1.0 Definition of Nephrotic syndrome (NS)

NS is one of the most common manifestations of glomerular disease, characterized by heavy proteinuria and hypoalbuminemia or hypoproteinemia and its progressive forms can lead to Chronic Kidney Disease (CKD) and/or End-Stage Renal Disease (ESRD) (1). It is caused by increased permeability of serum protein through the damaged basement membrane in the renal glomerulus. Though, the disease has been reported in both children and adults, it is 15 times more common in children than adults (2). NS is classified into either primary glomerulopathy, accounting 90% or secondary glomerulopathy, associated with systemic diseases such as Henoch Schonlein purpura, systemic lupus erythematosus, hepatitis B infection, collagen and vascular diseases, encompassing the remaining 10% (3). Thus, NS is a constellation of renal and extra renal manifestations that can be caused by a multitude of systemic diseases (secondary) as well as by primary insults to the kidney (primary). Primary NS is the most frequent form of NS in children representing more than 90 percent of cases between 1 and 10 years of age and 50 percent after 10 years of age.

1.1 Structure and function of the kidney

The two kidneys are a pair of bean-shaped organs situated in the retroperitoneal space. The kidney size in an adult human averages 11-12cm in length, 5.0-7.5 cm in width and 2.5-3.0 cm in thickness. The weight of each kidney varies from 125 to 170g in the adult male and 115-155g in an adult female. The kidney is covered by three layers; that is from interior to the exterior (i) fibrous capsula, (ii) adipose capsula and (iii) renal fascia. A bisected kidney surface has two distinct regions the outer cortex has glomeruli and proximal tubules; the inner medulla rich in henle loops and collecting ducts. The structural and functional unit of kidney is nephron, each kidney has about one million nephrons (4). Every nephron consists of one renal corpuscle and its associated tubules. The renal corpuscle is further divided in to glomerulus, bowmans capsule and juxtaglomerular apparatus. One glomerulus composed of 5-7 capillary branches originating from the afferent artery, whose surface is extensively covered by glomerular podocytes. The lumen of the glomerular capillary is lined by a thin fenestrated
Introduction

Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

Between the podocytes and endothelium is a layer of a mesh-like structure, the glomerular basement membrane (GBM), which prevents leakage of plasma macromolecules (Figure 1.1).

![Figure 1.1: Schematic diagram of kidney structure (5)](image)

A: showing a single nephron with the glomerulus containing a tuft of capillaries and tubule showing of Loop of Henle.

B: showing a diagrammatic section of the glomerulus with the capillary tuft within the Bowman’s capsule separated by the Bowman’s space. The afferent and efferent arteries are seen on either side of the cut section of the tubule.

C: showing GBM containing the GMB with the inner endothelial layer of cells with their fenestrae and outer layer of the interdigitating foot process of the epithelial cells called podocytes.

Kidney has physiologically multiple functions. It is vital for maintaining the stable homeostasis of the human body; regulates body fluid and maintains osmolarity, electrolytes and excretion of wastes such as creatinine, urea, and uric acid. Furthermore, the kidney can produce and secrete erythropoietin and then regulate the maturation process of erythrocytes in the bone marrow. It also secretes rennin, stored in juxtaglomerula cells, used to adjust blood volume, blood vessel contraction and promote the secretion of other hormones. The kidney is a vital site for the action of 1,25-dihydroxyvitamine D3 (6).
One of the kidney’s most important functions is filtration of the blood by glomeruli, allowing excretion of fluid and waste product, while retaining all blood cells and proteins within the blood stream. During glomerular filtration, plasma fluid traverses several cellular and extracellular layers that make up the complex structure of the ultrafiltration unit. From inside outward, it consists of endothelial fenestrae, GBM, and epithelial foot processes with intervening slit diaphragms (Figure 1.2). The integrity of each of these structural elements is essential for the maintenance of normal ultrafiltration (6).

![Diagram of GFB](image)

**Figure 1.2: Schematic diagram of GFB** (7)

A. Diagrammatic section of GFB showing the endothelial layer with fenestrae and covered by endothelial glycocllyx, the GBM and the outer layer of the foot process interconnected by the filtration diaphragm.

B. Diagrammatic section showing molecular structure of the two foot processes and the interconnecting slit diaphragm.

The filtration of macromolecules across the glomerular filtration barrier (GFB) is restricted by two mechanisms: charge-selectivity and size selectivity (8). The endothelial cells and GBM have a net negative charge; it is due to presence of polyamines such as heparin sulphate proteoglycans. By electrostatic repulsion, negatively charged plasma proteins are restricted from penetrating and passing through the glomerular capillary wall. The glomerular capillary wall has size-selective pores with an approximate radius of 40 to 45Å. Under normal conditions, molecules greater than 42Å in diameter or more than 200 kDa are unable to cross the filtration barrier. (8). That is either a reduction in
negative charges of the GBM or structural injury results in an increase in the number of large pores in the GBM resulted in NS (9).

1.2 Clinical features of NS

Clinically, NS is characterized by a triad of massive proteinuria (>40 mg/m² per hour), hypoalbuminaemia (≤ 2.5 mg/dl), hyperlipidaemia (serum cholesterol >200 mg/dl) (10, 11) presence of edema and hypovolemia (12). Structural and functional abnormalities in the GFB resulting in severe proteinuria are responsible for the clinical manifestation (13). Alterations on the perm selectivity barrier of the glomerular capillary wall are not able to restrict the loss of protein such as albumin to less than 100 mg/m² body surface per day. Primarily, intermediate size (60-200 kDa) plasma proteins are lost, which leads to a marked change in plasma protein composition and resulted into fall of plasma oncotic pressure and rises in viscosity. The changes in plasma protein composition and oncotic pressure determine most of the secondary consequences of NS (12,14). The dysfunction of tubular reabsorption causes tubular proteinuria.

The main markers of tubular proteinuria are β2-microglobulin and globulins (15). If the levels of plasma proteins are increased, they can be filtered in excess of the reabsorption capacity of the tubules and then be present in the urine; this is called overflow proteinuria. Proteinuria is referred to as secretory proteinuria or histuria when the urinary proteins originate from surrounding tissues or other organs via excretion and secretion (13, 16). The proteinuria in childhood NS is relatively selective, constituted primarily by albumin. In the milder forms of NS, plasma albumin levels are reasonably preserved (>25 g/L) and the plasma volume is expanded. Severe NS is characterized by marked hypoalbuminemia (< 20 g/L and can fall below 10 g/L), severe edema and occasionally hypovolemia that is reflected by normal or low blood pressure (14).

In the first few years of life, children with NS often show periorbital swelling with or without generalized edema. Edema is the predominant feature resulting from an imbalance between the hydrostatic and colloidal osmotic pressure in the intravascular and extracellular compartments. As serum albumin falls below 20 g/L, compensatory mechanisms, such as activation of the renin-angiotensin-aldosterone axis with an
increased tubular reabsorption of sodium, further increase the formation of edema. Apart from these clinical features, oliguria, abdominal tenderness, fever, hematuria, uremia and thrombosis were found in descending orders in NS patients. Diabetes mellitus and hypertension are risk factors (17). Some patients may present with complications of NS as their initial manifestation like thromboembolism, septicaemia, skin sepsis, infections, peritonitis, malnutrition, anemia, and hypocalcemia (18).

1.3 Classification

Traditionally, NS is classified into primary and secondary subtypes (3); the former is due to primary glomerular diseases, while the latter is associated with specific etiologic events or a complication of other diseases. On the basis of their etiology, glomerular injury can be divided into either acquired (caused by metabolic toxins or environmental infection) or hereditary (genetics/familial). These two categories may have significant overlap with each other and in some cases, the cause of the disease is less apparent.

The disease further classified based on the (i) age of onset (ii) histopathology findings and (iii) patients response to steroid therapy. Classification based on the various criteria has summarized and shown below in Figure 1.3.

![Classification of NS](image)

**Figure 1.3: Disease classification**
1.3.1 Primary NS:

Primary NS is a group of diseases with the typical characteristics of NS, with unknown etiology. They are further categorized based on histopathology.

1.3.1.1 Histopathological classification:

According to histologic lesions, primary NS are categorized into minimal change nephrotic syndrome (MCNS), focal segmental glomerulosclerosis (FSGS), diffuse mesangial proliferative glomerulonephritis (DMPG) and membranous nephropathy (MN) (14).

1.3.1.2 Minimal Change Nephrotic Syndrome (MCNS):

MCNS is called as a Nil disease, as no significant lesions are detected in glomeruli (renal) morphology (3, 19). The disease is also called minimal change lesion or minimal change disease (MCD), to stress the relative paucity of glomerular lesions under light microscopy. The glomeruli appear largely normal, with mild increase of cellularity in the mesangial area and enlargement of epithelial cells. Proximal tubules may contain fine lipid droplets. The glomeruli lack deposits of immunoglobulins and complement. However, effacement and retraction of the epithelial podocytes have been observed under electron microscopy.

The obliteration of the slit pore membrane complex is also noted in most glomeruli and glomerular capillaries (20). Several factors have been considered to explain the dysfunction of the glomerular permselectivity in MCNS. Proposed etiologic factors include (i) charge neutralization of GBM-related molecules by circulating cationic proteins, (ii) glomerular permeability factors, (iii) lymphokines, (iv) allergens, and (v) overproduction of oxygen radicals (21, 22). MCNS is the most common form of NS in children, accounting for 80% of all cases in children aged 4 to 8 years and for 20% of all cases in adults (23). The subcategories of MCNS include nil disease, focal glomerular obsolescence, mild mesangial thickening, mild mesangial hypercellularity, and focal tubular changes.
1.3.1.3 Focal Segmental Glomerulo Sclerosis (FSGS):

FSGS is characterized by the presence of sclerosis in a part of the glomerular tuft (24). That is pathological findings revealed the presence of focal and segmental sclerotic lesions or scars, affecting a portion of glomeruli, usually those in the deeper, juxtamedullary cortex due to the higher perfusion pressure. Electron microscopy examination revealed the effacement of podocytes and the detachment of foot processes from the GBM, especially when heavy proteinuria is observed (25). This sub type is a heterogeneous disorder and the severe form of all types of glomerulopathy in children leading to ESRD. It occurs both in children and adults in 7-15% and 15-20 % of the primary NS, respectively (26).

1.3.1.4 Diffuse Mesangial Proliferative Glomerulonephritis (DMPG):

Diffuse mesangial proliferative glomerulonephritis (DMPG) is characterized by mesangial expansion due to increased mesangial matrix and cellularity without peripheral capillary basement membrane abnormalities. Immunofluorescence findings include a range of combinations of IgG, IgM, complement fractions and fibrinogen. In many cases, IgA predominates, an association considered predominantly and designated as IgA nephropathy. Waldherr et al. (27) reported that in the serial biopsies of DMPG, there was a progression from ‘minimal change’ to ‘diffuse proliferative GN’ and vice versa. This author has also reported that there was a close relationship between ‘diffuse mesangial proliferative GN’ and ‘minimal change’.

1.3.1.5 Membranous Nephropathy (MN):

The renal biopsy features of MN on light microscopy revealed almost normal glomeruli with diffuse thickening of the glomerular capillary walls (12). MN is caused either by the accumulation of immune complexes within the kidney itself (primary membranous Nephropathy) or kidney injury associated with or caused by another illness (secondary MN) such as systemic lupus erythematosis, hepatitis B and C, cancers of the lung or colon.
1.3.2 Secondary NS and systemic diseases:

These groups of diseases are caused by known etiologies (Table 1.1). Nevertheless, the secondary NS have the similar histologic patterns as to that of the primary NS, though they may exhibit some difference inclusion bodies (25).

<table>
<thead>
<tr>
<th>Table 1.1: Causes of secondary NS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medications</strong></td>
</tr>
<tr>
<td><strong>Infections</strong></td>
</tr>
<tr>
<td><strong>Neoplastic</strong></td>
</tr>
<tr>
<td><strong>Allergens</strong></td>
</tr>
<tr>
<td><strong>Multisystem disease</strong></td>
</tr>
<tr>
<td><strong>Heredofamilial and metabolic disease</strong></td>
</tr>
</tbody>
</table>

1.3.3 Classification based in Steroid response/Treatment:

Of the various drugs used in the management of NS, steroid administration remains the primary option. Steroid responsiveness is the most important prognostic indicator of NS and appears to be the single most important clinical parameter in differentiating patients of primary NS (28). Based on the response, the patients are classified into either Steroid-Sensitive Nephrotic Syndrome (SSNS) or Steroid-Resistant Nephrotic Syndrome (SRNS). The vast majority of patients with primary NS (>90 percent) respond to glucocorticoid therapy and are termed as SSNS. NS patients who do
not respond to glucocorticoid therapy are classified as SRNS. Among NS patients substantial inter-individual differences have been encountered in terms of responsiveness to the steroid therapy such as steroid resistance and pattern of disease relapse (29). A summary of patient response to therapy and their classification is shown Table 1.2.

<table>
<thead>
<tr>
<th>Table 1.2: A summary of NS classification based on their response to steroid therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remission</td>
</tr>
<tr>
<td>Relapse</td>
</tr>
<tr>
<td>Frequent relapses</td>
</tr>
<tr>
<td>Steroid dependence</td>
</tr>
<tr>
<td>Steroid resistance</td>
</tr>
<tr>
<td>Early non responder</td>
</tr>
<tr>
<td>Late non responder</td>
</tr>
</tbody>
</table>

1.3.3.1 Steroid-Sensitive Nephrotic Syndrome (SSNS):

About 80% of children with NS respond to corticosteroid therapy with complete remission of proteinuria and edema are said to be steroid sensitive (SSNS) or steroid responders. Among this steroid responsive group, the clinical course is variable, with up to 60% having frequent relapses upon stopped treatment or becoming dependent on steroid therapy to maintain them in remission. Although SSNS is largely viewed as a relatively benign chronic disease in childhood, many children have frequent relapses or
become steroid-dependent. Patients who are steroid-responsive have favorable long-term outcome with a very low risk of chronic renal disease.

1.3.3.2 Steroid-Resistant Nephrotic Syndrome (SRNS):

SRNS is defined as a child with NS who fails to show a complete remission of symptoms after using the full prescribed steroid treatment. About 10–20% of children (< 10 years of age) failed to respond to corticosteroids are classified as steroid resistant. The steroid resistance can be grouped into primary resistance (failure of complete remission after treatment for the first time) and secondary resistance (initially responds well to steroid regimen for a period of time, after which shows recurrence and failure of complete response) (30). FSGS, MPGN and MCD were the morphologic lesions seen in 70%, 44% and 7% of children with SRNS, respectively (31). The children with SRNS tend to progress to CKD (Stage V)/ESRD due to the progressive damage of the GFB. Molecular studies performed in children with sporadic primary SRNS have identified mutations in several genes encoding proteins involved in maintaining the integrity of GFB (32). Therefore, mutational analysis in SRNS would help in preventing unnecessary exposure to immuno-suppressants and their adverse effects, besides helping in prognostication.

1.3.4 Classification based on age on onset:

Based on the age of onset it is further classified as (a) congenital (before 3 months of age) (b) infantile (3-12 months) (c) early childhood onset (13 months to 5 years), (d) late childhood onset (6 to 12 years), (e) adolescent onset (13 to 17 years) and (f) adult onset (> 18 years) types (33,34). Moreover, age of onset may also be predictive of the underlying histologic lesion causing NS. While, the MCNS is seen in 80% of children diagnosed with NS before 6 years of age, it was 18% of those with FSGS and 2% of those with MPGN present for the same age. Within 5 years of diagnosis, 21% of children with FSGS developed ESRD and another 23% developed CKD. Thus in a child diagnosed as having FSGS, the risk of developing CKD or ESRD within 5 years is almost 50%. (33,34)
1.4 Epidemiology:

The annual incidence of NS has been estimated to range from 2 to 7 new cases per 100,000 children, and the prevalence is 1 in 6000 children (35). There is a male preponderance, at a ratio of 2:1 to females among young children. However, this gender disparity wanes by adolescence, making the incidence in adolescents and adults equal among males and females (12). Scrutiny of literature demonstrates the existence of ethnic differences in the incidence of NS (Table-1.3).

<table>
<thead>
<tr>
<th>Country</th>
<th>Study period</th>
<th>Incidence (100,000/year)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benghazi, Libya</td>
<td>1968-1982</td>
<td>11.6</td>
<td>Elzouki et al. (36)</td>
</tr>
<tr>
<td>Leicestershire, UK</td>
<td>1973-1982</td>
<td>Asians 9.4 Non-Asians 1.3</td>
<td>Feehally et al. (37)</td>
</tr>
<tr>
<td>Former Yorkshire Regional Health Authority, UK</td>
<td>1987-1998</td>
<td>2.3</td>
<td>McKinney et al.(38)</td>
</tr>
<tr>
<td>Erie county, USA</td>
<td>1946-1961</td>
<td>2.0</td>
<td>Schlesinger et al. (39)</td>
</tr>
<tr>
<td>Birmingham, UK</td>
<td>1979-1983</td>
<td>Asian 16.9 European 2.6</td>
<td>Sharples et al.(40)</td>
</tr>
<tr>
<td>Metropolitan area of Kansas City, USA</td>
<td>1984-1995</td>
<td>Caucasian 1.8 African American 3.6</td>
<td>Srivastata et al. (41)</td>
</tr>
<tr>
<td>New Zealand</td>
<td>2001-2004</td>
<td>1.9</td>
<td>Wong et al.(42)</td>
</tr>
<tr>
<td>Australia</td>
<td>Jul 1998 to Dec 2000</td>
<td>1.15</td>
<td>Hodson et al.(43)</td>
</tr>
<tr>
<td>Farwaniya and Jahra, Kuwait</td>
<td>1981-1985</td>
<td>7.2 (&lt;10years) 6.0 (&lt;12years)</td>
<td>Zaki et al. (44)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>2003-2006</td>
<td>1.52</td>
<td>El Bakkali et al. (45)</td>
</tr>
</tbody>
</table>
NS is six times more common among Asian children than in Caucasian children in the United Kingdom (40). In contrast, it is relatively less common among African children (3.6/100000). Similarly, despite 70-80% of cases of NS occur in children less than 6 years, the peak age of presentation is 2 years. A familial occurrence of NS is also a well-recognized phenomenon and there is an increased familial incidence, particularly among identical twins. In a report of 1877 children with NS in Europe, 3.3% of children were found to have affected family members, most often siblings (46). The disorder tends to occur in the siblings at the same age and with similar biopsy findings and clinical outcomes (46).

Consistent with the incidence, ethnic variation has been reported on the histological variant and the response to immunosuppressive treatment. In particular, Hispanic and black patients are more likely to have steroid unresponsive than are white patients (47). More black children develop CKD stage 4 and 5 a higher mortality compared to white children. Obiagwu et al. (48), reported 40% prevalence of FSGS in northern Nigerian population as compared with that of 50% documented in biopsied patients in Port Harcourt (48, 49). In India and Pakistan, the most common histological diagnosis in children was FSGS (50, 51) despite MCD was high for past three decades ago (52). An increase in FSGS than other sub-types was also found in Saudi Arabia (53), Iran (54) and South America (55). However, the frequency of MN and MPGN are essentially unchanged from the low percentages noted in the 1960s and 1970s (56, 57).

1.5. Pathogenesis:

All forms of NS are characterized by abnormalities in the podocyte and injury of this cell type typically leads to marked proteinuria (58). Podocytes are highly differentiated polarized epithelial cells. They have voluminous cell body, which bulges into the urinary space. Altered podocyte morphology includes cell swelling, retraction and fusion of foot processes, resulting in the formation of a diffuse cytoplasmic sheet along the GBM; other features include vacuolization in podocyte cytoplasm, occurrence of occluding junction with disruption of slit diaphragms, apoptosis, widening and
Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

Introduction

effacement of foot processes detachment of the podocyte from the GBM (Figure 1.4 a-d), decrease in the total length of slit-pore junction, displacement of the junction away from the GBM and regional denudation of the GBM (58).

Figure 1.4: Altered morphology of podocyte in NS (59)

Actin cytoskeletal proteins play an important role in the regulation of the plasticity of the podocyte and in the maintenance of the filtration barrier. Alpha actinin 4 (α-ACTN4) is an actin bundling protein that is responsible for the integrity of the podocyte cytoskeleton associated with cell motility. Kos et al. (60), generated mice that were defective of ACTN, which developed severely damaged podocytes and progressive kidney disease. The deficiency in ACTN4 increased the fluidity of podocytes and also
altered the cell motility and cell adhesion. This study showed that ACTN4 is important for cell movement and also normal podocyte function (60).

Adhesion of the podocyte to the GBM is also controlled by the expression of two adhesion complexes consists of integrins and dystroglycans (61). Reduced expression of dystroglycans is reported to associate with MCNS and coincidence with proteinuria suggesting the detachment of the podocytes (62). In FSGS, the expression of dystroglycans was reported to show no changes. However, focal areas of externally denuded GBM have been observed both in the glomeruli from patients with FSGS (63) and MCNS (64). In addition, a decrease in podocyte number has been shown to associate with the development of glomerulosclerosis and detachment. Thus, the podocytopenia is resulted due to podocyte injury, followed by either apoptosis or detachment.

Research on pathogenesis has also emphasized the importance of T lymphocyte dysregulation and vascular permeability factors that might alter podocyte function and permselectivity. A plasma factor may alter glomerular permeability, especially among patients with SRNS. Cultured T cells isolated from nephrotic patients have been reported to synthesize a factor or factors that produce transient proteinuria when injected into rats (65) or impair synthesis of glycosaminoglycans from podocyte (66). The association of NS with primary immunological disorders such as lymphoma, leukemia, thymoma, kimura’s disease, and castleman’s disease, and therapeutic agents such as interferon further support the role of immunonological factors on the development of NS.

NS is considered as a primary immune disease associated with immune-regulatory imbalance between T helper 1 cell (Th1) and T helper 2 cell (Th2) cytokines (29). Patients with MCNS display a defect in delayed type hypersensitivity response, which suggest an abnormal Th1 dependent cellular immunity. Genetic studies in patients with inherited NS have identified mutations in the genes that encode several podocyte proteins. Many genes that are highly expressed at GFB, podocytes and slit diaphragms, have been implicated in the pathogenesis of NS (67-69).
1.6 Genetic studies in NS:

The structure and functions of the glomerulus is maintained by the regulated expressions of many genes and their protein products. An undefined proportion of patients who are classically defined as steroid resistant harbor genetic mutations in podocyte specific genes. There are conflicting reports about the effectiveness of immune-based therapy in the setting of these mutations.

Mutations in those genes are reported to alter the cellular functions and resulted into NS (Table 1.4). Some of the important genes and their mutations implicated in the NS are *NPHS1* (Nephrin), *NPHS2* (Podocin), *CD2AP* (CD2 associated protein), *TRCP6* (Transient receptor potential ion channel 6), *ACTN4* (α-actinin-4), *LAMB2* (Laminin subunit beta 2), *WT1* (Wilms tumor 1) and *LMX1B* (LIM-homoeodomain protein). Identification of gene mutations can avoid the use of immunosuppressive medications with its consequence economic and toxic side effects.

![Molecular anatomy of the podocyte foot process](image)

**Figure 1.5: Molecular anatomy of the podocyte foot process** (58)
<table>
<thead>
<tr>
<th>S.No</th>
<th>Type of NS</th>
<th>Gene/chromosome</th>
<th>Protein</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CNS of Finish type</td>
<td>NPHS1 / 19q13.1</td>
<td>Nephrin</td>
<td>Key component of podocyte slit diaphragm</td>
</tr>
<tr>
<td>2.</td>
<td>Familial/ Sporadic SRNS</td>
<td>NPHS2 / 1q25.q31</td>
<td>Podocin</td>
<td>Establishment of podocyte slit diaphragm</td>
</tr>
<tr>
<td>3.</td>
<td>Familial FSGS</td>
<td>CD2AP / 6p12</td>
<td>CD2-associated</td>
<td>Cytoskeletal remodeling cell motility &amp; endocytosis</td>
</tr>
<tr>
<td>4.</td>
<td>Early-onset familial NS</td>
<td>PLCE1 / 10q23</td>
<td>Phospholipase C epsilon</td>
<td>Involved in cell growth and differentiation gene expression</td>
</tr>
<tr>
<td>5.</td>
<td>Reported in multiple diseases</td>
<td>MDR1 / 7q21</td>
<td>Multiple drug resistance, P-glyco protein</td>
<td>Functions as an energy-dependent drug efflux pump and reduces the intracellular concentrations of a wide range of drugs and xenobiotics</td>
</tr>
<tr>
<td>6.</td>
<td>Studied in Multiple diseases</td>
<td>ACE / 17q23</td>
<td>Angiotensin converting enzyme</td>
<td>Key role in renal pathophysiology. Regulate the blood flow and rate of filtration and indirectly contribute the NS</td>
</tr>
</tbody>
</table>

1.6.1 *NPHS1*:

*NPHS1* gene, encoding the protein nephrin is an essential component of the slit diaphragm between the podocyte (70). The *NPHS1* has a size of 26 kb, containing 29
exons and is located on chromosome 19q13.1 (71) (Figure 1.6). Its gene product, the nephrin molecule is a transmembrane cell adhesion protein, consist of 1241 amino acids with an estimated molecular size of 130 kDa. It contains eight extracellular Ig domains, one type III fibronectin motif, a transmembrane domain, and an intracellular domain, which contain nine tyrosine residues. Nephrin forms a zipper-like filter structure in the centre of the slit diaphragm between the podocytes and plays an important role in cell-cell signalling.

Figure 1.6: Structure and location of NPHS1 (67,72)

Nephrin is important for maintaining cell-cell contacts and for organizing the cytoskeleton of the podocytes. This notion is supported by studies, which revealed that nephrin was shown to bind actin, possibly via CD2AP, thus providing a link between the cytoskeleton and the slit diaphragm (73). In another study, nephrin was shown to co-precipitate with CD2AP, ZO1, CASK, P-cadherin and P120 catenin in a large multiprotein complex—an indication of possible interaction with these scaffolding and cell-adhesion proteins (73). Apart from structural function, nephrin has a role in signaling pathways; the intracellular domain of protein contains several tyrosine residues, which are potential targets for phosphorylation (73). Fyn, a member of src family kinase has been shown to phosphorylate nephrin (74, 75) which seems to be essential for the maintenance of slit diaphragm, as Fyn deficient mice have been reported to develop massive proteinuria (76). Mutations in NPHS1 lead to disruption of the filtration barrier.
and cause massive proteinuria. Despite, the frequency of \(NPHS1\) mutations differing among the populations (Table 1.5).

Mutations in nephrin have been shown to result in congenital nephrotic syndrome (77) in Finnish population. Patients with congenital nephrotic syndrome of the Finnish type present a developmental failure to form the podocyte slit diaphragm absent foot processes and massive proteinuria beginning before birth. The two common mutations in the \(NPHS1\) are Fin-major and Fin-minor. Of which, the former is a two base pair deletion (121-122 del CT) in exon 2 resulting in a frameshift and an early stop codon. The gene product is a truncated protein consisting of only 90 amino acids. The latter mutation is a nonsense mutation (3325 C→T) in exon 26, where a substitution of one nucleotide leads to a stop codon and consequently to a truncated protein of 1109 amino acids. Including those first/common mutations, a total of 68 mutations, such as deletions, insertions, splicing, nonsense and missense mutations have been reported in the nephrin gene (67-69).

<table>
<thead>
<tr>
<th>Population</th>
<th>Type of mutation</th>
<th>Mutation Frequency (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finish</td>
<td>Fin_major &amp; Fin_minor</td>
<td>98%</td>
<td>Lenkeri et al. (67)</td>
</tr>
<tr>
<td>African</td>
<td>Missense</td>
<td>66%</td>
<td>Lenkeri et al. (67)</td>
</tr>
<tr>
<td>Japanese</td>
<td>Nonsense</td>
<td>&lt;1%</td>
<td>Sako et al. (78)</td>
</tr>
<tr>
<td>European</td>
<td>Missense, nonsense, deletions, insertion, splice site mutations</td>
<td>39-55%</td>
<td>Heeringa et al. (79)</td>
</tr>
<tr>
<td>Turkish</td>
<td>Homozygous</td>
<td>39-55%</td>
<td>Heeringa et al. (79)</td>
</tr>
<tr>
<td>North American</td>
<td>Fin_major</td>
<td>39-55%</td>
<td>Kestila et al. (71)</td>
</tr>
<tr>
<td>Pakistan</td>
<td>Missense</td>
<td>5.5%</td>
<td>Abid et al. (34)</td>
</tr>
</tbody>
</table>

\(NPHS1\) mutations account for 39-55% cases of childhood NS and 40% of all cases of congenital nephrotic syndrome outside Finland indicating that this gene might cause a broad spectrum of clinical severity and age distribution in NS (16, 80). NS caused by \(NPHS1\) mutations constantly shows resistance to corticosteroid therapy and low
recurrence after transplantation. Primary congenital nephrotic syndrome is typically caused by mutations in genes encoding for components of the GFB.

*NPHS1* mutation specifically affects the kidney and is characterized by proteinuria already *in utero* and a severe NS soon after birth. It was reported that for an unknown reason, the placenta is disproportionately large weighing more than 25% of the birth weight in practically all newborns with *NPHS1* mutation (81). Since the discovery of the first podocyte gene *NPHS1* and its mutations in Finnish-type NS, mutations in seven other genes (*NPHS2, NPHS3/PLCE1, WT1, CD2AP, ACTN4, TRPC6, INF2*) strongly expressed in podocytes have been implicated in SRNS (80). A transmembrane protein structurally related to nephrin, designated Neph1, with podocyte specific gene expression in the kidney has been reported (82). Genetic deletion of Neph1 in mice caused foot process defacement and massive proteinuria leading to death within 2 months, a phenotype similar to that observed following nephrin deletion of mutation. Neph1 located at the slit diaphragm and binds nephrin via its extracellular domain (82).

1.6.2 *NPHS2*:

The gene, *NPHS2*, contains 8 exons located on chromosome 1q25-q31 (71) as denoted in Figure 1.7. The gene encodes a putative 383-amino acid protein of approximately 42 kD named podocin (83). Podocin molecule is an integral protein consists of membrane domain forming a hairpin structure with two cytoplasmic ends at the C- and N-terminus.

![Figure 1.7: Structure and location of NPHS2 (72,84)](image-url)
Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

The \textit{NPHS2} is exclusively expressed in podocytes, localized to the insertion site of slit diaphragm and has been shown to play a critical role in facilitating nephrin signaling (85). Mutations in the \textit{NPHS2} cause both familial (85) and sporadic SRNS (86, 87) and FSGS (88, 89). Mutations in the \textit{NPHS2} cause a recessive form of SRNS with an early childhood onset of the disease and renal morphology of FSGS. Despite the frequency of \textit{NPHS2} mutations differing among the populations (Table 1.6), it has been found in 20-40\% of congenital nephrotic syndrome of European origin and in adult onset form of FSGS (80, 90).

<table>
<thead>
<tr>
<th>Population</th>
<th>Type of mutation</th>
<th>Mutation frequency (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indian</td>
<td>Nonsense and missense</td>
<td>4</td>
<td>Vasudevan et al. (91)</td>
</tr>
<tr>
<td>Turkish</td>
<td>Missense</td>
<td>29</td>
<td>Berdeli A et al. (92)</td>
</tr>
<tr>
<td>African</td>
<td>Missense</td>
<td>3</td>
<td>Yu et al. (93)</td>
</tr>
<tr>
<td>Japanese</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Maruyama K et al. (94)</td>
</tr>
<tr>
<td>Chinese</td>
<td>Missense</td>
<td>4</td>
<td>Yu et al. (93)</td>
</tr>
<tr>
<td>Korean</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Cho YH et al. (32)</td>
</tr>
<tr>
<td>European</td>
<td>Missense</td>
<td>20-40</td>
<td>Pereira et al. (95)</td>
</tr>
<tr>
<td>North American</td>
<td>Missense and nonsense</td>
<td>40</td>
<td>Lowik et al. (96)</td>
</tr>
<tr>
<td>Pakistan</td>
<td>Missense</td>
<td>3.4</td>
<td>Abid et al. (34)</td>
</tr>
</tbody>
</table>

There are different \textit{NPHS2} mutations, comprising nonsense, frameshift and missense mutations, to segregate with the disease, demonstrating a crucial role for podocin in the function of the GFB. The phenotype in mice lacking podocin strongly supports this concept (82). They develop extensive podocyte lesions and proteinuria before birth and then die from uremia after a few days of life (82). It is noteworthy that \textit{NPHS2} \textsuperscript{−}/\textsuperscript{−} mice do not develop FSGS but present pathologic features of diffuse mesangial sclerosis and tubular dilatation with vacuolization of epithelia more reminiscent of the human pathology due to the mutation of nephrin. \textit{NPHS2} \textsuperscript{−}/\textsuperscript{−} mice showed extensive
effacement of foot process and lack podocin and nephrin (82). A comparable alteration of podocin-nephrin interaction was demonstrated by Huber et al. (97), utilizing cells transfected with intact nephrin and podocin R138Q and R138X mutants. The authors also have demonstrated that mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains.

Other genes, such as WT1, TRPC6, ACTN4, FSGS2 and LMX1B (nail patella syndrome) have been implicated in the causation of NS in children and adolescents (80, 90). Of those a gene encoding alpha-actinin-4 has been shown to be associated with autosomal dominant FSGS. Mutations in WT1 are associated with Denys-Drash syndrome (characterized by male pseudo hermaphroditism, NS and Wilms’ tumour) and Frasier syndrome (male pseudo hermaphroditism, FSGS and gonadoblastomas). NS with FSGS has also been reported in patients with mitochondrial cytopathies, presenting with isolated NS or in association with myopathy, encephalopathy and lactic acidosis.

In addition to the above monogenic causes polymorphisms in many other genes (ACE, cytokines or growth factors, apolipoprotein E (APOE), paraoxonase 1 (PON1), multidrug resistance protein 1 (MDRI) and glucocorticoid receptor (NR3C1) including NPHS1 and NPHS2 (67, 93, 98) have also been studied for their association with the risk or progression of NS. Alterations in these genes may act as modifiers of the clinical course of NS. Of the many genes, a large body of studies has been focused on the polymorphism in the ACE as a probable genetic risk factor that has been implicated in the development and progression of NS (99, 100).

1.6.3 ACE:

ACE is a carboxyl terminal dipeptidyl exopeptidase and a key enzyme of Renin-Angiotensin-System (RAS). It converts the inactive angiotensin I into a vasoactive and aldosterone-stimulating peptide angiotensin II (100, 101). The ACE maps to chromosome 17q23, spans 21 kb, and comprises 26 exons (Figure 1.8). ACE shows a polymorphism at intron 16 which is characterized by the presence or absence of 287-bp element. The
Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

Introduction

region is highly polymorphic and genotypes are D/D, I/D, and I/I among subjects. There is an association between those genotypes and the levels of ACE (102).

![Figure 1.8: Structure and location of ACE (72,103)](image)

Over expression of ACE might be responsible for the elevated plasma angiotensin II level (104), a key regulator for circulation of ACE to different tissues and organ system. The functional receptors of angiotensin II have been demonstrated on glomerular podocytes (104) and an elevated angiotensin II level makes deleterious effects on renal hemodynamics and induces the expression of other growth factors, leading to glomerulosclerosis (100). Angiotensin II may contribute to increase systemic and glomerular blood pressure, proliferation and matrix production by renal cells, tubule interstitial fibrosis, and glomerulosclerosis (105, 106). A number of mechanisms have been shown to underlie the pathogenesis of angiotensin II effects on the transglomerular passage of protein. These include modulation of the efferent arteriolar tone, intraglomerular pressure, and glomerular plasma flow as well as changes in the ultrafiltration coefficient and size-dependent barrier functions (107,108).

Previous studies have shown that children with NS were found to have an increased prevalence of homozygosity for the deletion allele (D) of the ACE (109,110). DD homozygous subjects are associated with elevated circulating and tissue ACE activity.
Introduction

Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

compared to I/I or I/D genotypes (111,112). Therefore, it has been thought that the DD genotype may link to the ACE-related pathophysiology of renal diseases, and predict a patient response to steroid therapy. Hori et al. (113), reported that the frequency of DD genotype was higher in FSGS patients than in healthy subjects. Lee et al. (109) reported that FSGS patients with DD genotype showed a lower responsiveness to corticosteroid therapy and a higher incidence of chronic renal failure than those with other genotypes. It is possible that increased angiotensin II in the DD genotype may be causing steroid resistance through increased production of nephrin and nephrin N-glycosylation at the podocyte. While, administration of angiotensin II induces proteinuria in rats (114), ACE inhibitors and angiotensin receptor blockers reduce proteinuria in patients with NS (108,115). Thus, the reduction of proteinuria with ACE inhibitors and ARB in patients with proteinuria stresses the role of RAS in the pathogenesis of these syndromes.

1.6.4 MDR1

The MDR1, is located on chromosome 7q21.12; containing 29 exons encode a protein of 1280 amino acids (Figure 1.9). MDR1 encodes for P-glycoprotein (P-gp), a pivotal member of ATP-binding cassette (ABC) transporters and plays a very important role in the processes of absorption, distribution, metabolism, excretion of a wide variety of drugs, as well as in the drug-drug interaction (116, 117). It is highly polymorphic and over 50 single nucleotide polymorphisms (SNPs) have been reported (118, 119).

Figure 1.9: Structure and location of MDR1 (72,120)
Earlier studies have reported that certain SNPs in the \textit{MDR1} are associated with altered drug disposition (120, 121). SNPs may change the protective role of P-gp and thus influence disease risk (122). C3435T SNP (rs1045642), is a silent mutation and located in exon 26, affects the expression and function of P-gp (123), showed an increased risk of some diseases such as renal tumor, Crohn’s disease, ulcerative colitis, Parkinson’s disease, HIV infection (124). \textit{MDR1} expression was significantly elevated in SRNS patients (particularly relapse cases) than SSNS patients whether in activity or in remission (125). Individuals homozygous for 3435T had significantly lower \textit{MDR1} and P-gp expression levels than homozygous C3435 carriers (125). Two additional SNPs, G2677T/A in exon 21 and C1236T in exon 12, were found to be in linkage disequilibrium with \textit{MDR1} C3435T and also seem to influence P-gp function (29). Mutations of the gene encoding for the podocyte proteins nephrin, podocin, CD2AP and α-actinin-4 resulting in structural changes in the slit diaphragm (126) or factors that modulate the disease response to pharmacological interventions, such as the expression of P-glycoprotein (P-gp), a product of multidrug resistance gene-1 (\textit{MDR1}) gene (127).

\textbf{1.7 Diagnosis:}

Appearance of edema is first symptom to suspect a NS. However laboratory investigations are needed to delineate the etiology of the edema as it is a general symptom in many pathological conditions. The laboratory tests should confirm (i) nephrotic-range proteinuria, (ii) hypoalbuminemia, and (iii) hyperlipidemia. Therefore, initial laboratory testing should include the following: urinalysis, urine protein quantification (by first-morning urine protein/creatinine or 24-hour urine protein), serum albumin and lipid panel. Once the presence of NS has been established, the next step is to determine its type (primary or secondary to a systemic disorder). Therefore, in addition to the above tests, parameters such as complete blood count, metabolic panel (Serum electrolytes, BUN and creatinine, calcium, phosphorus, and ionized calcium levels), testing for HIV, hepatitis B and C, complement studies (C3, C4), antinuclear antibody (ANA), anti-double-stranded DNA antibody (in selected patients), 25-OH-vitamin D; 1,25-di(OH)-vitamin D; free T4; and thyroid-stimulating hormone (127) are carried out routinely.
The optional tests include (1) Genetic studies (molecular testing) (2) kidney ultrasonography and (3) chest radiography. In addition to those laboratory investigations, renal biopsy remains the gold standard in the diagnosis of NS in adults and not in children. However, renal biopsy is generally limited to steroid-unresponsive and steroid-dependent patients (127, 128).

1.8 Treatment and Management:

Steroids are the mainstay of therapy for children with NS. The initial therapy for childhood NS comprises oral glucocorticoids (prednisone 60 mg/m² per day for 4 weeks, with the maximum dosage of 80 mg). This is followed by prednisone 40 mg/m² on alternate days for 4 weeks, with a steroid taper over 3 to 6 months (12). Most patients respond to steroid therapy and a high proportion of them relapses but continues to respond throughout the subsequent course of the disease (38). To prevent relapses, they were also administered levamisole (2 mg/kg on alternate days). Cyclosporine may be useful in steroid-dependent patients with signs of steroid toxicity and after a failure of a course of alkylating agent (28). Almost 85% of patients respond to cyclosporine, but they relapse after tapering or stopping the drug. In SRNS patients, there is no study showing a clear-cut beneficial effect of alkylating agents, as the remission rate after treatment is close to the rate of spontaneous remission. Cyclosporine in association with prednisone may be effective, but the risk of nephrotoxicity seems to be higher than in steroid-dependent patients (35).

Roughly 95% of patients with MCNS and 20% with FSGS achieve remission after an 8-week course of prednisone (60 mg/m² daily for 4 weeks followed by 40 mg/m² on alternate days for 4 weeks) (129). Traditionally, patients receive divided doses but once-daily treatment also seems to be effective and majority of those patients (75%) respond within 2 weeks (12, 129). Given the high relapse rate for MCNS patients, there has been a shift in the past decade to longer courses of corticosteroid treatment for first episodes of NS in an effort to decrease the relapse rate (12). A mainstay of therapy in steroid-resistant FSGS is the use of ACE inhibitors, which reduce proteinuria and the rate
Introduction

Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

of decline in glomerular filtration rate (GFR) in a variety of forms of glomerular disease (130). Another therapeutic agent is mycophenolate mofetil, which in sporadic reports and open-label trials resulted in relapse in <50% of patients (131). Non-steroidal anti-inflammatory agents also have been used, usually in association with ACE inhibitors. This therapeutic approach, although it sometimes reduces proteinuria, has the disadvantage of causing a decline in GFR and a rise in serum potassium values (132). Pathophysiologic consequences of NS such as hypovolemia, acute renal failure, edema, hypercoagulation, and infections should be treated symptomatically (12).

1.9 Prognosis:

Treatments for NS and its complications have reduced the morbidity and mortality once associated with the syndrome. Currently, the prognosis for patients with primary NS depends on its cause. Infants with congenital nephrotic syndrome have a dismal prognosis: survival beyond several months is possible only with dialysis and kidney transplantation. Vital renal prognosis is dependent on non-modifiable variables such as the cause of NS, renal function and age at presentation, as well as the extent of renal interstitial lesions on the biopsy. Steroid responsiveness is the major determinant of prognosis in NS. Approximately 85 to 90% of patients with NS respond to steroid treatment with complete remission of proteinuria, while 10 to 15% have partial or even no response to steroid therapy (133). Only approximately 20% of patients with focal glomerulosclerosis undergo remission of proteinuria; an additional 10% improve but remain proteinuric. Many patients experience frequent relapses, become steroid-dependent, or become steroid-resistant. End-stage renal disease (ESRD) develops in 25-30% of patients with FSGS by 5 years and in 30-40% of these patients by 10 years.

The prognosis may worsen because of an increased incidence of renal failure, complications of secondary to NS or treatment-related conditions, such as infectious complications of immunosuppressive drug therapy. The prognosis of MCD is better in children than adults. At least 70% of children with MCD enter adult life without renal injury or urinary abnormalities. In contrast, a much less favourable outcome is expected if the NS is associated with FSGS or MPGN (134, 135). Adults with MCD also have a
good prognosis, with more than 90% surviving 10 years or more without the development of ESRD. Although adults with MCD resemble children in many respects, they go into acute renal failure more frequently and are more likely to have hypertension and diminished renal function. Adults respond slower and slightly less often to both steroids and cytotoxic agents but relapse less frequently and have more stable remissions after cyclophosphamide treatment (136). When compared with adults with FSGS, patients with MCD have better prognosis.

1.10 Scope of the Investigation:

From the foregoing literature, it is understood that etiology of the NS is diverse and the burden of the disease varies among the population. Despite the presence of effective treatment options, response to the therapy is differed among the patients even of the same sub-group. Thus, not only the risk but the response to treatment also reported to be influenced by mutations and SNPs in candidate genes *NPHS1* and *NPHS2* as well as associated pathways that regulate the kidney functions such as *ACE* and *MDRI*. While, limited studies on the candidate gene mutations has been reported in Indian population, identification of such mutations and SNP would aid better management of SRNS patients.
1.11 Hypothesis of the present study is represented in flowchart 1.

Flowchart 1: Hypothesis of the study

1.12 Aim and Objective:

To investigate mutations in \( NPHS1 \), \( NPHS2 \) and polymorphisms in \( ACE \) and \( MDR1 \) and its association with the pathogenesis of NS.

Objectives of the study

- To identify the mutations in \( NPHS1 \) and \( NPHS2 \) of NS patients (SSNS and SRNS) and to compare it with healthy control subjects.
- To identify SNP of \( ACE \) (I/D) and \( MDR1 \) (rs1045642, rs2032582, rs1128503) in NS patients and healthy subjects as an association factor.
References:


Introduction


Introduction


