heeringa et al. 2008). On the other hand, inter-ethnic variations are striking and only a small proportion of Israeli Jewish and Asians show similar mutations (Yu, Ding et al. 2005); (Maruyama, Iijima et al. 2003); (Cho, Lee et al. 2008). Children with *NPHS2* mutations have early onset of disease, rapid progression to ESKD and associated cardiac defects in some cases (Frishberg, Feinstein et al. 2006).

Most patients with two *NPHS2* pathogenic mutations develop NS before the age of six years, present mostly with FSGS, do not respond to immunosuppressive therapy, reach ESKD before the first decade of life, and have a minimal risk for recurrence of FSGS after kidney transplantation (Caridi, Bertelli et al. 2001); (Karle, Uetz et al. 2002); (Hinkes, Vlangos et al. 2008).

The *NPHS2* gene mutations have also been identified in 51% of CNS cases of European origin and also in adult onset forms of FSGS (Hinkes, Mucha et al. 2007); (Tsukaguchi, Sudhakar et al. 2002) The incidence of *NPHS2* gene mutations in familial SRNS was found to be 40% in European and American children, 29% in Turkish and 0% in Japanese and Korean children (Lowik, Groenen et al. 2009).

Mutations in the *NPHS2* gene cause an autosomal recessive form of SRNS with an early onset of the disease and renal histology of FSGS (Boute, Gribouval et al. 2000). The *NPHS2* gene mutations have also been identified in 51% of CNS cases of European origin and in adult onset forms of FSGS (Hinkes, Mucha et al. 2007); (Tsukaguchi, Sudhakar et al. 2002).

Hinkes *et al* (Hinkes, Mucha et al. 2007) conducted an impressive multinational collaboration, on 430 patients with SRNS, the vast majority of whom were the only affected family member, although the series did include 23 families with more than one affected member. The patients were screened for mutations in *NPHS2* by direct sequencing of all eight exons of the gene. Eighty-two patients (19% of the total) had mutations in *NPHS2*. In the families with more than one affected member, the proportion with *NPHS2* mutations rose to 39%. In patients with two *NPHS2* mutations, the authors report that approximately 40% had one truncating (frameshift or nonsense) mutation and an additional 30% had homozygous *R1308Q* mutations (the “founder” *NPHS2* mutation identified by Boute *et al*) (Boute, Gribouval et al. 2000). These two groups of individuals nearly all developed NS at an early age (6 yr, with a mean age of onset 2 yr). The remaining 30% of patients with other mutations or variants in *NPHS2* had later onset disease without any further specific link between any given genotype and age of onset (although the numbers of patients with each genotype were small). Mutation type did not affect rate of deterioration, time from onset to ESKD being the same in all groups. Although this represents real progress, even within the groups with early presentation there was still a wide range of age of onset. Also, 80% of the collection with steroid-responsive NS did not have any abnormality of

NPHS2, so their proteinuria remains unexplained; clearly, there is more work to be done. The power of large multinational studies such as this one will be essential if analyses of genotype–phenotype relationships in NS are to yield informative conclusions. Ideally, genetic analysis should be more widely available as a diagnostic and prognostic aid in patients presenting with NS; however, at present, clinicians will need further guidance from geneticists about the interpretation of genotype–phenotype relationships.

The polymorphism R229Q is one of the most commonly reported podocin sequence variations. The arginine residue at protein position 229 is highly conserved across species, and arginine-to-glutamine substitution R229Q has been reported to alter functional properties of podocin *in vitro* and possibly *in vivo* (Zhang, Marlier et al. 2004). It has been found repeatedly with slightly increased frequency in SRNS compared to healthy controls (Weber, Gribouval et al. 2004); (Franceschini, North et al. 2006). On the other hand, subsequent studies reported similar frequencies of this polymorphism in SRNS and in normal subjects (5.13 and 3.75 %, respectively) (Ruf, Lichtenberger et al. 2004). Further, the influence of the R229Q functional variant on microalbuminuria was investigated. First, the R229Q functional variant was associated with microalbuminuria in the general population (Pereira, Pereira et al. 2004). In the second study, the R229Q variant did not appear to alter the risk of proteinuria in general population and in a diabetic population (Tonna, Needham et al. 2008). It is important to understand the role of the R229Q function in ESRD that will help us understand aspects of the podocyte and glomerular physiology and also the role in disease prediction and prevention.

APOL1 and *MYH9* genes are located on chromosome 22. *APOL1* gene encodes a secreted high-density lipoprotein that binds to apolipoprotein A-I. Apolipoprotein A-I is a relatively abundant plasma protein and is the major apoprotein of HDL (High Density Lipoprotein). The 1000 Genomes Project database led to two distinct sets of variants in the *APOL1* gene, termed

alleles 'G1' and 'G2', being discovered as the main cause of the association of the 22q12.3 region with end-stage renal disease in African Americans, as well as the underlying cause of all the associations originally reported in 2008 (Genovese, Friedman et al. 2010); (Tzur, Rosset et al. 2010). Both the G1 and G2 alleles alter the amino acid sequence of the APOL1 protein. Notably, the altered protein product confers innate resistance against *Trypanosoma brucei rhodesiense*, a sub-Saharan parasite that causes African trypanosomiasis (or 'sleeping sickness'), thus providing a plausible biologic explanation for the high frequency of these alleles in individuals of African descent.

Mapping by admixture linkage disequilibrium (MALD) localized an interval on chromosome 22, in a region that includes the *MYH9* gene, which was shown to contain African ancestry risk variants associated with certain forms of ESKD (Kao, Klag et al. 2008); (Kopp, Smith et al. 2008). *MYH9* encodes nonmuscle myosin heavy chain IIa, a major cytoskeletal nanomotor protein expressed in many cell types, including podocyte cells of the renal glomeruli. Dense mapping of *MYH9* identified individual single nucleotide polymorphisms (SNPs) and sets of such SNPs grouped as haplotypes that were found to be highly associated with a large and important group of ESKD risk phenotypes, which as a consequence were designated as *MYH9*-associated nephropathies (Bostrom and Freedman 2010). These included HIV-associated nephropathy (HIVAN), primary nonmonogenic forms of FSGS and hypertension affiliated chronic kidney disease not attributed to other etiologies (Bostrom and Freedman 2010). The *MYH9* SNP and haplotype associations observed with these forms of ESKD yielded the largest odds ratios (OR) reported to date for the association of common variants with common disease risk (Winkler, Nelson et al. 2010). Two specific *MYH9* variants (rs5750250 of S-haplotype and rs11912763 of F-haplotype) were designated as most strongly predictive on the basis of Receiver Operating Characteristic analysis (Nelson, Freedman et al. 2010). However, despite intensive efforts including re-sequencing of the *MYH9* gene no suggested functional mutation

has been identified (Nelson, Freedman et al. 2010); (Winkler, Nelson et al. 2010). This led to Winkler *et al* re-examining the interval surrounding *MYH9* and to the detection of novel missense mutations with predicted functional effects in the neighbouring *APOL1* gene, which are significantly more associated with ESKD than all previously reported SNPs in *MYH9*.

To date there has been one published report by Kasembali *et al* (Kasembeli, Duarte et al. 2015) in HIV associated nephropathy in Black South Africans in adult patients. This study showed a strong association of *APOL1* variants and biopsy-confirmed HIVAN driven by interaction between *APOL1* and untreated HIV infection. Qulu *et al* (Qulu 2016) at my institution undertook a study to determine the role of genetic variants at the *APOL1* locus in the development of FSGS in South African children with idiopathic and HIVAN. For idiopathic FSGS the odds of carrying two copies of G1 and/or G2 risk alleles were 2.4(95%CI 0.26 to 30, $p=0.38$) and Inf = 0.03 ($p=0.45$) for the HIV-associated FSGS in a recessive model. For HIV-associated FSGS, the allele frequencies were 10% for the G1 risk allele and 10% for the G2 deletion and 7% of the HIV with FSGS population carried high-risk genotypes compared to the controls (0%). A majority of the HIV-associated FSGS (71.4%) showed absence of the *APOL1* G1 and G2 risk alleles whilst forty-seven percent of patients with idiopathic FSGS (HIV-) showed absence of the *APOL1* G1 and G2 risk alleles. This study demonstrated that *APOL1* risk variants are not predictors of biopsy proven HIV associated FSGS and idiopathic FSGS in the paediatric Black South African population from the KwaZulu-Natal region of South Africa.

Aim

The aim of our study was to determine the existence of mutations in the *NPHS2* gene in Black and Indian children with SRNS in KwaZulu-Natal, South Africa, using DNA genotyping and to identify any other candidate genes that may play a role in the genetics of SRNS.

Chapter two - Methods and Results

Title: A Single *NPHS2* Mutation Accounts for 27% of Steroid Resistant Nephrotic Syndrome in Black South African Children

2.1 Introduction

Nephrotic syndrome (NS), an important kidney disease in children, is the consequence of damage to the glomerular filtration barrier leading to significant proteinuria, hypoalbuminemia, hyperlipidemia, and edema. NS is classified by its response to steroid therapy as steroid sensitive (SSNS) or steroid resistant (SRNS) nephrotic syndrome.¹ Although the majority of children with idiopathic NS respond to glucocorticoids and have a favorable prognosis, approximately 20% are SR, which is associated with repeated hospital admissions, treatment with immunosuppressive drugs with risk for adverse events, and often progressive loss of kidney function.² Among children with idiopathic NS, the most common histological diagnosis in Black children in South Africa (SA) is focal segmental glomerulosclerosis (FSGS) while minimal change disease (MCD) is more frequently diagnosed in White and Indian children.³⁻⁵ Indian children living in South Africa with SRNS respond better to oral cyclophosphamide treatment than Black children, who are less likely to achieve complete remission (69% vs. 20% in Indians and Blacks, respectively).^{6,7} The basis for these disparities has not been explained, but has been hypothesized to be due in part to genetic differences predisposing to SR-FSGS.⁸

Among the many mutations in over 30 genes causing SRNS in families or associated with sporadic SRNS, mutations in *NPHS2* represent the most frequent cause in children.⁹⁻¹² In one worldwide study of 430 families with NS, 18% were homozygous or compound heterozygous for deleterious *NPHS2* mutations; of these the most common mutation was the European founder R138Q mutations, found in 7% of the families.¹³ Among children with SRNS and congenital NS

enrolled in the PodoNet registry cohort recessive mutations in *NPHS2* were identified 138/1088 (12.6%) of children undergoing targeted mutational analysis; however, only 3 parents reported African ancestry.¹⁴ There are few reports of causative mutations in *NPHS2* among African ancestry children in the USA with FSGS, possibly reflecting fewer children enrolled in studies or the absence of *NPHS2* founder mutations.^{14,15,16}

NPHS2 encodes podocin, a member of the stomatin protein family, which is a transmembrane protein that recruits nephrin to lipid rafts in the plasma membrane. Podocin is essential for the maintenance of the podocyte ultrastructure and the podocyte slit diaphragm.¹⁷⁻²⁰ Protein altering mutations in *NPHS2* weaken or abrogate the podocin-nephrin interaction at the plasma membrane leading to a failure of the kidney filtration barrier and resultant SRNS. Truncating (R138X) and certain missense mutation (e.g. R138Q and V260E) are associated with SRNS with an earlier age of onset, generally before the age of 6 years.^{13,21}

Genetic coding variants in *APOL1*, encoding apolipoprotein L1, are recessively associated with FSGS, with a median age of onset of 35 years, and with chronic kidney disease in persons of sub-Saharan African ancestry.^{22,23} *APOL1* variants are present only on African-ancestry haplotypes and restore the ability of APOL1 protein to kill *T.b. rhodensiense*, the causative agent of acute African human trypanosomiasis.²² In an African American pediatric study of chronic kidney disease, carriage of high-risk genotypes was associated with a lower estimated glomerular filtration rate (eGFR) at study entry and with FSGS histology and were enriched in children with NS.²⁴ The association of *APOL1* with NS in children in sub-Saharan Africa has not been investigated.

The genetic basis for the racial disparity in SRNS and FSGS histology of children with NS from sub-Saharan Africa is unknown, and no genetic studies have been reported to date. Considering the increased risk of Black children for SRNS in South Africa, we hypothesized that *APOL1* renal risk variants, which are found only on African ancestry haplotypes, or founder mutations in *NPHS2*, might be responsible for the higher rate of SR in Black children compared to Indian or White South African children with NS.

2.2 Materials and Methods

2.2.1 Subjects

Unrelated Indian and Black children (n=64) with sporadic SRNS (n=49) or SSNS (n=15) treated between January 2005 to December 2011 at two referral hospitals in Durban, KwaZulu-Natal Province, South Africa, were invited to participate in the study. The majority of black Africans in KwaZulu-Natal Province identify as Zulu (>95%).

2.2.1 Inclusion criteria

All children with primary NS were given oral prednisone (2mg/kg, maximum 60 mg) for 6 weeks followed by the same dose on alternative days for another 6 weeks, reduced to none over 2-5 months. Failure to respond to oral steroids after 6 weeks was taken as SR in accordance with standard criteria.^{2,25} Second line treatment included oral cyclophosphamide (2mg/kg with a maximum dose of 100 mg daily) given as a daily dose for 8-12 weeks with angiotensin-converting enzyme inhibitor (0.5mg/kg, maximum 10mg) and oral prednisone (1mg/kg, maximum 60mg) given on alternative days. Children with SRNS who did not respond to oral cyclophosphamide plus low dose oral prednisone received intensive treatment with intravenous methylprednisolone (n=11) or intravenous (IV) cyclophosphamide (n=7) or both methylprednisolone and IV cyclophosphamide (n=2) or tacrolimus (n=14), or both IV

cyclophosphamide plus tacrolimus (n=2) together with low-dose prednisone on alternative days. Only children not responding to oral steroids underwent kidney biopsy. Kidney biopsies were interpreted using light microscopy, immunofluorescence and electron microscopy. Children with primary SSNS or SRNS and with an eGFR of $>60\text{ml/min}/1.73\text{m}^2$ using the modified Schwartz formula,²⁶ were eligible for entry into the study.

2.2.3 Exclusion criteria

Children with eGFR <60 at diagnosis were excluded because they were not given immunosuppression therapy. Other exclusion criteria included those for whom histology was indeterminate or who were lost to follow-up or refused to participate in the study. The following tests were performed to exclude secondary forms of NS: antistreptolysin O titer (ASOT), hepatitis B and C screen, blood culture, Widal tests, Wasserman reaction, antinuclear factor, and testing for Epstein Barr virus, HIV, parvo virus and cytomegalovirus.

2.2.4 Control group

Black (n=55) and Indian (n=49) healthy blood donors were enrolled as control groups. Proteinuric remission was defined as a protein to creatinine ratio (PCR) <0.2 mg/mg and serum albumin $>30\text{g/dL}$; partial remission was defined as a $\text{PCR}<1.9$ mg/mg and serum albumin $>25\text{g/dL}$, but not meeting full remission criteria. For replication of *NPHS2* results, we enrolled an additional 20 Black sporadic SRNS cases with biopsy-proven primary FSGS, and 18 age-matched Black controls with no serological evidence of HIV infection, with normal kidney function, and no proteinuria.

2.2.5 Informed Consent

Written informed consent was obtained from the parents of children, and children over 7 years assented to be in the study. The Biomedical Research Ethics Committee of the University of

KwaZulu-Natal, SA and the National Cancer Institute, NIH, USA approved this study. (In Addendum attached)

2.2.6 Sequencing and genotyping

APOL1 SNPs defining G1 (rs73885319 and rs60910145) and G2 (rs717185313) were genotyped by ABI Custom TaqMan SNP Genotyping Assays (ABI, Foster City, California). *NPHS2* Exons 1 through 8 were Sanger sequenced using the ABI 3700 Analyzer under standard conditions. Primer sequences were from Boute et al.,⁹ with an additional primer pair in genomic regions flanking *NPHS2* exon 1 (primers in Supplementary Appendix). To determine relatedness and the age of the *NPHS2* V260E mutation, DNA from 9/14 V260E homozygous patients (those with remaining DNA) and 71 Black cases and controls who did not carry the mutation were genotyped using the Human Exome Chip (HumanExome-12 v1-2, Illumina, San Diego, CA).

2.3 Statistical and Bioinformatics Methods

We used Fisher's exact test for categorical tests for *APOL1* and *NPHS2* variants, including a burden test comparing the number of singleton *NPHS2* missense variants in cases and controls. We compared the age distributions between Indian and Black SRNS, and between Black SRNS with and without V260E homozygosity, with the Mann-Whitney test. All statistical tests and simulations were done in R (<http://www/R-project.org>).

We determined relatedness among *NPHS2* V260E homozygotes by estimating the age of the most recent common ancestor for the V260E mutation among subjects using coalescence (Supplementary Methods).²⁷ Briefly, we examined homozygosity of Chromosome 1 SNPs typed on the Illumina Exome chip 12 v1-2, by plotting heterozygous and homozygous SNPs for each subject in the region around *NPHS2*. Subjects inheriting the mutation from a recent common ancestor will have inherited identical segments of chromosome around the mutation from their

two parents, creating an extended block of homozygosity, with length determined by recombination since the common ancestor. We compared the observed lengths of homozygosity with simulation results for different numbers of generations since the common ancestor using the Mann-Whitney test. We further tested for consanguinity among V260E homozygotes by the coefficient of relatedness test implemented in PLINK.²⁸

2.4 Results

Sixty-four unrelated children with sporadic idiopathic NS were enrolled between 2005 and 2011 (Table 2.1). Mothers of the affected children reported no affected siblings and no familial history of kidney disease. SR was significantly more frequent in Black (97%) than in Indian (58%) children with NS (OR 21; 95% CI: 2.8, 960; $p=0.0002$); for children with SRNS the median age of presentation was similar for Indian (86 months) and Black children (83 months). Among 49 children with SRNS undergoing biopsy, FSGS was the most common histopathology in both Black (80%) and Indian (74%) children. Children responding to oral steroids were not biopsied.

As previous studies identified *NPHS2* autosomal recessive mutations as a major cause of familial SRNS in children, we sequenced *NPHS2* exons in SSNS and SRNS cases and controls. Supplementary Table 1 lists the *NPHS2* variants identified in Indian and Black cases and controls. We observed seven missense variants: P20L, G42R, A61V, R229Q, A242V, and V260E, all of which have been previously reported, and a novel variant, P369S, which has not been reported in the 1000 genomes database or other public databases and is predicted to be benign. *NPHS2* V260E (Hg19 coordinate,1:179523626 A/T) is a known pathogenic mutation in the homozygous state previously observed in several families from regions of the former Omni empire.¹¹ The *NPHS2* V260E mutation disrupts podocin trafficking to the podocyte plasma membrane by producing a protein that is retained in the endoplasmic reticulum.¹⁹ *NPHS2* R229Q is a known pathogenic polymorphism for FSGS, but only in compound heterozygosity with

certain other *NPHS2* pathogenic mutations in trans configuration.²⁹ Other than *NPHS2* V260E, no missense variants were observed in the homozygous or compound heterozygous state in Black or Indian SSNS or SRNS cases. *NPHS2* mutations in the heterozygous state are not predicted to cause disease, and gene burden tests for these variants did not reveal an excess of variants in SSNS or SRNS cases versus controls ($p > 0.5$).

Notably, the *NPHS2* V260E mutation was present in the homozygous state in 8 of 30 (27%) Black SRNS cases; V260E homozygosity was specifically associated with biopsy-confirmed FSGS (Table 2.2), accounting for 33% of children (8/24) with SR-FSGS. *NPHS2* V260E homozygosity was observed only among Black children with SRNS. One Black child in the control group was heterozygous for *NPHS2* V260E; the variant was not observed in any of the Indian NS cases or controls (Tables 2.2 and 2.3).

To replicate the V260E/E association with SR-FSGS, we sequenced DNA from a second group of 20 unrelated Black children with SR-FSGS, and 18 race- and age-matched controls. Of the children with SR-FSGS, 6/20 (30%) were homozygous for V260E whereas none of the controls in this cohort carried the mutation. Combining the discovery and replication SR-FSGS cases, V260E homozygosity accounted for 14/44 (32%) of Black children with SR-FSGS, $P < 10^{-6}$. (Table 2.3). Black SR-FSGS cases homozygous for V260E had an earlier age of onset compared to SRNS cases homozygous for the V260 allele (median age 34 months versus 78 months, respectively, $p = 0.009$) (Figure 2.1). The deleterious mutation was found in the heterozygote state in 1/73 discovery and replication controls, for a nominal observed population allele frequency of 0.7%, (95% binomial CI: 0.02%, 4%) (Table 2.3) indicating that this variant is modestly rare in this southern African population. The ExAC Browser (exac.broadinstitute.org) reports a population frequency of 0.0001926 ($< 1/10,000$) in Africans and 0 in Asians, Europeans, Latinos, and South Asians.

2.4.1 Response to therapy

We measured response to therapy by urinary protein-creatinine ratio (uPCR) and serum albumin levels in children with SRNS. No subjects homozygous for V260 E (N = 14) had either full or partial response to additional immunosuppressive therapy (Table 2.4). In contrast, among the 32 subjects homozygous for the V260 reference allele, 9 had complete remission, 6 had partial remission, and 17 had no treatment response. Six children carrying 260E/E were further treated with tacrolimus (n=3) or intravenous cyclophosphamide (n=3), but none showed complete or partial remission. The differences in treatment response between *NPHS2* 260 EE and 260VV were statistically significant for complete remission (p=0.04) and combined complete and partial remission, p=0.002.

Eight Black children, all with FSGS and five of whom were homozygous for *NPHS2* V260E, progressed to end stage kidney disease (eGFR<15 mL/min/1.73m²).

2.4.2 Founder effect and age of variant

The association of *NPHS2* V260E with familial SR-FSGS suggested that our subjects might have cryptic consanguinity by descent from a recent common ancestor. Inbreeding coefficient tests implemented in PLINK showed no evidence of very recent genetic relatedness among the nine children; however, we considered that a moderately recent founder effect would cause a smaller degree of consanguinity that would be revealed by extended regions of homozygosity surrounding *NPHS2*. We determined extent of homozygosity by genotyping 9 individuals—all of the individuals with sufficient DNA remaining—carrying two copies of *NPHS2* V260E, and 71 individual lacking the mutation, using the Illumina Exome array, which provides good coverage of this region. Two of the 9 V260E homozygous children had stretches of homozygosity around *NPHS2* of 13 and 14 Mb, with the 7 remaining ranging from 1.9 to 3.6 Mb. Comparing all of these lengths with simulated lengths of homozygosity resulting from recombination over the number of generations since the common ancestor indicated that the time to a common

ancestor was unlikely to be less than 14 generations ($p < 0.05$). However, it is likely that the longer lengths of homozygosity are due to a more recent common ancestor of the parents of these children, and that the shorter lengths of homozygosity are more indicative of the time to the overall common ancestor; analysis limited to these individuals yielded 31 generations as the most likely number of generations since the overall common ancestor, 95% CI 19,47.

We also tested for association between *APOL1* and NS. No Indian cases or controls carried either the G1 or G2 variant. Among the Black controls, the allele frequencies for the G1 and G2 alleles were 8.9% and 10.1%, respectively. The *APOL1* association is largely recessive, with carriage of two risk alleles (G1/G2, G1/G1, and G1/G2) required for *APOL1*-associated FSGS. Limiting analysis to individuals not carrying two copies of *NPHS2* V260E, 2 of 73 controls and 1 of 30 FSGS cases carried two *APOL1* risk alleles, OR = 1.2, (95% CI 0.02, 24.3), $p = 1$, Fisher's exact test (Supplementary Table 2).

2.5 Discussion

We have shown that one pathogenic recessive mutation, *NPHS2* V260E, accounted for 27% of SRNS and 32% of SR-FSGS among unrelated Black children presenting with NS. This was an unexpected result since published data suggest that *NPHS2* mutations are identified in <15% of children with sporadic FSGS and rarely in black children; further, it was unexpected that a single variant would account for approximately 30% of SR-FSGS.^{15,16} Children homozygous for *NPHS2* V260E were steroid resistant with FSGS, did not respond to intensive treatment, and developed disease at a younger age compared to non-carriers, consistent with the severity of disease previously reported for the *NPHS2* V260E pathogenic mutation.¹¹ *NPHS2* V260E, like the European founder mutation, *NPHS2* R138Q, disrupts the trafficking of the altered podocin protein to the plasma membrane by its retention in the endoplasmic reticulum. Nephron localization is essential for recruitment of nephrin to lipid rafts and structural integrity of the podocyte slit diaphragm.^{18,19}

To elucidate the history of this mutation and to determine if consanguinity accounted for the high rate of V260E homozygosity, we genotyped 9 subjects, those with sufficient remaining DNA and homozygous for V260E, and 71 Black cases and controls without the mutation, with a genome-wide array. Overall, parents of children carrying the V260E mutation showed no sign of recent consanguinity, but rather showed evidence, in the length of extended homozygous segments around *NPHS2*, of descent from a common ancestor 31 generations removed from the present, indicating that the high frequency of this variant in SR-FSGS is not due to a recent common ancestor, and suggesting a moderately rare but widespread presence on chromosomes among the Black Zulu population.

V260E resembles the founder nonsense mutation *NPHS2* R138X, which was identified in SRNS patients from two consanguineous kindreds of Israeli-Arab origin. The mutation was also found in 6/18 children from unrelated, but highly inbred families, all of whom shared the same

haplotype.³⁰ However, unlike Israeli-Arabs in the prior study, consanguineous relationships are uncommon among Zulu and the affected children did not share haplotypes from a recent common ancestor. These studies, and others, affirm the importance of sequencing efforts to detect disease-causing founder variants that may account for population-specific disparities in disease prevalence, presentation, or treatment response.

Previous observations of SRNS caused by *NPHS2* V260E have been in families from regions where consanguineous marriage is practiced (i.e., Southwest Asia, Saudi Arabia or the Indian Ocean islands, with several areas associated with the Omani empire of the late 17th to 19th centuries, prompting the hypothesis that the mutation spread with travel and migrations associated with this empire.^{11,31} The Omani empire was a power on the East African coast from the 1690s to the mid-19th century, and in the early 19th century traded extensively with the African Great Lakes region, where Bantu ancestors of the Zulu population lived.³² However, the age of the mutation in the studied population suggests an appearance prior to this time and makes it more likely that it was introduced by Africans to the Omani Empire. However, it remains to be determined whether *NPHS2* V260E in our population is related to previously observed V260E, or is an independent mutation.

Our study is limited in that the number of individuals is small, and the results for the studied population, Black Africans of the Zulu ethnic group of the KwaZulu-Natal Province, may not be generalizable to other Black ethnic groups. However, the frequency of the association in our study, and the age of the mutation, suggest that the mutation may be present in a broader population that share ancestry with Zulu, a population of over 20 million.

Screening for the *NPHS2* V260E mutation has the potential to inform the differential diagnosis, prognosis, and treatment in Black African children presenting with NS. Identification of this mutation as a part of differential diagnosis would be a cost-effective alternative to kidney biopsy

in homozygous carriers, and would identify a large subset of patients who are unresponsive to immunosuppressive agents (specifically, oral steroid treatment and cyclophosphamide). This would spare children with *NPHS2* V260E homozygosity the potentially severe adverse effects of toxic agents while at the same time reducing health care costs for an overburdened health care system (Figure 2.2). In view of the poor response to immunosuppressive treatment in patients with V260E homozygosity, this group of patients should have maximal renoprotection using angiotensin converting enzyme inhibitors or angiotensin II receptor blockers. Clinical trials will be necessary to determine optimal treatment for homozygous carriers of *NPHS2* V260E and particularly responsiveness to treatment with non-glucocorticoid immunosuppressive agents.

2.6 Conclusion

In summary, the high frequency of *NPHS2* V260E homozygosity among unrelated children with sporadic SRNS makes genetic testing for this mutation in Black African children presenting with NS a potential application of precision medicine: for subjects carrying two copies of *NPHS2* V260E a simple genetic test replaces a stressful kidney biopsy and a potentially toxic course of treatment. The applicability of this approach may extend beyond the 20 million Zulu living in southern Africa, as the variant is likely to be present in other Bantu populations sharing ancestry with the. Further studies are vital to define the extent of *NPHS2* V260E in children with SRNS, and other kidney diseases, in this and related Black African populations.

2.7 Acknowledgements.

We thank the families and children for their participation in this study. We thank Elizabeth Binns-Roemer for excellent technical assistance. This project has been funded in whole or in part with Medical research Council, South Africa, National Research Foundation, Federal funds

from the National Cancer Institute, National Institutes of Health, under contract HHSN26120080001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This Research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and by NIDDK intramural Research program.

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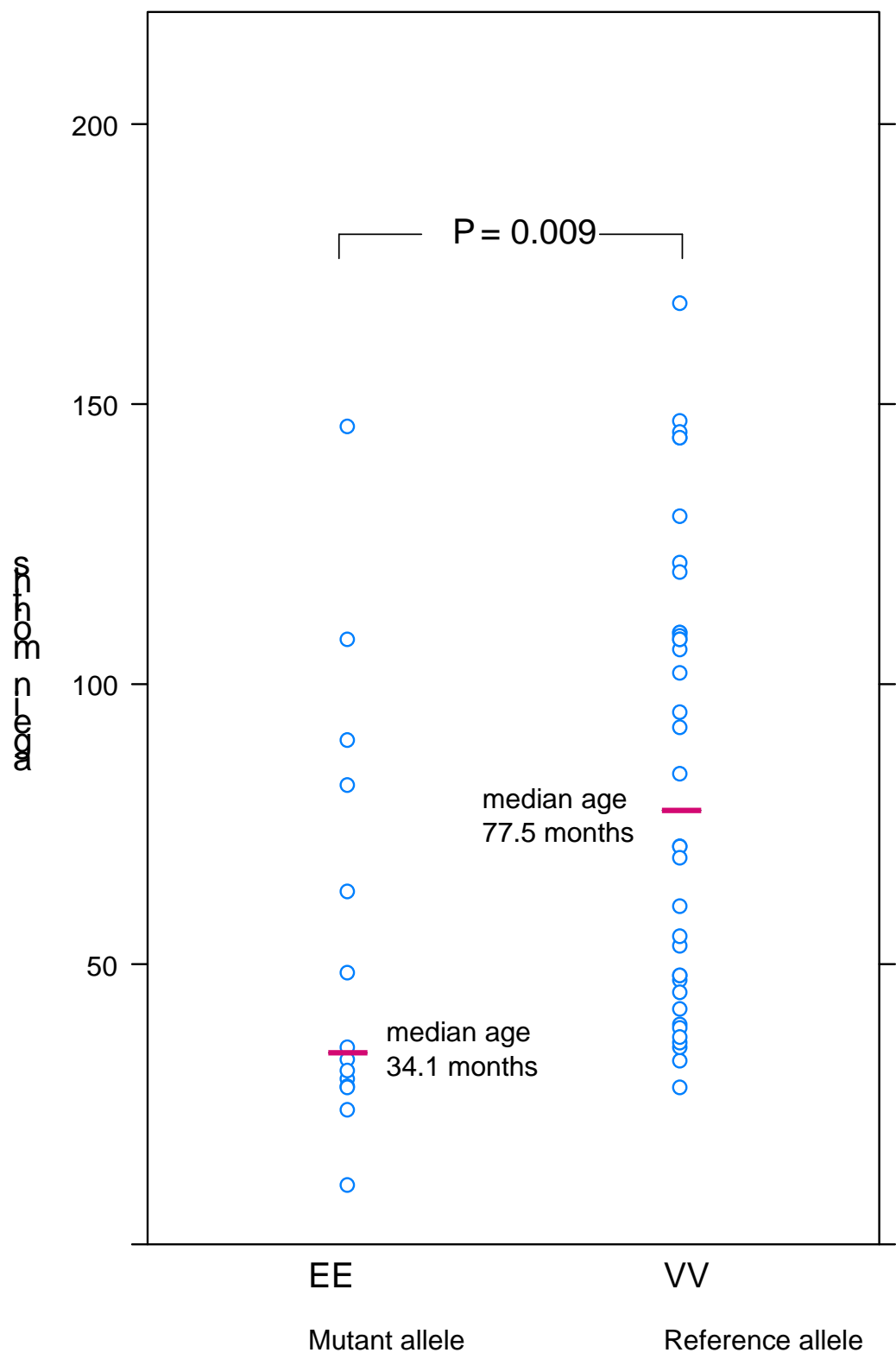


Figure 2.1. Comparison of distributions of ages of onset of steroid resistant nephrotic syndrome (SRNS) between carriers of two copies of the reference allele (N = 37) and carriers of two copies of the mutant allele (N = 14) at *NPHS2* V260E; p value from the Mann-Whitney test. There were no heterozygotes for this locus in the SRNS group. Data are from the combined discovery and the replication cohorts.

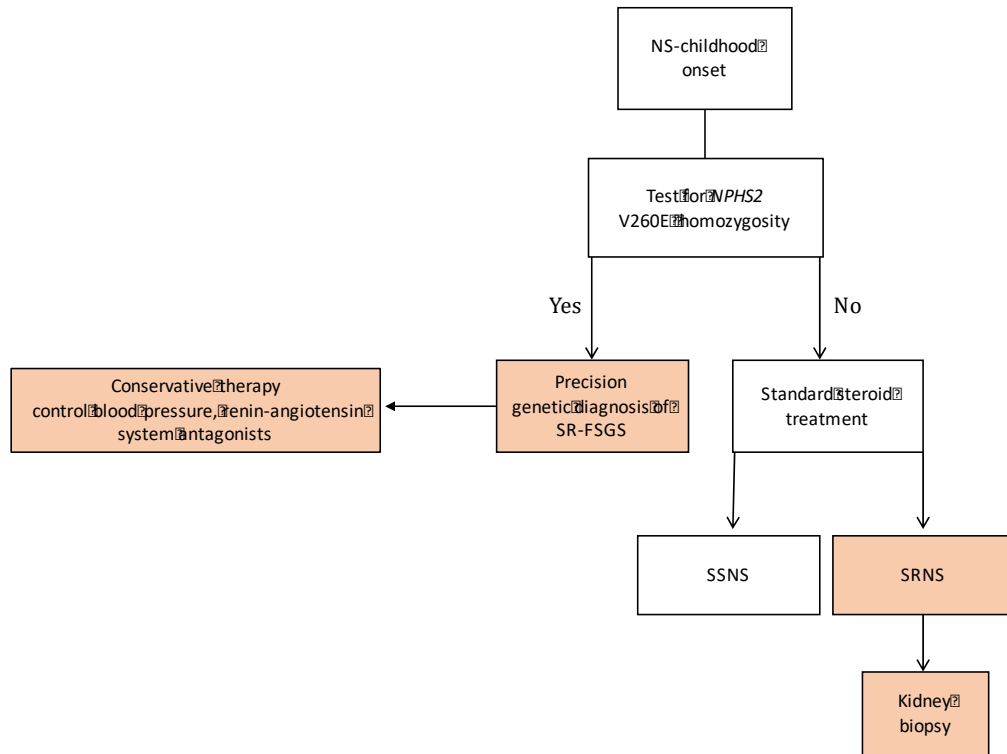


Figure 2.2: Proposed targeted genetic approach for Black African children presenting with nephrotic syndrome.

Table 2.1. Demographic and clinical characteristics of children with nephrotic syndrome in the discovery and replication cohorts.

	Discovery			Replication
Disease entities	Indian nephrotic syndrome cases N= 33 (%)	Black nephrotic syndrome cases N=31 (%)	Statistics: Black vs. Indian	Black SR-FSGS Cases N=20
Steroid sensitive nephrotic syndrome	14 (42.4)	1 (3.2)	OR 21 (2.8, 960)) p = 0.0002	0
Steroid resistant nephrotic syndrome	19 (57.6)	30(96.8)		20
Age range (months)	37-169	24-168	p = 0.34	11-149
Median age (months)	86	83		97
Males	13 (31.6)	13 (43.3)	2.8 (0.7, 11.5) p=0.14	13 (62%)
Females	6 (68.4)	17 (56.7)		7 (38%)
Histology of steroid resistant nephrotic syndrome				
Focal segmental glomerulosclerosis	14 (73.7)	24 (80.0)	1.4 (0.3-6.8), p=0.7	20 (100%)
Interstitial nephritis	1 (5.3)	2 (6.7)	ND	0
Mesangioproliferative glomerulonephritis	1 (5.3)	2 (6.7)	ND	0
Mesangial sclerosis	0	1 (3.3)	ND	0
Proliferative glomerulonephritis	0	1 (3.3)	ND	0

Glomerulonephritis	2 (10.5)	0	ND	0
Minimal change disease	1 (5.3)	0	ND	0

Shown are the numbers (percent) of steroid sensitive (SSNS) and steroid resistant nephrotic syndrome (SRNS). Black children were much more likely than South Asian Indian children to have SRNS.

Table 2.2. NPHS2 V260E in Black and Indian SSNS and SRNS with nephrotic syndrome in the discovery cohort.

	Black cases N= 31		Indian cases N=33	
	260V/V N= 23	260E/E N=8	260V/V N=33	260E/E N=0
Steroid sensitive NS	1	0	14	0
Steroid resistant NS¹	22	8	19	0
FSGS	16 (73%)	8 (100%)	14 (74%)	0
Other histologies	6 (27%)	0	5 (26%)	0

¹ Only children who showed no remission in proteinuria after a 4 week course of corticosteroid treatment underwent renal biopsy. Shown are the number of *NPHS2* V260E genotypes in the nephrotic syndrome discovery cohort. No steroid sensitive case carried the *NPHS2* 260E mutation and the mutation was not observed in Indian cases. *NPHS2* V260E/E was specifically associated with FSGS histology.

Table 2.3 Association of *NPHS2* V260E restricted to the subset of Black children with steroid resistant FSGS from the discovery (n=24) and replication cohorts (n=20).

Discovery cohort			
<i>NPHS2</i> genotype	Controls	Steroid resistant FSGS	OR (95%CI), P_{FET}
p. 260 V/V	54 (98%)	16 (67%)	Reference
p. 260 E/V	1 (2%)	0	Not significant
p. 260 E/E	0	8 (33%)	Infinite (4.9, infinite) 3 x 10 ⁻⁵
Replication cohort			
p. 260 V/V	18 (100%)	14 (70%)	Reference
p. 260 V/E	0	0	--
p. 260 E/E	0	6 (30%)	Infinite (1.2, Infinite) 0.02
Combined cohorts			
p. 260 V/V	72 (99%)	30 (68%)	Reference
p. 260 V/E	1 (1%)	0	Not significant
p. 260 E/E	0	14 (32%)	Infinite (7.2, Infinite) 3 x 10 ⁻⁷

NPHS2 genotypes are summarized for individuals with SR-FSGS and controls. *NPHS2* sequencing results were available for 44 FSGS cases and 73 controls. *NPHS2* 260 E/E were compared to *NPHS2* 260V/V; FET, Fisher exact test.

Table 2.4: Response to therapy by *NPHS2* genotype for all black NS cases.

NPHS2 genotype	Treatment Response			
	No Response N (%)	Partial Remission N (%)	Full Remission N (%)	Any Remission N (%)
p. 260 VV	17 (53)	6 (19)	9 (28)	15 (47)
p. 260 EE	14 (100)	0	0	0
P value		0.16	0.04	0.002

In contrast to patients with *NPHS2* 260 V/V, patients with *NPHS2* E/E showed no response to standard or extensive treatment. Complete remission was defined as urine protein/creatinine ratio (PCR) < 0.2g/mmol and serum albumin > 30g/dL. Partial remission was defined as not meeting the criteria for full remission, with PCR < 1.9g/mmol and serum albumin > 25g/dL.

Supplementary Appendix

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Supplementary Methods

Sequencing and genotyping

Exons 1 through 8 were sequenced using ABI Prism BigDye Terminator on the ABI 3700 Analyzer at standard conditions using primers from Boute et al. for exons 2-7.¹ A new primer was designed within genomic regions flanking NPHS2 exon 1 (TGTAACGACGGCCAGTCGACTCCACAGGGACTGC -NPHS2_ex1_Forward) and (CAGGAAACAGCTATGACC CCTTAGTTACCACCTGGA - NPHS2_ex1_Reverse). M13F (TGTAACGACGGCCAGT) and M13R (CAGGAAACAGCTATGACC) sequence tags were added to the primers to aid in sequencing. Exon 1 fragments were amplified using a TaKaRa LA Taq kit (CloneTech, Mountain View, CA), using GC Buffer I according to the manufacturer's suggestion. PCR conditions were the following: 94 C for 1 minute followed by 30 cycles of 94 C for 1 minute; 57 C for 2 minutes 30 sec, followed by an extension at 72° C for 10 minutes. For a coalescence analysis to determine the age of the variants we genotyped DNA from 9 V260E homozygous patients and 71 individuals who did not carry the mutation, using the Human Exome Chip (HumanExome-12 v1.2, Illumina, San Diego, CA).

Bioinformatics Methods

Estimating time since the most recent common ancestor: Supplemental Figures 1 and 2 plot homozygous (top line) and heterozygous (bottom line) SNPs genotyped on the Illumina Exome chip, vs. genomic position relative to NPHS2 V260E, for a region of 10 million base pairs on either side of the locus. Figure S1 plots data for 9 individuals homozygous for the E (risk) allele, while Figure S2 plots data for individuals homozygous for the V (common) allele. The one (control) subject heterozygous for the mutation did not have DNA available for the exome chip. Figure S3 shows histograms of the length of homozygosity around V260E for individuals carrying (top) and lacking (bottom) the mutation. Overall the lengths of homozygosity are much longer for individuals carrying the mutation, indicating sequence identity for these individuals for the chromosomal region around V260E inherited from the individual's two parents, which indicates a lack of recombination in either chromosome, over this region, since the most recent common ancestor of the individual's parents carrying the mutation.^{2,3}We wish to test the hypothesis that there is a recent common ancestor for all the copies of the mutation carried by the subjects; the number of generations since this ancestor must be at least as long as the maximum of the number of generations since the common ancestor of the two parents, over all of the individuals. A direct coalescence determination of the time to a single common ancestor would require the actual haplotypes for this region for all individuals, which we have not determined.

Simulation program for modeling length of homozygosity: To estimate the time to a common ancestor for pairs of parents, we performed a coalescent simulation, simulating recombination in succeeding generations following the putative common ancestor. We assume that recombinations occur as a Poisson process at the rate of 1/100mB/generation; recombination data from deCODE indicates genome average rates of recombination in the region around NPHS2 (0.99 and 0.73 centimorgan per megabase for females and males,

respectively),⁴ and on scales greater than 3 Mb African and European recombination rates are essentially identical.⁵ We model a length of chromosome extending in each direction from the center (V260E in NPHS2), with initial length 100mB. For each recombination that shortens the segment, the new length is recorded. The model tracks two chromosomes, each descending from the common ancestor, for n generations, then assumes that an individual has inherited one copy of each. On each side of the mutation the length of homozygosity—length of shared chromosome—will be the minimum of the lengths of the unrecombined chromosomes. The sum of these is the length of homozygosity.

We simulated this process for numbers of generations running from 6 to 48. We assume that the two chromosomes inherited by the proband are the same number of generations removed from the common ancestor; a small deviation from this will not significantly affect the simulation results. For each number of generations we ran 100,000 simulations.

The simulation assumes that the proband's parents have no common ancestors more recent than the assumed founder of the mutation in the population. Having more recent common ancestors can result in longer segments of homozygosity; if the overall common ancestor is many generations removed in time, we would expect that some individuals' parents would have more recent common ancestors. Among the 9 individuals carrying two copies of V260E AA, two had distinctly longer segments of homozygosity (Figure S3), suggesting a more recent ancestor.

We estimate the time to the overall common ancestor by comparing the observed lengths of homozygosity with the simulation predicted lengths, for the simulated range of number of generations, with a Wilcoxon rank test. Using all 10 individuals, the rank test indicates that the number of generations is unlikely to be less than 14 ($p < 0.05$), with the most likely number of generations being 23. Assuming the two individuals with longer homozygosity to

have more recent common ancestry than the overall, which is more plausible, the number of generations is unlikely to be less than 21, with 31 being the most likely number.

Figure S1: Length of homozygosity around p.V260E in 9 subjects carrying p. 260 E/E

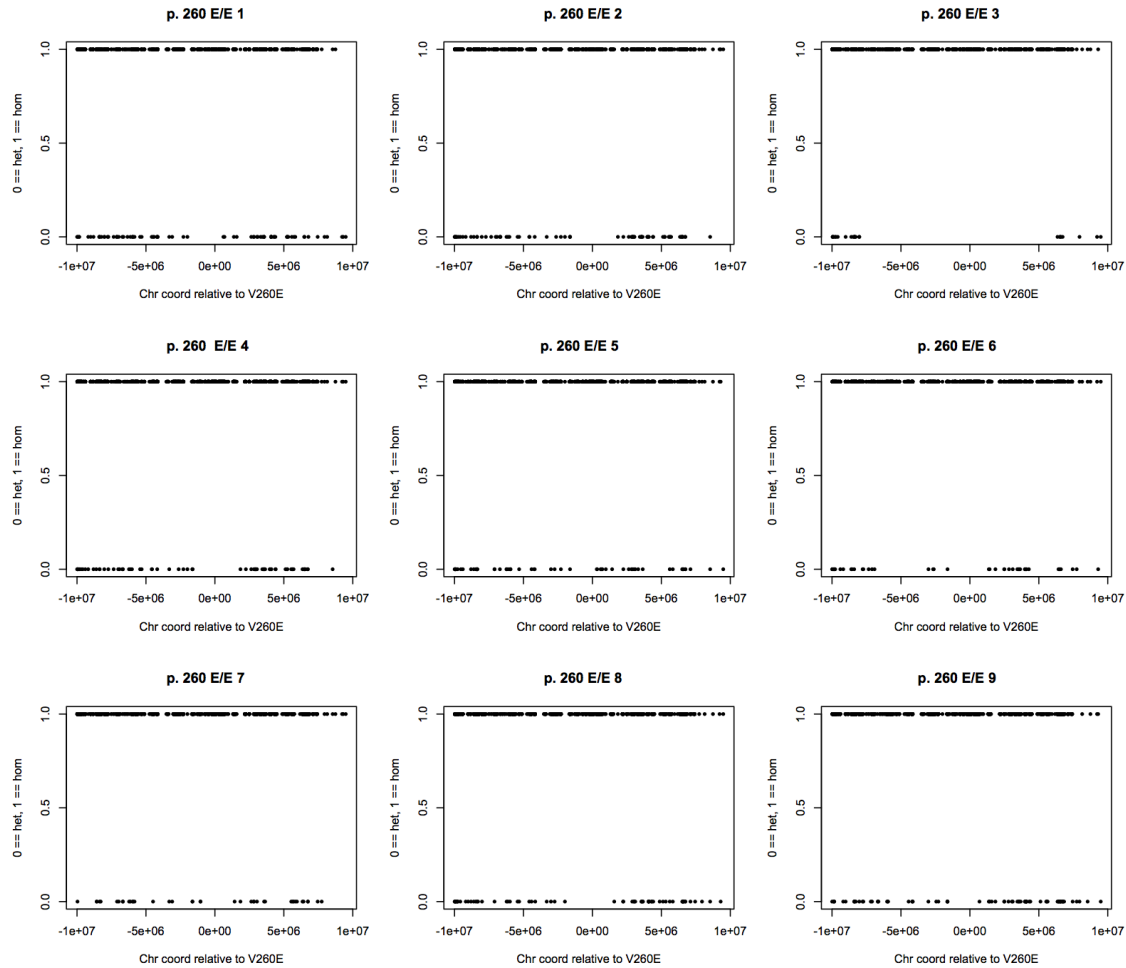


Figure S1: Heterozygous and homozygous loci around NPHS2 V260E from genotyping by the Illumina Exome V2.1 chip, for nine individuals carrying p.260 E/E (homozygous for the variant allele). The region shown is 10 mB before and after V260E; x axis coordinates are base positions relative to this locus. Top dots represent homozygous loci, bottom heterozygous; the region lacking heterozygous loci indicates the region where the subjects' two parental chromosomes carry identical haplotypes.

Figure S2: Length of homozygosity around p. V260E in 40 subjects carrying p. 260 V/V

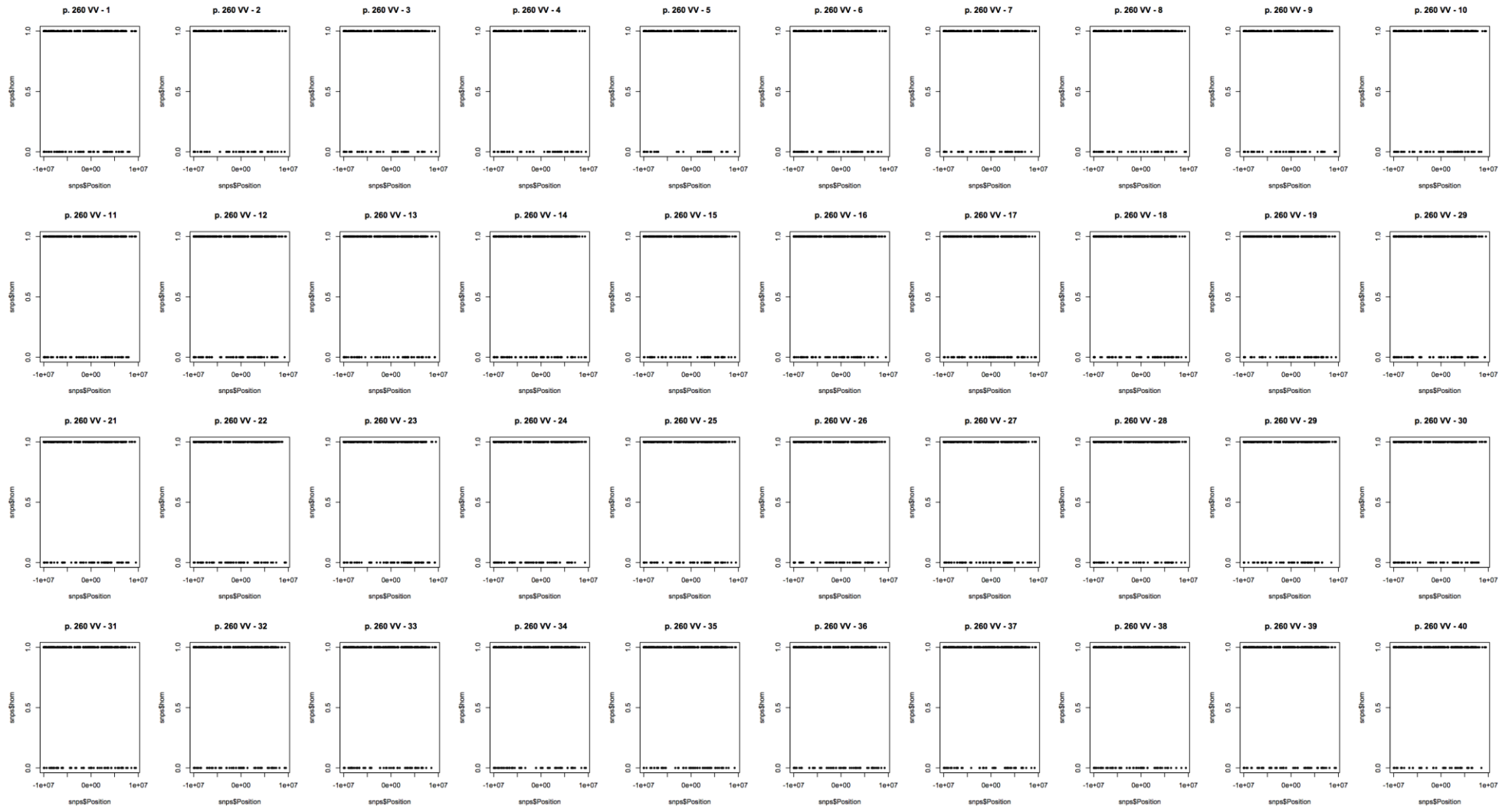


Figure S2: Heterozygous and homozygous loci around NPHS2 V260E from genotyping by the Illumina Exome V2.1 chip, for 40 individuals (representative of 71 total) carrying p.260 V/V (homozygous for the wild type allele), for comparison with Figure S1. In contrast to the individuals homozygous for the V260E mutant allele, for most individuals here there is no visible extent of homozygosity around the locus.

Figure S3

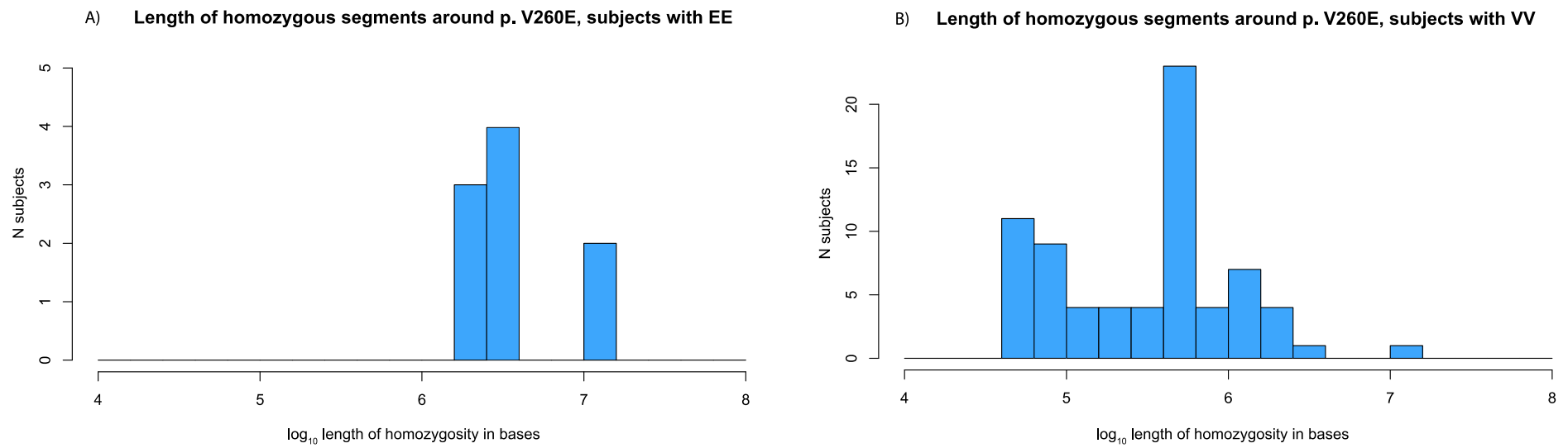


Figure S3: Histograms of the extent of homozygosity around NPHS2 V260E, for individuals homozygous for the mutant allele, and for individuals homozygous for the wild type allele, as shown in Supp. Figures 1 and 2.

Supplementary Table 1: NPHS variants detected in Indian and Black African children with nephrotic syndrome, and controls.

Variant	Allele	All SSNS No=19		Indian SRNS No =19		Indian Controls No =49		Black SRNS No =35		Black Controls No=55		1000GP or dbSNP	SIFT prediction	PolyPhen prediction	Reported association with FSGS	Ref
		Het	Hom	Het	Hom	Het	Hom	Het	Hom	Het	Hom					
-52 5'UTR	c.-52 C>G	0	0	0	0	0	0	4	0	14	5	rs78541594			Uncertain significance	6,7
-51 5'UTR	c.-51 G>T	2 20%	0	6 35%	0	10 24%	0	5 19%	8 36%	12 22%	0	rs12406197			No	7
p. P20L	c.59C>T	1 7%	0	1 6%	0	0	0	0	0	0	0	rs74315344	Deleterious	Possibly damaging	Yes, pathogenic	1,8
p. G34G	c.102G>A	5 36%	0	4 22%	0	9 20%	0	7 25%	1 4%	16 29%	3 5%	rs1079292			No	9
p. G42R	c.124G>A	0	0	0	0	0	0	0	0	4 7%	1 2%	rs559836164	Tolerated	Benign	No	
p. S48S	c.144C>T	0	0	0	0	1 2%	0	2 7%	0	5 9%	0	rs111306764			No	
p. A61V	c.182C>T	0	0	0	0	1 2%	0	1 3%	0	0	0	rs201050491	Tolerated	Benign	Yes	10

p. R229Q 4%	c.686G>A	0	0	0	0	1 2%	0	1 (3%)	0	0	0	rs61747728	Tolerated	Possibly damaging	Yes, deleterious with 2 nd NPHS2 mutation	11
p. A242V	c.725C>T	1 7%	0	0	0	1 2%	0	4 13%	0	4 8%	0	rs61747727	Deleterious	Probably damaging	Yes, pathogenic	12
p. V260E	c.779T>A	0	0	0	0	0	0	0	8 27%	1 2%	0	CM042098 reported in HGMD	Damaging	Probably damaging	Yes, pathogenic	12
p. A297A	c.891G>A	0	0	0	0	1 2%	0	1 3%	0	0	0	rs5005771			No	12
p. A318A	c.954T>C	8 61%	1 8%	11 55%	4 20%	27 54%	16 32%	12 38%	5 9%	32 56%	15 26%	rs1410592			No	9
p. L346L	c.1038A>G	0	0	3 5%	0	3 6%	0	5 16%	0	4 7%	0	rs3818587			No	1
p. P369S	c.1105C>T	0	0	0	0	1 2%	0	0	0	0	0	This study	Tolerated	Benign	No	This study

Supplementary Table 2: APOL1 risk alleles in SR-FSGS cases and controls

Number APOL1 risk alleles (RA)	Restricted to <i>NPHS2</i> E/E		Restricted to <i>NPHS2</i> V/V or V/E ¹	
	FSGS N (%)	Controls	FSGS N (%)	Controls N (%)
0	7 (50)	0	17 (56.7)	48 (65.8)
1	5 (35.7)	0	12 (40)	23 (31.5)
2	2 (14.2)	0	1 (3.3)	2 (2.7)

¹One control was heterozygous for *NPHS2* V260E; Because *NPHS2* E/E genotype is completely penetrant, the association of *APOL1* variants with FSGS is restricted to those with the reference genotype (*NPHS2* V/V). OR 1.2 (95%CI: 0.2, 24.3), P (FET)=1, for association of *APOL1* 2RA versus 1 or 0 RA.

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Addendum

Ethics Approval Letter



11 June 2004

Professor R Bhimma
Department of Paediatrics
Nelson R Mandela School of Medicine

Dear Professor Bhimma

PROTOCOL : The genetic basis of steroid resistant nephrotic syndrome in non-Caucasian patients. K Asharam, Paediatrics. Ref.: H043/04

The Research Ethics Committee and the Higher Degrees Committee considered the abovementioned application and made various recommendations. These recommendations have been addressed and the protocol was approved by consensus at a full sitting of the Research Ethics Committee at its meeting held on 8 June 2004 pending permission being received from the Hospital Managers at IALCH and KEH. These documents have now been received and the study may begin as at today's date – 11 June 2004.

This approval is valid for one year from 8 June 2004. To ensure continuous approval, an application for recertification should be submitted a couple of months before the expiry date.

Yours sincerely

PROFESSOR A DHALI
Chair : Research Ethics Committee

c.c. Ms K Asharam, Paediatrics
Mrs L Adendorff, Postgraduate Educ

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INFORMED CONSENT FOR PATIENTS WITH STEROID RESISTANT NEPHROTIC SYNDROME

INFORMATION GIVEN TO PARTICIPANTS:

Your child has a kidney disease because he/she is losing protein through the kidneys, which is also causing swelling of the body. This disease is called 'Nephrotic Syndrome'. We use steroids to treat this disease but some children become resistant to steroids and may subsequently develop chronic kidney failure. Previous studies have shown changes in certain genes leading to the development of steroid resistant nephrotic syndrome.

To date there have been no studies in Africa on the genetic aspects of steroid resistant nephrotic syndrome. In this study we want to find what changes occur in these genes that lead to the development of steroid resistant nephrotic syndrome.

This study will take place at King Edward VIII Hospital (KEH VIII) and Inkosi Albert Luthuli Central Hospital (IALCH). Professor Adhikari, Professor Bhimma, Dr Richard Naidoo, and Ms Kareshma Asharam will conduct the study. The aim of this study is to determine the genetic basis of steroid resistant nephrotic syndrome (SRNS) in black-African and Indian children.

To conduct this study we require a kidney biopsy sample and blood samples from your child for genetic analysis. You will be given a detailed explanation of how a kidney biopsy is done and the risks involved before you sign consent for the procedure. Samples will be stored for further genetic testing when required.

For further enquiries please contact

Prof R Bhimma - Tel.: 031-2604351 or

Ms Kareshma Asharam - Tel.: 031-2604489

Your participation in this study will not alter the management of your child's disease and no experimental treatment will be used. You can withdraw from the study at any stage and this will not in anyway prejudice your child's management.

You will be given a signed and dated copy of this consent form.

SIGNATURES

I agree to let my child's doctor use and give out my child's health information in the way it is described in this consent form until the end of the research study.

I have read this consent form, and I agree for my child _____ to take part in this study as it is explained in this consent form.

Date

Signature of Parent (s) or Surrogates (s)

I give my permission to have some of the samples of my child's blood and kidney biopsy specimen frozen and stored for possible future testing, as discussed.

Date

Signature of Parent

I certify that I have explained the above to _____ and believe that the signature(s) was affixed freely. I also agree to answer any questions that may arise.

Date

Signature of P.I. or person presenting

Printed Name of Person Providing Oral Translation: _____

Relationship of Translator to Subject, Parent, or Surrogate: _____

You will be given a signed and dated copy of this assent form.

SIGNATURES

I have read this assent form, and I _____ agree to take part in this study as it is explained in this assent form.

Date

Signature of Child (only those 8-17 years old)

I give my permission to have a sample of my blood and kidney tissue frozen and stored for possible future testing, as discussed.

Date

Signature of Child (only those 8-17 years old)

Please indicate how assent was obtained by initialling the applicable line:

I certify that I have explained the above to _____ and believe that the signature was affixed freely. I also agree to answer any questions that may arise.

Written assent was not obtainable because _____. However, I certify that I have explained the above to _____ and believe that verbal assent was freely given. I also agree to answer any questions that may arise.

Date

Signature of the Principal Investigator

or person presenting information

Verbal assent could not be obtained because

INFORMED CONSENT FOR PATIENTS WITH STEROID SENSITIVE NEPHROTIC SYNDROME

INFORMATION GIVEN TO PARTICIPANTS:

Your child has a kidney disease ‘Nephrotic Syndrome’. We use steroids to treat this disease and your child has responded to this treatment. However some children become resistant to steroids and may subsequently develop chronic kidney failure. Previous studies have shown changes in certain genes leading to the development of steroid resistant nephrotic syndrome.

To date there have been no studies in Africa on the genetic aspects of steroid resistant nephrotic syndrome. In this study we want to find what changes occur in these genes that lead to the development of steroid resistant nephrotic syndrome.

This study will take place at King Edward VIII Hospital (KEH VIII) and Inkosi Albert Luthuli Central Hospital (IALCH). Professor Adhikari, Professor Bhimma, and Ms Kareshma Asharam will conduct the study. The aim of this study is to determine the genetic basis of steroid resistant nephrotic syndrome (SRNS) in black-African and Indian children.

To conduct this study we require a 5mls (1 teaspoon) of blood samples from your child for genetic analysis. The bloods will be taken once off, a study number will be assigned to the sample. Part of the sample will be used for further genetic testing when required. You will be made aware of the results as soon as all data is collated and analysed.

For further enquiries please contact

Prof R Bhimma - Tel.: 031-2604351 or

Ms Kareshma Asharam - Tel.: 031-2604489

Your participation in this study will not alter the management of your child's disease and no experimental treatment will be used. You can withdraw from the study at any stage and this will not in anyway prejudice your child's management.

You will be given a signed and dated copy of this consent form.

SIGNATURES

I agree to let my child's doctor use and give out my child's health information in the way it is described in this consent form until the end of the research study.

I have read this consent form, and I agree for my child _____ to take part in this study as it is explained in this consent form.

Date

Signature of Parent (s) or Surrogates (s)

I give my permission to have some of the samples of my child's blood and kidney biopsy specimen frozen and stored for possible future testing, as discussed.

Date

Signature of Parent

I certify that I have explained the above to _____ and believe that the signature(s) was affixed freely. I also agree to answer any questions that may arise.

Date

Signature of P.I. or person presenting

Printed Name of Person Providing Oral Translation: _____

Relationship of Translator to Subject, Parent, or Surrogate: _____

You will be given a signed and dated copy of this assent form.

SIGNATURES

I have read this assent form, and I _____ agree to take part in this study as it is explained in this assent form.

Date

Signature of Child (only those 8-17 years old)

I give my permission to have a sample of my blood and kidney tissue frozen and stored for possible future testing, as discussed.

Date

Signature of Child (only those 8-17 years old)

Please indicate how assent was obtained by initialling the applicable line:

I certify that I have explained the above to _____ and believe that the signature was affixed freely. I also agree to answer any questions that may arise.

Written assent was not obtainable because _____. However, I certify that I have explained the above to _____ and believe that verbal assent was freely given. I also agree to answer any questions that may arise.

Date

Signature of the Principal Investigator

or person presenting information

Typed Name of Principal Investigator

or person presenting information

Verbal assent could not be obtained because
