NPHS2 Gene Mutation Screening in Palestinian Children With Steroid-Resistant Nephrotic Syndrome

الكشف عن طفرات في جين NPHS2 لدى الأطفال الفلسطينيين المصابين بمتلازمة الكلامية المقاومة لعلاج الستيرويد

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Molecular Biology

A Proposal Submitted in Partial Fulfillment for the Degree of Master of Science in Biological Sciences- Medical Technology

OCT/2017
نتيجة الحكم على أطروحة ماجستير

بناءًً على موافقة عمادة البحث العلمي والدراسات العليا بجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحث/ عطا جابر محمد ضيف الله، لقبول درجة الماجستير في كلية العلوم قسم العلوم الحياتية - تحليل طبيبة، ومواضيعها:

الكشف عن مutations في جين NPHS2 لدى الأطفال الفلسطينيين المصابين بمتلازمة الكلالانية

NPHS2 gene mutation screening in Palestinian children with steroid-resistant nephrotic syndrome

وبعد المناقشة التي تمت اليوم الأربعاء 04 ربيع الأول 1439 هـ الموافق 22/11/2017م المساء:

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وإن لم تقبل هذه الدرجة فإنها تمثل بنتيجة بشر للأمر ونورًا على علمه في خدمة دينه ووطنه.

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Abstract

**Background:** Nephrotic syndrome (NS), an uncommon childhood disorder, is characterized by edema, massive proteinuria, hypoalbuminemia, hyperlipidemia, and may progress to end stage renal disease. Based on the response to steroid therapy, NS is divided into steroid-sensitive (SSNS) and steroid-resistant (SRNS). SRNS is where the patient does not respond to prednisone treatment.

SRNS inherited, as an autosomal recessive disorder with *NPHS2* being the most frequently mutated gene. *NPHS2* gene encodes podocin protein, which has an important role in glomerular ultrafiltration and controlling slit membrane permeability.

**Objective:** The spectrum and frequency of *NPHS2* mutations in the Palestinian population have not been explored before. The aim of this study is to screen 20 SRNS Palestinian patients for *NPHS2* mutations and to compare our findings with those reported in other populations.

**Methods:** Twenty SRNS patients were recruited from the Ranteesy pediatric hospital. All the eight exons of the *NPHS2* gene were PCR-amplified from patients genomic DNA using appropriate primers. Direct sequencing of the purified PCR fragments was then ensued by automated Sanger sequencing method. Nucleotide changes were verified by comparing obtained sequences with the reference gene sequence stored in the NCBI database.

**Results:** Analysis of the obtained sequences identified previously known mutations in 3 (15%) of the patients. Two of the mutations, G130K and R229Q were missense mutations and the third, R138X was a nonsense mutation. All mutations were present in homozygous form.

**Conclusions:** The current study reports the identification of G130K, R138X, and R229Q *NPHS2* mutation in SRNS patients in Gaza-Palestinian. The mutations thus identified would spare patients from the unnecessary and harmful immunosuppressive steroids and help physicians and patients' families take proper decisions regarding patient management and their future offspring.

**Keywords:** SRNS, NPHS2, Sequencing, Gaza-Palestine.
المتلازمة الكلائية (NS) اضطراباً غير شائع يصيب الأطفال، ومن أعراضه الاستسقاء، البروتيني، نقص ألبومين الدم، ارتفاع الكليو في الدم، ويمكن أن يؤدي إلى فشل الكلي. ويتم تقسيمه إلى قسمين بالنظر إلى مدى استجابة العلاج للستيرويد: المتلازمة الكلائية المستجيبه للستيرويد (SSNS) والمتلازمة الكلائية المقاومة للستيرويد (SRNS)، حيث لا يستجيب المريض في النوع الثاني للعلاج بالبريدنون.

وتوزع المتلازمة الكلائية المقاومة للستيرويد (SRNS) كاضطرابات صبغية جسدية ضارة (صفة متنحية)، حيث تتشكّل الطفرات الجينية غالباً في جين (NPHS2) والذي له دور مهم في ترشيح التثبيت الكبيبي وتحكم ترشيح الغشاء الفلستي.

الهدف الدراسة: تعزف الدراسة إلى استكشاف مدى انتشار طفرات (NPHS2) بين السكان الفلسطينيين، وذلك من خلال فحص 20 مصاباً بالمتلازمة الكلائية المقاومة للستيرويد (SRNS)، ومقارنة النتائج التي تم التوصل إليها مع الطفرات الموجودة في مختلف وحدات المريض في مجموعات أخرى من السكان.

المنهجية الدراسة: تم اختيار عشرين مريضاً بالمتلازمة الكلائية من مستشفى الرنتيسي للأطفال، حيث تم فحص الأكسونات الثمانية الخاصة بجين (NPHS2) (للحصول على PCR)، حيث تم مضاعفتها باستخدام تقنية البلمرة المتسلسلة (RT-PCR) للحمض النووي للفحص البصري. ومن ذلك بعد ذلك تحليل النتائج المباشر للأجزاء المنقاة بواسطة تقنية Sanger باستخدام معرفة DNA من خلال مقارنة التتابعات التي تم ملاحظتها مع تلك الموجودة في الجين المرجعي، والمغزنة في قاعدة بيانات (NCBI).

النتائج: أكدت نتائج تحليل التتابعات التي تم الحصول عليها وجود طفرات جينية معروفة سابقاً في 3 مرضى (15%) من العينة، حيث كانت اثنان منها (R229Q و G130K) من ضمن الطفرات المغلقة، بينما كانت الثالثة (R138K) طفرة هرانية. وكانت جميع الطفرات موجودة في شكل متماثل.

الاستنتاجات: توصلنا إلى تحديد وجود طفرات (R138K و R229Q و G130K) في جين (NPHS2) لدى مرضى المتلازمة الكلائية المقاومة للستيرويد (SRNS) في غزة - فلسطين. ومن شأن هذه الطفرات أن توفر على المرضى عناية صحية عالية الدقة إلى استخدامات المشتقة من المنتجات التي تساهم في الأطباء والعائلات المرضى على اتخاذ القرارات المناسبة فيما يتعلق بإدارة حياة المرضى وأبنائهم في المستقبل.

الكلمات المفتاحية: SRNS، NS، NPHS2، غزة - فلسطين.
Dedication

I dedicate my modest work to most dear people, my parents who bestowed me courage and support.

To my beloved wife who gave me all the chance to achieve this work, to my daughter and all my brothers and sisters. Be proud!
I am grateful to Allah, who always offer me life, power, peace of mind, and courage to accomplish this study.

Foremost, I would like to express my sincere gratitude to my advisor Prof. Dr. Fadel A. Sharif for the continuous support of my Master study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master study.

I want to express my deep thanks to the Faculty of Health Sciences and the Department of Medical Technology in the Islamic University.

I want to express my deep thanks to the Rantessay Hospital and the Department of Nephrology, doctors, nursing staff and special thanks to the Dr. Mostafa Elaila for helping me in the completing this study.

I want to express my deep thanks to Trans-Orient Company, especially the head of the company Hammam EL-rayes for donating all materials used in this study.

Special thanks to the IUG Genetics lab, in particular, Mr. Mohammed Ashour and Mr. Shadi El-Ashi, who offered the support and equipment I have needed to produce and complete my thesis.
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<td>CD2 Associated Protein</td>
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<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<td>CNS</td>
<td>Congenital Nephrotic Syndrome</td>
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<td>Cs</td>
<td>Capillary Space</td>
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<td>DCT</td>
<td>Distal Convoluted Tubule</td>
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<td>DdTTPs</td>
<td>Dideoxynucleotides</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>E</td>
<td>Endothelium Cell</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>FSGS</td>
<td>Focal Segmental Glomerulosclerosis</td>
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<tr>
<td>GC</td>
<td>Glucocorticoid</td>
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<tr>
<td>GEC</td>
<td>Glomerular Endothelial Cell</td>
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<td>GFR</td>
<td>Glomerular Filtration Rate</td>
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<td>GMB</td>
<td>Glomerular Basement Membrane</td>
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<td>GN</td>
<td>Glomerulonephritis</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>INS</td>
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<td>IVF</td>
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<td>KDIGO</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>NS</td>
<td>Nephrotic Syndrome</td>
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<td>P</td>
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<td>PCT</td>
<td>Proximal Convoluted Tubule</td>
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<td>Pre-implantation Genetic Diagnosis</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>SRNS</td>
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Chapter 1

Introduction
Chapter 1
Introduction

1.1 Overview

Nephrotic syndrome (NS) is considered as one of the main kidney diseases in children and is characterized by proteinuria, edema, hypoalbuminemia, and hyperlipidemia. The idiopathic nephrotic syndrome affects about 2 children per 100,000 aged 15 years and younger. About 10% to 20% of these children are resistant to steroids treatment (Otukesh, Otukesh, et al., 2009; Patrakka et al., 2002).

The generally used definition of steroid-resistant nephrotic syndrome (SRNS) is lack of remission after exposure of 8 weeks of prednisone 2mg/kg/d or 60 mg/m2/d for 4 weeks followed by 1.5mg/kg or 40 mg/m2 per dose alternate-day for 4 weeks (Gipson et al., 2009).

Genetic forms of SRNS do not respond to strengthened immunosuppressive treatment and progress inevitably to end-stage kidney disease but on the other hand, They have a minimal risk of post-transplant recurrence. Hence, genetic testing is of importance of clinical relevance in this complex group of patients (Jungraithmayr et al., 2011; Santín et al., 2010) Genetically, SRNS is caused by mutations in a number of different genes, the most common being NPHS2 (Lipska et al., 2013).

NPHS2 gene encodes podocin, a member of the stomatin protein family that is localized at the slit membrane in Bowman's capsules in the nephrons of the kidney. (Guaragna et al., 2015). The gene contains 8 exons which code for the 383 amino acid long podocin protein. Podocin localizes at the slit diaphragm and interacts directly with nephrin and CD2-associated protein.

Nephrin and podocin are key components of the slit diaphragm of the glomerular epithelial cell and play a necessary role in the normal function of the glomerular filtration barrier. Podocin deficiencies alter slit diaphragm permeability and can also alter the processing and localization of nephrin (Otukesh, Ghazanfari, et al., 2009).
So far, 126 NPHS2 pathogenic mutations have been identified worldwide. The mutations are distributed along the entire coding region of the gene and cause all kinds of alterations, including 53 missense, 17 nonsense, 11 small insertions, 26 small deletions, 16 splicing, two insertion/deletion mutations and one mutation in the stop codon (Bouchireb et al., 2014).

In the present study, 20 Palestinian patients suffering from SRNS were tested with the aim of detecting the spectrum of, and the common NPHS2 mutations in our population. Mutation detection was performed through automated Sanger sequencing of the eight exons and exon-intron boundaries of the NPHS2 gene.

Knowledge of the NPHS2 mutations in our population will improve the diagnosis and medical care of SRNS patients and could also be used as a basis for patient counseling, carrier screening and prenatal/preimplantation diagnosis programs in Gaza Strip.

1.2 Objectives of the study

1. The aim of the present study is to identify the spectrum of NPHS2 mutations in Palestinian patients suffering from SRNS.

2. Developing PCR-based techniques for rapid detection of the identified NPHS2 mutations in Gaza Strip.

3. Correlating severity of SRNS and disease onset age with the identified mutations.
1.3 Significance

The significance of this study can be summarized as follows:

1. Determining, for the first time in Gaza strip, the *NPHS2* mutations prevalent in our population.

2. Mutational analysis in SRNS would help in preventing unnecessary exposure to immunosuppressants and their adverse effects, besides helping in prognostication.

3. The results may help physicians in Gaza Strip to manage SRNS associated with *NPHS2* gene alterations and to reducing birth of affected children by employing prenatal diagnosis or in vitro fertilization (IVF) with preimplantation genetic diagnosis (PGD) techniques.
Chapter 2
Literature Review
Chapter 2
Literature Review

2.1 Nephrotic Syndrome

Nephrotic syndrome (NS) is one of the most common causes of chronic kidney disease (CKD) in the pediatric population. The cardinal features are edema, massive proteinuria, and hypoalbuminemia. Children with NS have a decreased quality of life \cite{McCaffrey2016}. They are at the risk of a wide range of complications associated with significant morbidity and experience mortality rates of up to 2.7\% \cite{Kawaguchi2014}.

NS can be divide into congenital NS (presentation before 3 months of age), infantile NS (presentation between 3 months to 1 year of age) and idiopathic nephrotic syndrome (INS). The largest of these groups is INS, and initial management involves an 8-12-week course of oral glucocorticoid (Gc) therapy \cite{Ehrich1993}. Approximately, 92\% of children with INS will enter remission during this initial treatment and are subsequently classified as having a steroid-sensitive nephrotic syndrome (SSNS), while 8\% fail to enter remission and are classified as having a steroid-resistant nephrotic syndrome (SRNS) \cite{Nephrologie1979}.

2.1.1 Congenital nephrotic syndrome

At least 170 mutations in the \textit{NPHS2} gene have been found to cause the congenital nephrotic syndrome. This condition is a kidney disorder that begins in infancy and typically leads to irreversible kidney failure (end-stage renal disease) by early childhood. Mutations in this gene appear to be the most frequent cause of the congenital nephrotic syndrome. Most \textit{NPHS2} gene mutations change single protein building blocks (amino acids) in the podocin protein. These mutations result in a reduction or absence of functional protein, which impairs the formation of normal slit diaphragms. Without a functional slit diaphragm, molecules pass through the kidneys abnormally and are excreted in urine. The filtering ability of the kidneys worsens from birth, eventually leading to end-stage renal disease \cite{Machuca2010}.
2.1.2 Infantile nephrotic syndrome

*NPHS2* gene mutations can cause other forms of nephrotic syndrome that develop later in life. In one form, called infantile nephrotic syndrome, signs and symptoms of the condition appear between 4 and 12 months of age. The features of this condition are similar to congenital nephrotic syndrome (described above), but they are often less severe. It is likely that *NPHS2* gene mutations that cause infantile nephrotic syndrome have less effect on podocin function than those that cause congenital nephrotic syndrome, accounting for the later onset of the disorder (Sadowski et al., 2015).

2.1.4 Steroid-sensitive nephrotic syndrome

Steroid-sensitive nephrotic syndrome, SSNS, is the most common form of NS in children. It usually first manifests at 2-6 years of age and is twice as likely in boys than in girls. It is characterized clinically by a rapid onset of edema. Most often urinalysis reveals the only proteinuria but some patients may have hyaline or waxy casts and hematuria. While SSNS is usually associated with minimal changes in the glomerular structure in light microscopy. (Patrakka et al., 2002).

In current clinical practice, children who enter disease remission during the initial course of Gc therapy do not require a kidney biopsy (unless there are atypical features), as initial clinical response to Gc therapy in children is of greater prognostic significance than histological findings (Vivarelli, Moscaritolo, Tsalkidis, Massella, & Emma, 2010).

Children with SSNS generally have a favorable outcome, and the results of older studies suggested they achieve long-term remission during teenage years (Trompeter, Hicks, Lloyd, White, & Cameron, 1985). However, recent data suggest that over 30% of SSNS children relapse in adulthood (Rüth, Kemper, Leumann, Laube, & Neuhaus, 2005).

The etiology of SSNS is still not well understood although several avenues have been researched. The popular proposition of the SSNS is that it has an immunological component as it is associated with abnormal T-cell response and
often appears together with disorders with the immunological bases, such as atopy, autoimmune diseases, and lymphomas (WEENING, 2004; Eddy & Symons, 2003).

It also often first appears after an infection although the mechanism of is this not known. Recently, especially respiratory tract virus infections are implicated (Eddy & Symons, 2003). T2-helper cell activation and production of cytokines such as IL4 and -13 (IL4 and IL13) that occurs in allergy, also occurs in animal models of SSNS (Ikeuchi et al., 2009; Kausman & Kitching, 2007).

However, a direct link between immune system and SSNS is yet to find. On the glomerular level, loss of Glomerular Basement Membrane (GBM) charge has been reported in some patients (Chugh, Clement, & Macé, 2012).

2.1.5 Steroid-resistant nephrotic syndrome

The most common histological diagnosis in SRNS is focal segmental glomerulosclerosis, a term which in practice (though not strictly correct) is used interchangeably with SRNS (Saleem, 2013).

Children with SRNS are treated with a range of other (non-Gc) immunomodulatory agents, including mycophenolate mofetil, cyclophosphamide, ciclosporin and rituximab (Lombel, Gipson, & Hodson, 2013).

More than 60% of children with SRNS who fail to achieve remission with pharmacological intervention will progress to end-stage renal disease (Abeyagunawardena et al., 2007). There are also differences in mortality rates according to the initial response to Gc therapy: 2.7 % in the INS population overall, 18.5% in children with SRNS, 6.3% in children with early relapse following initial remission and 0.4% in children without early relapse (McCaffrey et al., 2016).

Over the past 15 years, genetic discoveries have vastly improved our understanding of the molecular basis of NS. The PodoNet consortium has recently reported data from a heterogeneous population of 1655 children with SRNS with a median follow-up time of 3.7 years. In this series, SRNS was either congenital (6 %), infantile (7 %), adolescent onset (13 %) or childhood onset (74 %). At the time of the last follow-up, 11.7 % of the children required dialysis, 14.2 % had received a kidney transplant and 2.3 % were deceased (Trautmann et al., 2015). Remarkably, 23.6%
of children from this heterogeneous SRNS cohort had a genetic mutation, in contrast with another series where mutations were found in 66% of NS cases presenting under the age of 1 year (Hinkes et al., 2007).

The most common mutations in childhood NS are found in genes encoding nephrin (NPHS1), podocin (NPHS2) and Wilms tumour 1 (WT1) (Saleem, 2013). The increasing availability of genetic testing in SRNS will help clinicians minimize exposure to immunosuppressive agents since genetic mutations are associated with a poor response to immunosuppression (Bullich et al., 2015).

2.2 Nephron Structure

The nephron is the functional unit of the kidney. A nephron consists of a twisted tubule closed at one end, open at the other with a network of associated blood vessels. Each kidney of man is formed of about one million nephrons. It is in the nephrons that urine is separated from the blood, and some of the water and salts in it are reabsorbed. It is responsible for maintaining the pH and temperature of the bloodstream.

Each nephron has a length of about 3 cm. It is differentiated into four regions having different anatomical features and different physiological roles (Kriz & Kaissling, 1992). The four regions are Bowman's capsule, proximal convoluted tubule (PCT), a loop of Henle, and distal convoluted tubule (DCT). These four regions are illustrated in Figure 2.1.

![Nephron Structure](http://www.buzzle.com)

Figure (2.1): the nephron structure. (Adopted from: http://www.buzzle.com, 20015).
2.2.1 The Glomerulus

The glomerulus, the filtering unit of the kidney, is a specialized bundle of capillaries that uniquely situated between two resistance vessels (Figure 2.2). These capillaries are each contained within the Bowman’s capsule and they are the only capillary beds in the body that not surrounded by interstitial tissue. Therefore, a unique support structure needed to maintain flow in these essential capillary units. In fact, all of the major components of the filter itself are unique compared with related structures in other capillary beds. The proximal component layer of the glomerular filter itself is a fenestrated endothelium, the second layer of the filter, the glomerular basement membrane (GBM) and the distal layer of the glomerular filter is composed of the visceral epithelial cells or podocytes. Together, this elegant structure permits the formation of the primary glomerular filtrate that enters a space delimited by the visceral and parietal epithelial cells before modification during transit through the tubule (Pollak, Quaggin, Hoenig, & Dworkin, 2014).

Figure (2.2): Structure of the renal corpuscle, looking into the Bowman’s capsule at glomerular capillary tuft. modified from (Pollak et al., 2014).
2.2.2 The glomerular filtration barrier:

The glomerulus consists of a cluster of capillaries appearing in a looped formation supported by mesangial cells. While blood plasma passes the glomerular capillary loops, the local intra-capillary pressure drives plasma through the glomerular filtration barrier, which consists of three layers that each contributes to its perm-selective properties: the capillary endothelium, the glomerular basement membrane (GBM) and the single-celled layer of glomerular epithelial cells (GEC) or podocytes (Figure 2.3) (Tanaka, 2008).

The first layer, the glomerular endothelial cells separate the blood and tissue compartments. The endothelial cells are highly flattened cells and regulate vasomotor tone and hemostasis, characterized by the presence of individual fenestrae on the order of 70–100 nm in diameter. These cells drape the luminal aspect of the capillary and permit filtration. (Ballermann, 2005).

The second layer, the glomerular basement membrane (GBM). Interposed between the endothelial layer and the podocytes. It is composed of three layers identified by electron density. These layers are named the lamina rara internal, lamina densa, and lamina rara external. It is described as a dynamic gel-like acellular meshwork of glycoproteins and proteoglycans (Levidiotis & Power, 2005).

The third layer, the podocytes. They are highly specialized, terminally differentiated cells with cytoplasmic extensions, the so-called foot processes. Podocytes have an important role in size and charge selective permeability, but also in synthesizing and maintaining the GBM (Pavenstädt, Kriz, & Kretzler, 2003). Furthermore, the fenestration of endothelial cells depends most notably on vascular endothelial growth factor A secreted from differentiated podocytes. These remarkable cells help to create the filtration slit diaphragm and serve as support to help sustain the integrity of the free-standing capillary loops. (Ballermann, 2005).
Figure (2.3): A cross-section (electron microscopy, original magnification ×30,000) of the glomerular filtration barrier with the capillary space (Cs), urinary space (Us), endothelial cells (E), glomerular basement membrane (BM), and podocytes (P) (Löwik, Groenen, Levchenko, Monnens, & Van Den Heuvel, 2009a).

The foot processes of the podocytes attach to the outer surface of the GBM through cell membrane receptors (α3β1 integrins linked to talin, vinculin, and paxillin, and α- and β-dystroglycans) linked to utrophin; see Figure 2.4 (Kretzler, 2002). Adjacent foot processes interdigitate, forming a pore of about 25–40 nm in width. This pore, or slit, is covered by a membrane with a “zipper-like” structure (Rodewald & Karnovsky, 1974).

Recently, the structure of the slit membrane became the focus of many studies, and although the complete structure has not elucidated yet, several new proteins were found to be important for its function. Many components of the slit membrane are involved in the pathogenesis of (nephrotic range) proteinuria (Löwik, Groenen, Levchenko, Monnens, & Van Den Heuvel, 2009b).
2.3 Podocin

Podocin (NPHS2, OMIM 604766): It is a member of the stomatin protein family is exclusively expressed in the podocytes and localizes at the insertion of the slit membrane. Due to its similarity to stomatin, it is believed that podocin forms a hairpin-like structure with intracellular NH2- termini and COOH-termini (Roselli et al., 2002).

Podocin, like nephrin, associates with lipid rafts and recruits nephrin and CD2AP in these rafts ensuring a stable and proper functioning filtration barrier. The COOH terminal cytoplasmic tail of podocin interacts with nephrin and CD2AP (the mouse homolog) (Schwarz et al., 2001). This protein interaction greatly enhances nephrin-induced signaling in vitro (Huber, Köttgen, Schilling, Walz, & Benzing, 2001).

The COOH-terminal domain of podocin also binds NEPH-1; a podocyte slit membrane protein structurally related to nephrin (Sellin et al., 2003) NEPH-1 is involved in maintaining the structure of the filtration barrier and also interacts with nephrin (Liu et al., 2004).

Podocin dysfunction leads to alterations of the slit membrane assembly and to proteinuria in experimental models. NPHS2−/− mice develop proteinuria and...
massive mesangial sclerosis (different from focal segmental glomerulosclerosis "FSGS" seen in humans), the podocytes are enlarged and focally vacuolized. Sclerosis rapidly progresses with age. Besides the absence of podocin, no nephrin found in the foot processes as well. The podocin-deficient mice die a few days after birth (Roselli et al., 2004).

Early reports showed that patients with NPHS2 mutations had no recurrence of FSGS after renal transplantation. Now it is believed that patients with NPHS2 mutations have a lower risk for recurrent FSGS after renal transplantation compared to patients with idiopathic FSGS (Ruf et al., 2004; Weber et al., 2004).

2.4 NPHS2 Gene

Mutations in NPHS2 gene, located at 1q25-31, are the most common cause of SRNS in childhood. The coding region of the gene encompasses 1,149 bp, which 8 exons (Figure 2.5) and encodes a 383-amino acid protein with 42 kD, called podocin, which is expressed in fetal and mature kidney (Guaragna et al., 2017).

![Figure (2.5): A schematic diagram of NPHS2 gene. Exons represented by black boxes with corresponding numbers above and introns by lines between the exons. Gray boxes represent 5` and 3` untranslated regions. Modified from (Franceschini, North, Kopp, Mckenzie, & Winkler, 2006)]

2.4.1 Normal Function

The NPHS2 gene provides instructions for making a protein called podocin. Podocin primarily found in the kidneys, which are organs that filter waste products from the blood and remove them in urine. Specifically, podocin found in cells called podocytes, which are located in specialized kidney structures called glomeruli. Podocin is located at the cell surface in the area between two podocytes called the slit diaphragm. The slit diaphragm is known as a filtration barrier because it captures proteins in blood so that they remain in the body while allowing other molecules like sugars and salts to be excreted in urine. Podocin likely helps bring other proteins that are needed for a functional slit diaphragm to the podocyte cell.
surface. The protein also is involved with podocyte cell signaling and helping the cell adapt to changes that occur during the filtration process (Grahammer, Schell, & Huber, 2013).

2.4.2 NPHS2 mutations

NPHS2 mutations spread across the entire gene and lead to all kinds of alterations including missense, nonsense, frameshift, and splice-site mutations (Figure 2.6).

![Figure (2.6): NPHS2 reported mutations. A: Exon structure of the NPHS2 gene with geometric shapes indicating relative positions of different types of mutations. B: Podocin domain positions. PHB (Bouchireb et al., 2014).](image)

2.5 Genetics of SRNS

Steroid-resistant nephrotic syndrome (SRNS) occurs at an estimated incidence of 1 per 200,000 children. Genetic causes can be identified in nearly 50% of affected children with this highly heterogeneous disorder. Genetic forms of SRNS do not respond to intensified immunosuppressive treatment and progress inevitably to end-stage kidney disease, but, on the other hand, have a minimal risk of post-transplant recurrence. Hence, genetic testing is of eminent clinical relevance in this complex group of patients (Lipska et al., 2013).

Mutations in the NPHS2 gene, encoding podocin, are a major cause of autosomal-recessive steroid-resistant nephrotic syndrome (SRNS) in childhood,
accounting for up to 30% of sporadic and 20-40% of familial cases (Megremis, 2009 #83).

Molecular studies have implicated many genes in the pathogenesis of SRNS (Table 2.1). The table indicates the results of an international cohort of 526 patients of 1783 families, in whom a single-gene cause of SRNS was detected in 1 of 21 monogenic causes of SRNS (27 genes have been examined).

**Table (2.1): Genes implicated in SRNS**

<table>
<thead>
<tr>
<th>Gene Causing SRNS</th>
<th>Mode of Inheritance</th>
<th>SRNS Families Molecularily Diagnosed by Sanger Sequencing (n)</th>
<th>SRNS Families Molecularily Diagnosed by Multiplex PCR (n)</th>
<th>Total SRNS Families with Molecular Diagnosis (% of Families)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPHS2</td>
<td>AR</td>
<td>170 (42)</td>
<td>7</td>
<td>177 (9.93)</td>
</tr>
<tr>
<td>NPHS1</td>
<td>AR</td>
<td>93 (61)</td>
<td>38</td>
<td>131 (7.34)</td>
</tr>
<tr>
<td>WT1</td>
<td>AD</td>
<td>78 (50)</td>
<td>7</td>
<td>85 (4.77)</td>
</tr>
<tr>
<td>PLCE1</td>
<td>AR</td>
<td>23 (16)</td>
<td>14</td>
<td>37 (2.17)</td>
</tr>
<tr>
<td>LAMB2</td>
<td>AR</td>
<td>10 (6)</td>
<td>10</td>
<td>20 (1.12)</td>
</tr>
<tr>
<td>SMARCA21</td>
<td>AR</td>
<td>1 (0)</td>
<td>15</td>
<td>16 (0.89)</td>
</tr>
<tr>
<td>INF2</td>
<td>AD</td>
<td>2 (0)</td>
<td>7</td>
<td>9 (0.5)</td>
</tr>
<tr>
<td>TRPC6</td>
<td>AD</td>
<td>1 (1)</td>
<td>8</td>
<td>9 (0.53)</td>
</tr>
<tr>
<td>COQ6</td>
<td>AR</td>
<td>6 (5)</td>
<td>2</td>
<td>8 (0.45)</td>
</tr>
<tr>
<td>ITGA3</td>
<td>AR</td>
<td>3 (3)</td>
<td>2</td>
<td>5 (0.28)</td>
</tr>
<tr>
<td>MYO1E</td>
<td>AR</td>
<td>0 (0)</td>
<td>5</td>
<td>5 (0.28)</td>
</tr>
<tr>
<td>CUBN</td>
<td>AR</td>
<td>1 (1)</td>
<td>4</td>
<td>5 (0.28)</td>
</tr>
<tr>
<td>COQ2</td>
<td>AR</td>
<td>0 (0)</td>
<td>4</td>
<td>4 (0.22)</td>
</tr>
<tr>
<td>LMX1B</td>
<td>AD</td>
<td>0 (0)</td>
<td>4</td>
<td>4 (0.22)</td>
</tr>
<tr>
<td>ADCK4</td>
<td>AR</td>
<td>3 (3)</td>
<td>0</td>
<td>3 (0.17)</td>
</tr>
<tr>
<td>DGKE1</td>
<td>AR</td>
<td>0 (0)</td>
<td>2</td>
<td>2 (0.11)</td>
</tr>
<tr>
<td>PDSS2</td>
<td>AR</td>
<td>0 (0)</td>
<td>2</td>
<td>2 (0.11)</td>
</tr>
<tr>
<td>ARHGAP24</td>
<td>AD</td>
<td>0 (0)</td>
<td>1</td>
<td>1 (0.06)</td>
</tr>
<tr>
<td>ARHGDIA</td>
<td>AR</td>
<td>1 (1)</td>
<td>0</td>
<td>1 (0.06)</td>
</tr>
<tr>
<td>CFH</td>
<td>AR</td>
<td>0 (0)</td>
<td>1</td>
<td>1 (0.06)</td>
</tr>
<tr>
<td>ITGB4</td>
<td>AR</td>
<td>0 (0)</td>
<td>1</td>
<td>1 (0.06)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>392 (189)</strong></td>
<td><strong>134</strong></td>
<td><strong>526 (29.5)</strong></td>
</tr>
</tbody>
</table>

Table (2.2): AD, autosomal dominant; AR, autosomal recessive. The number in parenthesis show “molecularly solved” families with causative mutation the detected that was published before our cohort (Sadowski et al., 2015).
The main conclusions which can be drawn from molecular genetic studies on early onset nephrotic syndrome: First, two-thirds of nephrotic syndrome manifesting in the first year of life can be explained by mutations in 4 genes (*NPHS1, NPHS2, WT1, or LAMB2*). Second, *NPHS1* mutations occur in congenital nephrotic syndrome only. Third, infants with causative mutations in any of the 4 genes do not respond to steroid treatment; therefore, unnecessary treatment attempts can be avoided. Fourth, there are most likely additional unknown genes mutated in early-onset nephrotic syndrome (*Hinkes et al., 2007*).

### 2.6 Previous Molecular Studies

A study on Brazilian children, analysis of the *NPHS1, WT1* and *NPHS2* genes in 27 steroid-resistant revealed four missense mutations and one frameshift mutation. All encountered mutations were in the *NPHS2* gene. *WT1* and *NPHS1* gene analysis did not reveal any mutation (*Guaragna et al., 2015*).

A study on Saudi Arabia children to determine the frequency of inherited NS, 62 cases (representing 49 families with NS) were screened for mutations in *NPHS1, NPHS2, LAMB2, PLCE1, CD2AP, MYO1E, WT1, PTPRO* and *NEIL1*. Potential causative mutations were detected in 25 out of the 49 families studied (51%). The most common genetic cause of NS in the investigated cohort was a homozygous mutation in the *NPHS2* gene, found in 11 of the 49 families (22%). Mutations in the *NPHS1* and *PLCE1* genes allowed a molecular genetic diagnosis in 12% and 8% of families, respectively. New *MYO1E* mutations were evident in three families (6%). No mutations were found in *WT1, PTPRO* or *NEIL1* (Figure 2.7) (*Al-Hamed et al., 2013*).
A study on Indian children for evaluating the frequency of *NPHS2* mutations in 25 with sporadic SRNS, Sun et al. found a homozygous mutation at nucleotide position 211, C>T in only one patient, resulting in a stop codon i.e., p.R71X. This mutation in exon 1 leads to the formation of a truncated protein, rendering it non-functional (Sun et al., 2009).

A study on Israeli-Arab children from the 27 patients tested (familial and nonfamilies), 15 patients (55%) were homozygous for the mutation C412T (p.R138X) in *NPHS2* is the cause of SRNS. (Frishberg et al., 2002).
Chapter 3
Materials and Methods
Chapter 3
Materials and Methods

3.1 Study design

The current study is a mutation analysis study, in which the NPHS2 gene from SRNS patients was screened for potential pathogenic mutations.

3.2 Study Sample:

The target population of this study consisted of 20 Palestinian children residing in Gaza strip. All participants were ≤ 12 years old.

3.3 Study location

All experimental work was done at the Genetics laboratory of the Islamic University, Gaza strip.

3.4 Inclusion Criteria

Patients greater than 12 years, diagnosed with primary SRNS.

3.5 Ethical considerations

Informed consent was taken from all the subjects who agreed to participate in the study. The objective of the study was fully explained to all participants. The study was approved by the Helsinki ethics committee in Gaza Strip.

3.6 Specimen collection and processing

About 2.0 ml of venous blood drawn into sterile EDTA tubes and mixed gently, under quality control and safety procedures. The blood samples were used for DNA extraction and purification.
3.7 Materials

3.7.1 Equipment

The present work was carried out in the Genetics lab at the Islamic University of Gaza. The major equipments used in the study are listed in Table 3.1.

Table (3.1): The major equipment's used in the study

<table>
<thead>
<tr>
<th>#</th>
<th>Instrument</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Thermal Cycler</td>
<td>Biometra, Germany</td>
</tr>
<tr>
<td>2.</td>
<td>Electrophoresis chambers and tanks (horizontal)</td>
<td>BioRad, USA</td>
</tr>
<tr>
<td>3.</td>
<td>Electrophoresis power supply</td>
<td>BioRad, USA</td>
</tr>
<tr>
<td>4.</td>
<td>Microcentrifuge</td>
<td>Sanyo, UK</td>
</tr>
<tr>
<td>5.</td>
<td>Microwave Oven</td>
<td>L.G, Korea</td>
</tr>
<tr>
<td>6.</td>
<td>Digital balance</td>
<td>AE adnam, USA</td>
</tr>
<tr>
<td>7.</td>
<td>Freezer, refrigerator</td>
<td>ORSO, pharml-spain</td>
</tr>
<tr>
<td>8.</td>
<td>Micropipettes (0.1-2.5μl / 0.5-10μl / 5-50μl / 20-200μl / 100-1000μl)</td>
<td>Dragon-lab, USA</td>
</tr>
<tr>
<td>9.</td>
<td>Safety cabinet</td>
<td>N-Biotek,Inc</td>
</tr>
<tr>
<td>10.</td>
<td>Gel documentation system</td>
<td>Vision, Scie-Plas Ltd, UK</td>
</tr>
<tr>
<td>11.</td>
<td>Nano-drop spectrophotometer</td>
<td>Implen, Germany</td>
</tr>
<tr>
<td>12.</td>
<td>ABI PRISM 310 Genetic Analyzer</td>
<td>Applied Biosystems, USA</td>
</tr>
</tbody>
</table>
3.7.2 Chemicals, Kits, and Disposables

Chemicals, kits, and disposables that were used in this study are listed in Table 3.2.

**Table (3.2):** Chemicals, kits, and disposables

<table>
<thead>
<tr>
<th>#</th>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wizard ® Genomic DNA Purification Kit</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>2</td>
<td>PCR Go Taq® Green Master Mix</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>3</td>
<td>POP 6 electrophoresis polymer</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>4</td>
<td>PCR primers</td>
<td>Hy.labs (Rehovot, Israel)</td>
</tr>
<tr>
<td>5</td>
<td>PCR purification kit</td>
<td>Invitrogen, Germany</td>
</tr>
<tr>
<td>6</td>
<td>Cyclic sequencing kit</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>7</td>
<td>Nuclease-free water</td>
<td>(Sigma USA)</td>
</tr>
<tr>
<td>8</td>
<td>DNA molecular size marker (Ladder).</td>
<td>BioLab, New England</td>
</tr>
<tr>
<td>9</td>
<td>Ethidium bromide</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>10</td>
<td>Ethanol 70%</td>
<td>(Sigma USA)</td>
</tr>
<tr>
<td>11</td>
<td>Absolute Isopropanol</td>
<td>(Sigma USA)</td>
</tr>
<tr>
<td>12</td>
<td>Agarose</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>13</td>
<td>3730 Buffer(10X) with EDTA</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>14</td>
<td>Hi-Di Formamide</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>15</td>
<td>Centri-sep spin columns</td>
<td>Applied Biosystems, USA</td>
</tr>
<tr>
<td>16</td>
<td>EDTA tubes</td>
<td>Hy. Labs. Israel</td>
</tr>
<tr>
<td>17</td>
<td>Microfuge tubes for PCR - thin wall 0.2 mL, 0.5 mL, 1.5mL capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>18</td>
<td>Disposable tips</td>
<td>Labcon, USA</td>
</tr>
</tbody>
</table>
3.7.3 PCR primers

The PCR primers used for amplifying the *NPHS2* gene exons and the expected product size are indicated (Table 3.3). Primers sequences were adopted from (Boute et al., 2000b).

**Table (3.3):** Primers and expected PCR product size.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 A</td>
<td>5′-GCAGCGACTCCACAGGGACT-3′</td>
<td>5′-GGACCTCATCCACGTCCAC-3′</td>
<td>289 bp</td>
</tr>
<tr>
<td>Exon 1 B</td>
<td>5′-GGTGACGTGATGATCT-3′</td>
<td>5′-TCAGTGGTCTCAGGGGAT-3′</td>
<td>177 bp</td>
</tr>
<tr>
<td>Exon 2</td>
<td>5′-AGGCAGTAACTAAGAG-3′</td>
<td>5′-GCCCTAGAAATCTCTAG-3′</td>
<td>203 bp</td>
</tr>
<tr>
<td>Exon 3</td>
<td>5′-TTCTGGGAGTGATTAAAG-3′</td>
<td>5′-TGAAGAAATGGCAAGTCAG-3′</td>
<td>168 bp</td>
</tr>
<tr>
<td>Exon 4</td>
<td>5′-AAGGTGAAACCAACACGC-3′</td>
<td>5′-CGGTAGGTAGACATGAAA-3′</td>
<td>204 bp</td>
</tr>
<tr>
<td>Exon 5</td>
<td>5′-CATAGGAAAGGGCACAAG-3′</td>
<td>5′-TTTCAAGCATATGGCCAT-3′</td>
<td>292 bp</td>
</tr>
<tr>
<td>Exon 6</td>
<td>5′-CTCCCACGTGACACAT-3′</td>
<td>5′-AATTTAATGAACACAGAA-3′</td>
<td>155 bp</td>
</tr>
<tr>
<td>Exon 7</td>
<td>5′-CTAAATCATGGCTGCACACC-3′</td>
<td>5′-TTTCCTAAAGGGCAGTCTGG-3′</td>
<td>167 bp</td>
</tr>
<tr>
<td>Exon 8</td>
<td>5′-GGTGAGCCCTCAGGGAAATG-3′</td>
<td>5′-TTCTATGGCAGGCCCCTTA-3′</td>
<td>380 bp</td>
</tr>
</tbody>
</table>
3.8 Methods

3.8.1 DNA extraction

3.8.1.1 DNA purification

Genomic DNA extracted from peripheral blood leukocytes using a commercial kit (Wizard gDNA Purification Kit; Promega-USA) following the manufacturer instructions. The isolated DNA was stored at -20°C until analysis.

3.8.1.2 Quality and quantity of extracted DNA

The quality of the isolated DNA was determined by running 5 µl of each sample on ethidium bromide stained 1.0% agarose gel. The DNA samples then visualized on a Gel documentation system. Additionally, the DNA concentration of each sample measured by the use of Nano-drop spectrophotometer.

3.8.2 NPHS2 gene mutation screening

3.8.2.1 Primers reconstitution

Primers were received in a lyophilized state. Primer containers first centrifuged at 14,500 rpm for 3 minutes and then reconstituted with ultrapure nuclease-free water to create a stock solution of each primer with a final concentration of 100pmol/µl. The stock primer solution was then vortex mixed. Thirty-microliter aliquot taken from the stock primer and diluted with 270-µl nuclease free water to make 10pmol/µl working solution.

3.8.2.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was done with a total volume of 30µl. The components of the (PCR) for each exon are shown in Table 3.4. PCR tubes were then placed in the thermal cycler and PCR amplification was done according to the program provided in Table 3.5. The PCR annealing temperatures for the various NPHS2 exons are given in Table 3.6.
Table (3.4): PCR components

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>2</td>
<td>20 pmol</td>
</tr>
<tr>
<td>Revers primer</td>
<td>2</td>
<td>20 pmol</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
<td>100 ng</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>PCR master mix</td>
<td>15</td>
<td>1X</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table (3.5): Thermal cycling program for PCR amplification of the NPHS2 gene.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>T (°C)</th>
<th>Duration</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94 °C</td>
<td>3 min.</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30 sec.</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>See (Table 3.6)</td>
<td>30 sec.</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>40 sec.</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72 °C</td>
<td>5 min.</td>
<td>1</td>
</tr>
<tr>
<td>Storage of product</td>
<td></td>
<td></td>
<td>4 °C</td>
</tr>
</tbody>
</table>

Table (3.6): Exons annealing temperatures.

<table>
<thead>
<tr>
<th>Exons</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61 °C</td>
</tr>
<tr>
<td>2</td>
<td>53 °C</td>
</tr>
<tr>
<td>3 and 6</td>
<td>51 °C</td>
</tr>
<tr>
<td>4</td>
<td>57 °C</td>
</tr>
<tr>
<td>5</td>
<td>55 °C</td>
</tr>
<tr>
<td>7 and 8</td>
<td>59 °C</td>
</tr>
</tbody>
</table>

3.8.2.3 Purification of PCR products

Cleaning of PCR product with PCR purification kit (Invitrogen, Germany)
3.8.3. DNA sequencing

3.8.3.1. Automated Sanger sequencing

Automated Sanger sequencing was employed for sequencing the nucleotides of the eight exons of the NPHS2 gene. The amount of the DNA template of the amplified and purified exons was in the range of 3-20 ng.

3.8.3.2. BigDye® terminator cycle sequencing

Nucleotide sequencing of the exons was performed using the BigDye® Terminator v1.1 Cycle sequencing kit (Applied Biosystem) together with an ABI310 automated DNA sequencer (Applied Biosystem) following the manufacturer instructions. Reaction mixture composition for BigDye® Terminator v1.1 is given in table 3.7. The thermal cycler program is provided in table 3.8.

Table (3.7): To prepare the reaction mixtures.

<table>
<thead>
<tr>
<th>1</th>
<th>For each reaction add the following reagent to a separate tube:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reagent</td>
</tr>
<tr>
<td></td>
<td>Terminator ready reaction mix.</td>
</tr>
<tr>
<td></td>
<td>Template</td>
</tr>
<tr>
<td></td>
<td>Primer</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
</tr>
<tr>
<td></td>
<td>Total volume</td>
</tr>
</tbody>
</table>

Table (3.8): The thermal cycler program.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>96 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>50 °C</td>
<td>5 sec</td>
</tr>
<tr>
<td>60 °C</td>
<td>4 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>Hold until ready to purify</td>
</tr>
</tbody>
</table>
3.8.3.3. Purification

Desalting (salt interferes with electrokinetic injection) and elimination of remaining labeled ddNTPs. Gel filtration Centri-sep columns (Princeton/ Applied Biosystem) used for purifying the obtained BigDye® Terminator labeled products following the manufacturer protocol.

3.8.3.4 Sample preparation for injection

BigDye® Terminator labeled products were mixed with Hi Di formamid (1:1 v/v) heated at 95 °C for 2 min and then put on ice for 1 min. Samples are now ready for injection into the ABI310 automated DNA sequencer (Applied Biosystem).

3.8.3.3.4 Principle of Capillary Electrophoresis

During capillary electrophoresis, the extension products of the cycle sequencing reaction enter the capillary as a result of electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separate by size based on their total charge. Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device on the genetic analyzers detects the fluorescence. The Data Collection Software converts the fluorescence signal to digital data, then records the data in a *.ab1 file. Because each dye emits light at a different wavelength when excited by the laser, all four colors, and therefore all four bases, can be detect and distinguished in one capillary injection. The results are then display as exemplified in (Figure 3.1).

Figure (3.1). An illustrative diagram of fluorescent sequencing result.
3.9 NPHS2 Gene Sequence Analysis

Obtained NPHS2 exons sequences were analyzed for nucleotide changes by comparing them to the reference sequences in the NCBI database (NCBI human genome build 38.7) using the NCBI BLAST (basic local alignment search tool) tool. Detected sequence changes then searched for novelty using the NPHS2 variation table stored in the Ensembl (http://www.ensembl.org) database.
Chapter 4

Results
Chapter 4
Results

The study cohort consisted of 20 children suffering from SRNS (10 girls and 10 boys). The patients were recruited from the Ranteesy Specialized Pediatric Hospital, Gaza. The mean age at onset of proteinuria was 3.57 years (range, 2 months to 6 years). Three (15.0%) patients had familial NS and 17 (85.0%) had sporadic NS. All patients were tested for the presence of NPHS2 gene mutations by sequencing the eight coding exons and their intron boundaries.

4.1 Isolated Genomic DNA

The following figure 4.1 represents samples of patients genomic DNA. The bands in the figure shows that the isolated DNA is of good quality since it is intact and of high molecular weight.

Figure (4.1): A photograph of ethidium bromide stained 3% agarose gel showing the genomic DNA samples of different patients (lanes 2 to 8). The first lane contains a size marker.
4.2 NPHS2 PCR-amplified exons

Figure 4.2 below illustrates examples of NPHS2 exons (exons 2, 5, 8) amplified with PCR.

![Figure 4.2: A photograph of ethidium bromide stained 3% agarose gel showing the PCR products of NPHS2 exons 2, 5 and 8.]

4.3 DNA Sequencing results

4.3.1 Detected Mutations

4.3.1.1 Exon 3 (c.388G>A) mutation

The following figure (4.3) shows the partial electropherograms of the transition mutation (c.388G>A) detected in one of the recruited SRNS patients. The patient was homozygous for this mutation. Consultation of the Ensembl variant table for NPHS2 gene mutation showed that this is a previously known pathogenic mutation (http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG 00000 116218; r=1:179550539-179575952). This nucleotide alteration leads to a missense mutation; replacing a normal glutamic acid at position 130 with lysine (i.e., p.E130K) in the podocin polypeptide.
Figure (4.3): Partial electropherograms for exon 3 (c.388 G>A) mutation. The left and right boxes illustrate the normal and the mutant sequences, respectively (note that the electropherograms are those of the reverse strands).

4.3.1.2 Exon 3 (c.412C>T) mutation

Another SRNS patients harbored the transition mutation (c.412C>T) whose partial electropherograms are indicated in Figure 4.4. This pathogenic nonsense mutation has been described before. The patient was homozygous for this mutation which introduces a premature stop codon in place of the amino acid arginine at position 138 (p.R138X) in the podocin polypeptide.

Figure (4.4): Partial electropherograms for exon 3 (c.412C>T) mutation. The left and right boxes illustrate the normal and the mutant sequences, respectively (note that the electropherograms are those of the reverse strands).
4.3.1.3 Exon 5 (c.686G>A) mutation

The third mutation detected in our cohort of SRNS patients was the transition (c.686G>A) in exon 5. The partial electropherograms illustrating this mutation are indicated in Figure 4.5. This previously reported missense mutation replaces the amino acid arginine at position 229 by glutamine (p.R229Q). The mutation was evident in homozygous form.

![Figure (4.5): Partial electropherograms for exon 5 (c.686G>A) mutation. The left and right boxes illustrate the normal and the mutant sequences, respectively.]

4.4 Summary of the identified mutations

Three patients comprising 15% of the study group harbored NPHS2 gene mutations. Table 4.1 below summarizes the detected mutations.

Table (4.1): NPHS2 sequence mutations identified in Palestinian SRNS patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Effect on protein</th>
<th>Genotype</th>
<th>Proteinuria onset (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>3</td>
<td>c.388G&gt;A</td>
<td>p. Glu130Lys</td>
<td>Homozygous</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>5</td>
<td>c.686G&gt;A</td>
<td>p. Arg229Gln</td>
<td>Homozygous</td>
<td>45</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>c.412C&gt;T</td>
<td>p. Arg138X</td>
<td>Homozygous</td>
<td>60</td>
</tr>
</tbody>
</table>

4.5 Age of onset of proteinuria and NPHS2 mutations

The disease-causing mutation in NPHS2 was detected in SRNS patients whose age of onset of proteinuria was <5 years (Table 4.1).
4.6 NPHS2 nucleotide variants

Analyses of the obtained exons sequences revealed several nucleotide variants that proved to be benign as reported in the literature and Ensembl database. The detected variants and their pertinent characteristics are illustrated in Table 4.2.

Table (4.2): *NPHS2* detected variants.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Polymorphism</th>
<th>Effect</th>
<th>Heterozygous/ Homozygous</th>
<th>Patients (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.102A&gt;G</td>
<td>P.Gly34Gly</td>
<td>Heterozygous</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>1</td>
<td>c.235G&gt;A</td>
<td>P.Gly79Lys</td>
<td>Heterozygous</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>8</td>
<td>c.1152C&gt;T</td>
<td>P.Ala318Ala</td>
<td>Heterozygous</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>8</td>
<td>c.925G&gt;C</td>
<td>P.Ala309Pro</td>
<td>Heterozygous</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>8</td>
<td>c.954C&gt;T</td>
<td>P.Ala318Ala</td>
<td>Heterozygous</td>
<td>3 (15%)</td>
</tr>
</tbody>
</table>

4.7 Renal Histologic Findings

Renal biopsy performed previously on 80% of the recruited patients were as follows: Focal segmental glomerulosclerosis (FSGS) was seen in 7 (35%), Minimal-change disease (MCD) was a frequent finding in 6 (30%) and Membrano-proliferative glomerulonephritis (MPGN) was seen in 3 (15%). These findings were adapted for patients records and are summarized in Table 4.3.

Table (4.3): Renal Histologic findings

<table>
<thead>
<tr>
<th>Renal Biopsy</th>
<th>Patients (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSGS</td>
<td>7 (35%)</td>
</tr>
<tr>
<td>MCD</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>MPGN</td>
<td>3 (15%)</td>
</tr>
</tbody>
</table>
Chapter 5
Discussion
Chapter 5
Discussion

Identification of mutations associated with genetic disorders is important because it clarifies the cause of the disease, influences physicians’ decision on patient treatment and management, and helps the patient's family take very important decisions for their future progeny. Moreover, as in the case of SRNS, patients harboring pathogenic mutations can be spared the side effects of useless immunosuppressive therapy and could be considered for kidney transplantation.

This study, the first of its kind in Palestine, was conducted in order to identify \( \text{NPHS2} \) gene mutations in 20 SRNS patients residing in Gaza strip. Based on our results, the incidence of podocin mutations in the examined cohort approached 15%. This figure is comparable to the \( \text{NPHS2} \) mutation detection rate reported from studies on Turkish and Brazilian SRNS children (Guaragna et al., 2015; Kitamura et al., 2006) but lower than that documented in European and American children (Kitamura et al., 2006; Caridi, Perfumo, & Ghiggeri, 2005; Weber et al., 2004; Caridi et al., 2003). Interestingly, no pathogenic \( \text{NPHS2} \) mutations were found in Japanese and Korean SRNS children (Kitamura et al., 2006). This controversy could be due to the high heterogeneity in the nephrotic syndrome and the associated genetic loci (so far more than 40 loci have been implicated in childhood nephrotic syndrome (Ha, 2017)) and reflects ethnic differences in disease genes.

In the SRNS patients examined in this study we could identify three pathogenic genotypes (all in homozygous form) comprising three different \( \text{NPHS2} \) mutations (E130K, R138X, and R229Q) in 3 out of the 20 investigated familial SRNS patients. It is important to indicate here that all the three affected individuals were the offspring of consanguineous parents.

The first mutation (E130K) was encountered in homozygous form in male patient #7. This missense mutation results from a G to A transition in the coding region of exon 3 at position 388. The mutation was also evident in the affected brother of the patient. The mutation replaces an acidic amino acid (glutamic acid) with a basic amino acid (lysine) in the podocin polypeptide. Obviously, the two
amino acids have different characteristics and is expected to impair the function of
the podocin. Moreover, this amino acid is among the amino acids located in the PHB
(prohibitin homology). PHB encompasses the amino acids 125 to 284 and is required
for the binding of podocin to nephrin (Huber et al., 2003). This mutation was
reported before by Kari et al. (2015) who identified this mutation in homozygous
form in two patients of Arabic origin (Sadowski et al., 2015). The familial and
autosomal recessive nature of this mutation was confirmed by detecting the same
genotype in the affected brother of the patient.

The second mutation (R138X) was detected in homozygous form in female
patient #21. This nonsense mutation is due to a C to T transition at nucleotide 412 in
exon 3. The mutation was also evident in the affected sister of the patient. The
genetic alteration in this mutation introduces a premature (TGA) stop codon in place
of the amino acid arginine at position 138 in the podocin sequence thus leading to the
production of a non-functional truncated polypeptide. This amino acid is also located
in the PHB domain of podocin and was shown to be crucial for the function of
podocin (Huber et al., 2003). R138X mutation was first described by Boute et al.
(2000) (Boute et al., 2000a). Interestingly, this mutation has been shown by
Frishberg et al. (2002) to be prevalent among Arab SRNS patients (where 15 out of
their 27 patients i.e., 55% harbored this mutation in homozygous form) a result
which made the authors raise the possibility of this mutation as being a founder
mutation among Arabs (Frishberg et al., 2002). This same mutation was also
reported by Becker-Cohen et al. (2007) in homozygous form, again in a girl of
Arabic descent (Becker-Cohen et al., 2007), further potentiating the importance of
this mutation in patients of Arab ethnicity.

The third mutation R229Q was documented in homozygous form in meal
patient # 17 who also has an affected sister. This missense mutation occurs as a result
of G to A transition at nucleotide 686 in exon 5 and leads to replacing the amino acid
arginine by glutamine. Exon 5 also constitutes part of the PHB domain and the
missense mutation is expected to influence the function of podocin. R229Q is the
most frequently encountered mutation in diverse populations (Tsukaguchi et al.,
2002). Based on the conservation of Arg 229 residue in podocin homologs,
segregation of the mutation with the disease, and functional study of the mutated
podocin made Tsukaguchi et al. (2002) consider this relatively common genetic change as a disease-causing mutation (Tsukaguchi et al., 2002).

In addition to the three identified pathogenic mutation sequencing data revealed four previously known benign polymorphisms (Table 5.1). All the polymorphisms were detected in heterozygous form. Two of the polymorphism (Gly34Gly in exon 1 and Ala318Ala in exon 8) were neutral in that the same amino acid was encoded despite the genetic alterations and were reported by several authors (Megremis et al., 2009; Berdeli et al., 2007; Franceschini et al., 2006).

Table (5.1): NPHS2 nucleotide variants

<table>
<thead>
<tr>
<th>Exon</th>
<th>Polymorphism</th>
<th>Effect</th>
<th>Heterozygous/Homozygous</th>
<th>Patients (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.102A&gt;G</td>
<td>P.Gly34Gly</td>
<td>Heterozygous</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>1</td>
<td>c.235G&gt;A</td>
<td>P.Gly79Lys</td>
<td>Heterozygous</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>8</td>
<td>c.1152C&gt;T</td>
<td>P.Ala318Ala</td>
<td>Heterozygous</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>8</td>
<td>c.925G&gt;C</td>
<td>P.Ala309Pro</td>
<td>Heterozygous</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>8</td>
<td>c.954C&gt;T</td>
<td>P.Ala318Ala</td>
<td>Heterozygous</td>
<td>3 (15%)</td>
</tr>
</tbody>
</table>

The third variant was a missense Glu79Lys in exon 1. Although this variant changes the amino acid replaces the acidic Glu with a basic amino acid (lys) still it has no effect on the function of podocin and is considered a benign variant. This could be due to the position of this amino acid in the intracellular N-terminus of the polypeptide. The fourth polymorphism was also a missense variant (Ala309Pro) of unknown effect (NPHS2 gene database: https://databases.lovd.nl/shared/variants/). This variant is located in the last exon of the podocin gene and lies outside the PHB domain.

Despite being classified as variants, due to mainly their presence in healthy individuals and their occurrence in high frequencies in the population, many genetic variants are known to act as risk factor or as modifiers of the disease state.

In conclusion, mutations in the NPHS2 gene contribute to some familial SRNS in Palestine. Mutations in this gene, exons 3 and 5 in particular, should be searched for in SRNS children in order to avoid unnecessary immunosuppressive steroid that may escalate patient morbidity. Identification of the responsible mutations will also
help the affected families in planning for carrier screening, prenatal and even pre-implantation genetic diagnosis. Further genetic work should be done for \textit{NPHS2}-negative families in order to identify the mutations in other genes (e.g., \textit{NPHS1}, \textit{WT1}, and \textit{PLCE1}) strongly implicated in the pathogenesis of SRNS.
Chapter 6

Conclusion & Recommendations
Chapter 6

Conclusion & Recommendations

6.1 Conclusion

The current study reports the identification of \textit{NPHS2} mutations in 3 out of 20 (15\%) familial SRNS patients in Palestinian cohort. The mutations were identified through nucleotide sequencing of the coding exons and the exon-intron boundaries of the \textit{NPHS2} gene. Analysis of DNA sequences revealed the following, previously known, pathogenic mutations: G130K, R138X, and R229Q. All detected mutations were encountered in homozygous form, thus confirming the autosomal recessive inheritance pattern of the disease. Knowledge of gene mutations associated with SRNS spares patients from unnecessary and harmful immunosuppressive steroids and helps physicians and patients' families take proper decisions regarding patient management and their future offspring.

6.2 Recommendations

1. Starting genetic studies on nephrotic syndrome patients with screening the identified mutations, and sequencing of \textit{NPHS2} exon 3 and 5.

2. Searching for pathogenic mutations in other SRNS-related genes, such as \textit{NPHS1}, \textit{PLCE1} and \textit{WT1}.

3. Providing awareness for patients' families, in whom mutations were identified, about the available options to avoid having affected children such as prenatal diagnosis and pre-implantation genetic diagnosis.
References
References


http://www.ensembl.org


http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000116218;r=1:179550539-179575952


children with steroid-resistant nephrotic syndrome. *Genetic testing and molecular biomarkers*, 13(2), 249-256.


Appendix

NPHS2 sample 21  exon 3  p.R138X;c.412 C>T

Homo sapiens NPHS2, podocin (NPHS2), RefSeqGene (LRG_887) on chromosome 1
Sequence ID: NG_007535.1  Length: 32411  Number of Matches: 1

Range 1: 19550 to 19679  GenBank  Graphics

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>217 bits(117)</td>
<td>6e-53</td>
<td>126/130(97%)</td>
<td>2/130(1%)</td>
<td>Plus/Plus</td>
</tr>
</tbody>
</table>

Query 1  
TTTCTGGAGTGA-TTGAAAGGATTAATTTCTCTTTGTTGTTTACAAAGAGTATGAAAGA 59

Sbjct 19550  
TTTCTGGAGTGGATTGAAAGGATTAATTTCTCTTTGTTGTTTACAAAGAGTATGAAAGA 19609

Query 60  
GTAATTATATATCTGACTGGGACATCTGTCTCTGGAAAGGCAAGGCCTGCTGAAAAAAA 119

Sbjct 19610  
GTAATTATATATCTGACTGGGACATCTGTCTCTGGAAAGGCAAGGCCTGCTGAAAAAAA 19669

Query 128  
aCA-TCATTT  128

Sbjct 19670  ACACCTCTTTT  19679

exon end