Pharmacogenetic Study of Prednisolone Resistance in Childhood Nephrotic Syndrome Patients of Bangladesh

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DECLARATION

I do hereby declare that the materials embodied in this thesis entitled "Pharmacogenetic Study of Prednisolone Resistance in Childhood Nephrotic Syndrome Patients of Bangladesh" prepared for submission to the University of Dhaka, Dhaka, Bangladesh for the Degree of Doctor of Philosophy in Clinical Pharmacy and Pharmacology are the original research works of mine and have not been previously submitted for the award of any Degree or Diploma of the University of Dhaka or any other University or any other institute of learning.

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CERTIFICATE

This is to certify that the materials included in this dissertation are the original research work conducted by Most. Nazma Parvin, Registration No.: 117, Session: 2013-2014, Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh. The dissertation contains no material previously published or written by another person except when due reference is made in the text of the dissertation.

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ABSTRACT

Nephrotic syndrome is a common childhood kidney disease characterized by protein leakage from the blood to the urine through the glomeruli, resulting in proteinuria, hypoalbuminemia, generalized edema and hypercholesterolemia. The cause of nephrotic syndrome is still unknown. Till now no pharmacogenetic study of ABCB1, NR3C1 and CYP3A5 genes have been reported on the Bangladeshi pediatric prednisolone resistance nephrotic syndrome patients. Additionally, researchers studied several genetic polymorphisms of ABCB1, NR3C1 and CYP3A5 genes to explain their influence on different patients' resistance to steroids; however, the results were inconsistent. Therefore, we aimed to investigate the association of ABCB1 gene polymorphisms 1236T>C (rs1128503), 2677G>T (rs2032582) and 3435T>C (rs1045642), NR3C1 gene polymorphisms rs10482634 and rs6877893 and CYP3A5*3 (rs776746) polymorphism of the CYP3A5 gene with the risk of developing prednisolone resistance in nephrotic syndrome in Bangladeshi population. A case-control study was carried out on 180 nephrotic syndrome patients and the patients were recruited from different hospitals of Bangladesh. Among them, 30 had prednisolone resistance nephrotic syndrome were considered as cases and 150 had prednisolone sensitive nephrotic syndrome were considered as controls. After isolation of genomic DNA, genotyping was done by polymerase chain reaction-restriction fragment length polymorphism method. The risk of prednisolone resistance nephrotic syndrome was estimated as an odds ratio (OR) with 95% confidence interval (CI) using unconditional logistic regression models. A significant association was found between 2677G>T, 3435T>C SNPs of the ABCB1 gene, rs10482634 SNP of the NR3C1 gene and the prednisolone resistance risk in nephrotic syndrome patients.

From our present study, we found that the 1236T>C polymorphism of the ABCB1 gene was not significantly associated with prednisolone resistance risk in childhood nephrotic syndrome patients (p >0.05). The GT heterozygous of 2677G>T polymorphism was found significantly responsible for the development of prednisolone resistance nephrotic syndrome (OR = 3.9, 95% Cl = 1.11 to 13.70, p = 0.034) and it possesses 3.9 times higher risk compared to GG normal homozygous genotype. The TT mutant homozygous and combined heterozygous and mutant homozygous GT+TT genotypes were found to have 1.18 and 1.46 times more risk for

the development of prednisolone resistance compared to GG normal homozygous genotype, respectively, but these results were not statistically significant. In case of 3435T>C SNP of the ABCB1 gene, TC heterozygous genotype was found to have 0.38 times lower risk of developing prednisolone resistance compared with TT homozygous wild-type and it was statistically significant (OR = 0.38, 95% CI = 0.15 to 0.99, p = 0.047). The CC mutant homozygous genotype was found to be significantly associated (OR = 3.06, 95% Cl = 1.06 to 8.79, p = 0.038, respectively) with 3.06 times elevated risk of prednisolone resistance nephrotic syndrome whereas the combined heterozygous and mutant homozygous TC+CC genotype shown a lower risk of developing prednisolone resistance nephrotic syndrome but this finding was not statistically significant (OR = 0.71, 95% Cl = 0.32 to 1.58, p = 0.408).

In this present study, we also examined two polymorphisms of the NR3C1 gene. In case of rs10482634 polymorphism, the TC heterozygous and combined heterozygous and mutant homozygous TC+CC genotypes were significantly found to be responsible for the development of prednisolone resistance in childhood nephrotic syndrome (OR = 2.40, 95% Cl = 1.07 to 5.40, p = 0.033; OR = 2.36, 95% Cl = 1.06 to 5.21, p = 0.034, respectively) and they showed 2.40 and 2.36 times greater risk of developing prednisolone resistance compared to TT wild genotype, respectively. The CC mutant homozygous was not significantly associated with prednisolone resistance and it possesses 1.80 times higher risk for the development of prednisolone resistance nephrotic syndrome. For the rs6877893 SNP, we observed that there was no significant association between rs6877893 and the risk of prednisolone resistance in children with nephrotic syndrome (p >0.05).

For CYP3A5*3 polymorphism of the CYP3A5 gene, we found no significant association between CYP3A5*3 polymorphism and prednisolone resistance risk in childhood nephrotic syndrome patients (p >0.05).

This study demonstrates that the presence of GT heterozygous genotype of 2677G>T polymorphism, CC mutant homozygous genotype of 3435T>C polymorphism of ABCB1 gene as well as TC heterozygous and combined heterozygous and mutant homozygous TC+CC genotypes of rs10482634 polymorphism of the NR3C1 gene are associated with the risk of prednisolone resistance development in Bangladeshi nephrotic syndrome children.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A	Adenine
ABCB1	ATP-Binding Cassette sub-family B member 1
ACTH	Adrenocorticotrophic Hormone
ADRs	Adverse Drug Reactions
Ala	Alanine
ALL	Acute Lymphoblastic Leukemia
APN	Arbeitsgemeinschaftfur Padiatrische Nephrologie
ATP	Adenosine Tri Phosphate
BICH	Bangladesh Institute of Child Health
BMI	Body Mass Index
bp	Base pair
BSMMU	Bangabandhu Sheikh Mujib Medical University
C	Cytosine
ºC	Degrees Celsius
CAKUT	Congenital Anomalies of Kidney and Urinary Tract
cDNA	Complementary DNA
CD243	Cluster of Differentiation 243
CD2AP	CD2-associated protein
CI	Confidence Interval
cm	Centimeter
Co.	Company
cSNP	Coding Single Nucleotide Polymorphism
CYP	Cytochrome P450

DBD	DNA-Binding Domain
dbSNP	Single Nucleotide Polymorphism Database
dl	Decilitre
DMCH	Dhaka Medical College Hospital
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EDTA-Na_2	Ethylenediaminetetraacetic Acid Disodium
ESRD	End-Stage Renal Disease
EtBr	Ethidium Bromide
FP	Forward Primer
FSGS	Focal Segmental Glomerulosclerosis
G	Guanine
g	Gram
GC	Glucocorticoid
GCP	Good Clinical Practice
GFB	Glomerular Filtration Barrier
Gly	Glycine
GN	Glomerulonephritis
GR or GCR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Elements
h	Hour
HE	Heterozygote
HIV-1	Human Immunodeficiency Virus-1
hsp	Heat shock protein

HWE	Hardy-Weinberg Equilibrium
Hz	Hertz
ICH	International Conference of Harmonization
IgA	Immunoglobulin A
IgAN	Immunoglobulin A Nephropathy
IgMN	Immunoglobulin M Nephropathy
Ileu	Isoleucine
Inc.	Incorporated
INS	Idiopathic Nephrotic Syndrome
indels	Insertions and deletions
ISKDC	International Study of Kidney Diseases in Children
kb	Kilobase
kg	Kilogram
L	Liter
Ltd.	Limited
M	Molar
m	Meter
MDR1	Multidrug Resistance 1
MesPGN	Mesangioproliferative glomerulonephritis
mg	Miligram
$MgCl_2$	Magnesium Chloride
MH	Mutant Homozygote
min	Minute
ml	Millilitre
mM	Millimolar

MN	Membranous Nephropathy
MPGN	Membranoproliferative Glomerulonephritis
mRNA	Messenger Ribonucleic Acid
n	Sample size
Na	Sodium
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnological Information
NEB	New England Biolabs
MCNS	Minimal Change Nephrotic Syndrome
ng	Nanogram
NH	Normal Homozygote
NIKDU	National Institute of Kidney Disease and Urology
nm	Nanometer
nM	Nanomolar
NR3C1	Nuclear Receptor subfamily 3, group C, member 1
NS	Nephrotic Syndrome
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
OD	Optical Density
OR	Odds Ratio
PCR	Polymerase Chain Reaction
P-gp	Permeability glycoprotein
P^H	Potential of Hydrogen
PO	Medication to be taken orally
PLCE1	Phospholipase C Epsilon 1
PRNS	Prednisolone Resistance Nephrotic Syndrome

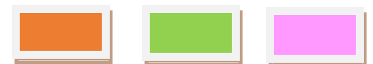
Pre-mRNA	Preliminary Messenger Ribonucleic Acid
PSNS	Prednisolone Sensitive Nephrotic Syndrome
pSNP	Perigenic Single Nucleotide Polymorphism
®	Registered Trademark
REase	Restriction Endonuclease
REs	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RP	Reverse Primer
rpm	Revolutions per minute
rs	DbSNP record ID number
RTI	Respiratory Tract Infection
S	Second
Ser	Serine
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Product and Service Solutions
SS	Steroid Sensitive
SSNS	Steroid Sensitive Nephrotic Syndrome
SRNS	Steroid Resistant Nephrotic Syndrome
SR	Steroid Resistance
T	Thymine
TAC	Tacrolimus
TAE	Tris-Acetate-EDTA

TBE	Tris-Borate EDTA
TE	Tris-EDTA
Tm/MT	Melting Temperature
TM	Trademark
TRPC6	Transient receptor potential cation channel, subfamily C, member 6
Tris	Tris (hydroxymethyl)-aminomethane
Tris-HCL	Tris-Hydrochloride
U	Unit
UK	United Kingdom
USA	United States of America
UTI	Urinary Tract Infection
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
VNTRs	Variable Number Tandem Repeats
vs	Versus
v/v	Volume per volume
wk	Week
WT1	Wilm’s Tumor
w/v	Weight per volume
yr	Year
>	Greater than
%	Percent
<	Less than
µl	Microlitre

μg	Microgram
μM	Micromolar
μ	Micro
α	Alpha
β	Beta
χ	Chi

CHAPTER ONE

INTRODUCTION



1. INTRODUCTION

1.1 Nephrotic Syndrome

There are different types of glomerular diseases among them nephrotic syndrome is one of the most frequent among children. It is also called nephrosis. It is described by a structural and functional deficiency in the glomerular filtration barrier (Bagga, 2009) which causes excessive loss of protein from blood to the urine through the glomeruli (Levine, 2003). When the blood protein is lost in the urine this leads to puffiness or swelling (edema), often to the eyelids, feet, ankles and eventually the abdomen. It is still unknown the exact cause of the nephrotic syndrome and it is usually triggered by time to time imbalance of the body's immune system. The reasons for the imbalance are certain types of chemical substances to disturb during the filtration through the kidney. These filters have started to pass the protein through the urine. In the body, nephrotic syndrome causes heavy proteinuria (proteinuria of greater than 3.5 g/ 24 h/ 1.73 m^2s or 40 mg/h/m^2 in children between 3 and 3.5 g/ 24 h is considered to be proteinuria in the nephrotic range), hypoalbuminemia (less than 2.5 g/dl), hyperlipidemia, hypercholesterolemia (>200 mg/dl), lowered plasma oncotic pressure, weight gain and edema follow continuous loss of large amounts of protein in urine and then it is buildup of body fluid (Gordillo and Spitzer, 2009).

Childhood nephrotic syndrome can affect the children at any age, from infancy to adolescence. It is more common in boys than in girls (2:1) and most commonly appears between the age of 2 and 6 years (Cameron, 1970). The prevalence worldwide is approximately 16 cases per 100000 children with an incidence of 2 to 7 per 100000 children (Tecile and Jessica, 2015).

According to genetic, racial, environmental differences as well as geographical locations, the pattern of childhood kidney diseases varies. It is observed that the spectrum of pediatric renal diseases starts from Congenital Anomalies of Kidney and Urinary Tract such as obstructive uropathy and other congenital urological manifestations to acquired kidney disorders such as glomerulonephritis, kidney stone diseases and urinary tract infections (Salusky *et al.,* 2009).

The first recorded description of nephrotic syndrome dates to the 15th century. Later, Volhard and Fahr popularized the term nephrosis, using it to describe a major classification of bilateral renal disease. Today nephrotic syndrome is recognized as a common chronic illness in childhood (Eddy and Symons, 2003).

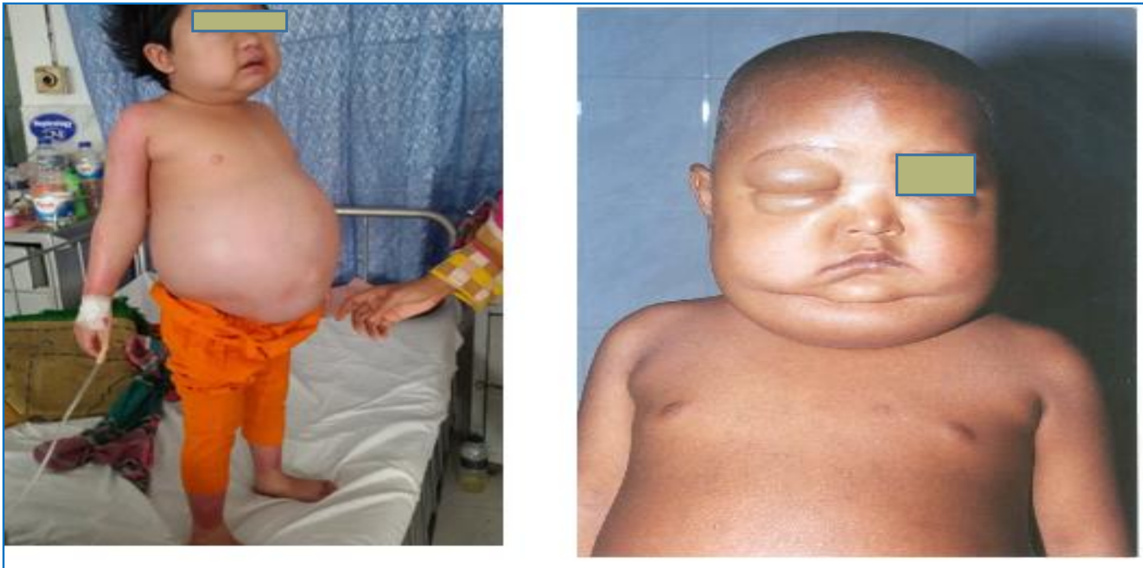


Figure 1.1: Clinical presentation of some nephrotic syndrome patients in Bangladesh Institute of Child Health

1.1.1 Historical Background

The history of nephrosis will return to the starting of the time. In the period of Alexandria, the urine was considered to be produced by kidneys. Hypocrites was saying that "Bubbles floating on the surface on the urine denote affection of the kidneys, and the disease will be long". In 1764, Cotungo described that a soldier had been suffering from massive edema whose urine was coagulated by heating. In 1836, Bright observed proteinuria in some edematous patients with different renal diseases. "Nephrosis" is a term which was used by Muller, to differentiate a group of kidney disorders. Initially, renal tubules were suspected as the source of blood protein leak. Ultimately 1n the 1940s, glomeruli became recognized as the source of protein leak. Munk noted the association of lipid droplets in the portion of urinary sedimentation in a patient with nephrosis and suggested the use of the term lipoid nephrosis. Clovin

and Goldberg used the term "Nephrotic Syndrome" to describe a patient who represents edema, proteinuria and hyperlipidemia. For the treatment of nephrotic syndrome the synthesized steroid hormones were used since 1950 (Rahman *et al.,* 2013).

1.1.2 Epidemiology

- **Incidence**

In both developing and developed countries, the glomerular disease is a common cause of the end-stage renal disease (Nawaz *et al.,* 2013). From country to country and even from region to region within a country, there is a wide variation of the pattern of glomerulonephritis reflecting the possible effects of environmental, socioeconomic, genetic factors as well as nephrology practice, service and facilities available in that region (Bagga and Srivastava, 2011). It constitutes an important cause of morbidity and mortality which imposes a considerable burden on the already strained health services in developing countries (Niaudet and Boyer, 2009). Still now in Bangladesh, the pattern of glomerulonephritis is not well documented. In our country, very few studies have been performed regarding this issue. On the national scale, there is an epidemiological study is not available yet. But Hossain (Hossain, 1997) reported that fifty to sixty percent of total indoor beds in the pediatric nephrology unit of Bangabandhu Sheikh Mujib Medical University of Bangladesh is occupied by the patient of nephrotic syndrome. Among Asian children, nephrotic syndrome is a common clinical condition (Feehally *et al.,* 1985). It also observed that the incidence of minimal change nephrotic syndrome is higher in the Indian subcontinent (Sharples *et al.,* 1985). Such incidence in Bangladesh is yet unknown. The incidence of all forms of nephrotic syndrome in childhood is 2-4 per 100,000 population, whereas in the Indian subcontinent it is estimated at 9-10 per 100,000 population (Bagga and Srivastava, 2011), but this figure will vary according to the ethnic mix of the population. There is a racial variation noticed in susceptibility with a reported in incidence in Asian children of 9-16/100,000 (Sharples *et al.,* 1985; McIntosh *et al.,* 2003) in comparison to 2 to 7 children in the USA (Robson and Leung, 1993), 2-4 new cases/100,000 children in the UK (McIntosh *et al.,* 2003; Neuhaus *et al.,* 1994). Mortality was observed 50/1000 before starting antibiotic and

steroid, which is now 5/1000 (Nash *et al.,* 1978). Among the nephrotic syndrome patients, about 90% of patients are steroid responsive (ISKDC, 1981), with a favorable long-term prognosis.

- **Age and Sex**

Nephrotic syndrome is more common in boys than in girls (2:1) and most commonly appears between the age of 2 and 6 years (Cameron, 1970). The common causes of congenital nephrotic syndrome (before 3 months age) and infantile nephrotic syndrome (age 3-12 months) are congenital infections or genetic mutations and are therefore noticeably illustrious from the more common type 'idiopathic' nephrotic syndrome in childhood (>12 months of age) (Teeninga, 2013). Children's age at the initial presentation time also has an important role in disease distribution frequency, we noticed that 70% minimal change nephrotic syndrome patients are younger than 5 years of age; on the other hand, 20% to 30% of adolescent patients have minimal change nephrotic syndrome. Focal segmental glomerulosclerosis is developing among children around 6 years of age and during the first year of life, congenital and infantile genetic disorders and congenital infections are more common than MCNS and FSGS and all cases of minimal change nephrotic syndrome or focal segmental glomerulosclerosis are idiopathic nephrotic syndrome (Viswanath, 2013).

- **Race**

Nephrotic syndrome occurs more in children of South Asia, where the condition is primary (idiopathic) in 95% of cases. Over the past three decades, it has been observed that the overall incidence of idiopathic nephrotic syndrome in childhood has been generally stable but the histological pattern is changing. In cases of adults and children, the incidence of focal segmental glomerulosclerosis seems to be increasing, even after treatment for changing in renal biopsy practices (Table 1.1). The histological variant and the response to immunosuppressive treatment are also affected by ethnic origin. In particular, the Hispanics and Blacks population are most likely shows to have steroid-resistance nephrotic syndrome than Whites populations (Viswanath, 2013).

- **Genetic Aspects**

Familial nephrotic syndrome is noticed in 2-8% cases and in most of the cases, siblings are involved. Familial cases appear to be inherited in a polygenic fashion. This situation is much common in monozygotic twins than in dizygotic, which recommends that it is the genetic factors that play a more important role than the environmental factors. It is reported that the steroid-responsive nephrotic syndrome patients more repeatedly have the combined incidence of HLA-DR3, HLA-DR7 and HLA-B8-DR3 whereas the steroid-resistance nephrotic syndrome patients more repeatedly have the combined incidence of HLA-B8-DR8 and DR7 (Rahman *et al.,* 2013).

1.1.3 Pathophysiology of Nephrotic Syndrome

Nephrotic syndrome (NS) is described by podocyte foot process effacement; but, it is still largely unknown and repeatedly debated the precise mechanism of nephrotic syndrome. Furthermore, massive proteinuria is seen in nephrotic syndrome results from increased permeability of the glomerular filtration barrier for proteins. This barrier is comprised of the fenestrated capillary endothelium, the glomerular basement membrane and intercalated foot processes of the visceral cells of the glomerulus, termed podocytes (Figure 1.2). Increased permeability for proteins can result from both structural changes within the glomerular filtration barrier and loss of negatively charged molecules (Saito *et al.,* 2009). Recent developments have demonstrated the podocyte and therefore the slit diaphragm in-between foot processes to be of key importance within the pathophysiology of nephrotic syndrome (Lane and Kaskel, 2009; Tesar and Zima, 2008). Nephrotic range proteinuria is associated with morphologic changes of podocytes, as well as retraction and flattening of foot processes. Throughout remission, podocytes have resumed their original shapes. The events triggering podocyte effacement are complex and not absolutely understood. Potential mechanisms resulting in podocyte effacement include impaired signal transduction inside the slit diaphragm, increased production of lysosomal proteases, loss of cell polarity and loss of negatively charged proteins from the glomerular filtration barrier (Shankland, 2006; Tesar and Zima, 2008; Faul *et al.,* 2007; Lane and

Kaskel, 2009). Changes within the cytoskeletal structure of the podocyte might result from both frailty of the podocyte and slit diaphragm architecture and from circulating plasma factors (Lane and Kaskel, 2009; Van Den Berg *et al.,* 2004; Van Den Berg *et al.,* 2000; Mathieson, 2003; Faul *et al.,* 2007). Steroid-sensitive nephrotic syndrome is presently thought to entail reversible conformational changes of podocytes, whereas irreversible conformational changes are associated with steroid-resistance and disease progression (Lane and Kaskel, 2009). Damage to the filtration barrier is caused by genetic defects primarily affecting podocytes. The underlying genetic defect of the patient is usually considered as a primary steroid-resistance nephrotic syndrome (Bierzynska *et al.,* 2017). Moreover, a considerable proportion of the patients is probably going to possess an immune-mediated circulating factor disease. These types of patients are usually considered as steroid-resistance, however, screening shows negative results for the known genes who are responsible for developing steroid-resistance nephrotic syndrome (Gallon *et al.,* 2012; Bierzynska and Saleem, 2017). Furthermore, polymorphisms of the gene encoding for the podocyte proteins, such as nephrin, podocin, CD2-associated protein and a-actinin-4, result in structural changes in the slit diaphragm and subsequent development of NS (Youssef *et al*., 2013; Fan *et al.,* 2006). Additionally, alternations of factors that modulate disease response to therapeutic regimens are another risk factor of NS and/or steroid-resistance, such as the expression of P-glycoprotein, a product of multidrug resistance gene 1 gene (Youssef *et al.,* 2013; Wasilewska *et al.,* 2007). Several studies addressed that gene polymorphisms of membrane transporters or metabolizing enzymes may influence the patient's responses to therapeutic regimens (Youssef *et al.,* 2013; Sakaeda *et al.,* 2004). However, steroid-resistance could also occur independently of the P-gp expression in NS patients (Youssef *et al.,* 2013; Jafar *et al.,* 2011).

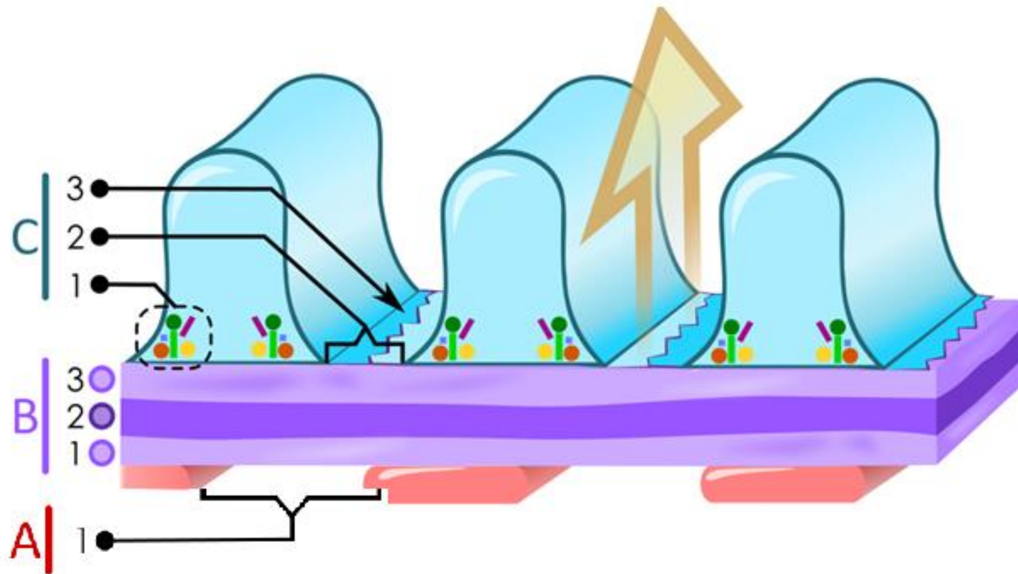


Figure 1.2: Scheme of filtration barrier in the kidney. **A**. The endothelial cells of the glomerulus: 1. pore (fenestra) **B.** Glomerular basement membrane: 1. lamina rara interna 2. lamina densa 3. lamina rara externa **C**. Podocytes: 1. enzymatic and structural proteins 2. filtration slit 3. diaphragm

1.1.4 Causes of Childhood Nephrotic Syndrome

Several factors are responsible for developing childhood nephrotic syndrome. The factors are-

1.1.4.1 Genetic Disorders

- **Nephrotic Syndrome Typical**

Focal segmental glomerulosclerosis, finnish-type congenital nephrotic syndrome, schimke immune-osseous dysplasia, diffuse mesangial sclerosis.

- **Proteinuria with or without Nephrotic Syndrome**

Nail-patella syndrome, alport's syndrome.

- **Metabolic Disorders with or without Nephrotic Syndrome**

α-1 antitrypsin deficiency, hurler's syndrome, fabry disease, sickle cell disease, lipoprotein disorders, alagille syndrome, glycogen storage disease, mitochondrial cytopathies, glutaric acidaemia.

• **Idiopathic Nephrotic Syndrome**

Minimal change nephrotic syndrome, focal segmental glomerulosclerosis, membranous nephropathy.

1.1.4.2 Secondary Causes

• **Infections**

Malaria, HIV-1, hepatitis B, hepatitis C, toxoplasmosis, syphilis.

• **Drugs**

Lithium, interferon, gold, heroin, penicillamine, nonsteroidal anti-inflammatory drugs, mercury and pamidronate.

• **Immunological or Allergic Disorders**

Food allergens, bee sting.

• **Associated with Malignant Disease**

Leukemia, lymphoma.

• **Glomerular Hyperfiltration**

Oligomeganephronia, morbid obesity (Viswanath, 2013).

1.1.5 Classification of the Nephrotic Syndrome

Nephrotic syndrome can be classified according to the following ways-

1.1.5.1. Classifications Based on the Clinical Presentation

• **Age at Presentation**

i) <3 months: is known as congenital nephrotic syndrome.

ii) 3-12 months: is known as infantile nephrotic syndrome.

iii) >12 months: is known as childhood nephrotic syndrome.

The absence or presence of symptoms representing glomerulonephritis: it is persisting as hematuria or hypertension and renal failure.

- **Initial Response to the Drug Prednisolone**

i) Steroid-Sensitive Nephrotic Syndrome (SSNS)

Comprises 80-90% of the syndrome. It is observed that the patient responds to the corticosteroids in the first 8 weeks of treatment.

ii) Steroid-Resistant Nephrotic Syndrome (SRNS)

Comprises 10-20% of the syndrome. The proteinuria persists after the 8 weeks of treatment. The lack of response is indicative of the seriousness of the glomerular damage, which could develop into chronic kidney failure.

1.1.5.2 Histopathological Classification

i) Minimal change disease/ Minimal change nephrotic syndrome
ii) Focal segmental glomerulosclerosis
iii) Membranous nephropathy
iv) Membranoproliferative glomerulonephritis
v) Mesangioproliferative glomerulonephritis
vi) Immunoglobulin A nephropathy
vii) Immunoglobulin M nephropathy

1.1.5.3 Pathogenetic Classification

1. Primary Nephrotic Syndrome

The nephrotic syndrome unassociated with the systemic disease is termed primary nephrotic syndrome. It accounts for 90% of childhood cases.

i) Idiopathic NS: cause unidentified
ii) Genetic

2. Secondary Nephrotic Syndrome

When it occurs as part of a systemic disease or is related to a drug or other toxin, it is termed as secondary nephrotic syndrome. It accounts for 10% of childhood cases.

i) Post-infectious diseases (e.g. group A beta-hemolytic streptococci)

ii) Different types of systemic diseases (e.g. systemic lupus erythematosus)

iii) Syndrome-associated diseases (e.g. nail-patella syndrome)

iv) Toxic substances (e.g. drug reaction)

v) Others

(Teeninga, 2013)

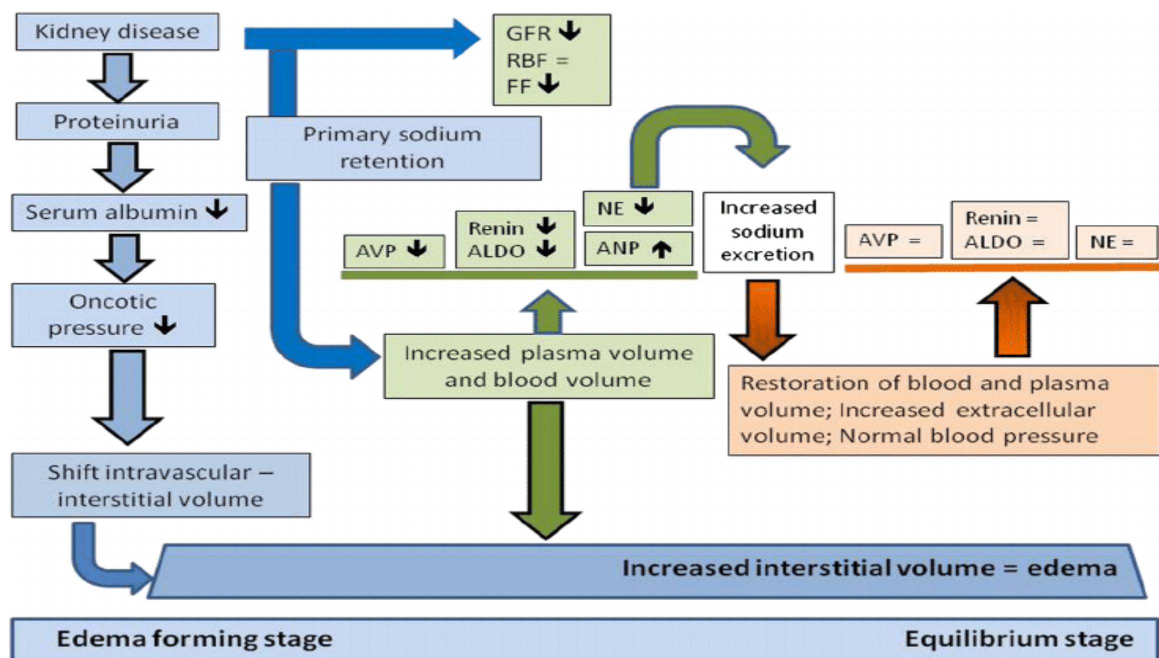


Figure 1.3: Different types of physiological changes during nephrotic syndrome

1.1.6 Definition of Steroid-Resistant Nephrotic Syndrome

Nephrotic syndrome can be clinically classified as a steroid-sensitive nephrotic syndrome and steroid-resistant nephrotic syndrome forms according to the responsiveness to oral glucocorticoid treatment, which is the first line of drug for childhood idiopathic NS (ISKDC, 1981). Among the Pediatric Nephrologists, steroid-

resistant nephrotic syndrome can be defined in two ways. The definition introduced by the International Study of Kidney Disease in Children and used by the Arbeitsgemeinschaftfur Padiatrische Nephrologie is widely accepted as follows (Brodehl *et al.,* 1982): “No urinary remission within 4 weeks of prednisone therapy 60 mg/m^2/day (2 mg/kg/day)”. The other definition, employed by the Society of French-Speaking Pediatric Nephrologists, (Niaudet, 1994) states: “No urinary remission following 4 weeks of prednisone 60 mg/m^2/day (2 mg/kg/day) followed by three intravenous pulses of methylprednisolone”. Remission is defined as the absence of proteinuria (the albumin of urine is nil or by dipstick or boiling test trace for three consecutive days). The rationale for both definitions is the understanding that the steroid-sensitive patients respond to the steroid within 4 weeks and only a small percentage will respond later and that is called late responders.

Thus, the definitions of resistance can be a minimum exposure of 8 weeks of prednisone 2 mg/kg/d or 60 mg/m^2/d for 4 weeks followed by 1.5 mg/kg or 40 mg/m^2 per dose alternate day for 4 weeks. At this point, steroid-resistance dictates the requirement for kidney biopsy to define the histopathology. Meanwhile, steroids may be continued for an additional 4 weeks, totaling 12 weeks, while awaiting pathology results (Gipson *et al.,* 2009). So, the definition of steroid-resistant nephrotic syndrome in children aged 1-18 years, who are unable to achieve a complete remission with corticosteroid therapy within 8 weeks is termed as steroid-resistant nephrotic syndrome. Approximately 10-20% will be classified as steroid-resistant (McKinney *et al.,* 2001; ISKDC, 1981). Recent reports indicate that the incidence of SRNS in children is rising and constitutes 23% approximately (Srivastava *et al.,* 1999; Bonilla-Felix *et al.,* 1999). Despite representing a smaller proportion of nephrotic syndrome cases, patients with steroid-resistant nephrotic syndrome have proven more difficult to treat, with 36%–50% progressing to the end-stage renal disease within 10 years (Mekahli *et al.,* 2009) of life.

When the lack of remission observed in the first episode then it is considered as initial resistance nephrotic syndrome. The patient who showed steroid-sensitive initially but steroid-resistance during a successive relapse then it is termed as late resistance (Bagga *et al.,* 2012).

1.1.7 Investigations of Steroid-Resistant Childhood Nephrotic Syndrome

Children diagnosed with steroid-resistant nephrotic syndrome (initial or late) should undergo renal biopsy before starting specific treatment. About 10-20% of patients with familial and sporadic SRNS and these patients are usually unresponsive to immunosuppressive medications, progress rapidly to end-stage renal disease. If facilities exist, mutational analysis should be offered to patients with: (i) congenital nephrotic syndrome (onset below 3 months of age) (ii) family history of SRNS, (iii) sporadic initial steroid-resistance that does not respond to therapy with cyclophosphamide or calcineurin inhibitors and (iv) patients with steroid-resistant FSGS (Kdigo, 2012). Baseline assessment of renal function, the blood level of albumin and cholesterol, and quantification of starting urinary protein loss (spot urine protein to creatinine ratio in young children; 24-h protein excretion in older children) guides the future evaluation of response to therapy. Patients should be evaluated for hepatitis B and C virus infection (Rahman *et al.,* 2013).

➢ Where available

- Renal histology by electron microscopy
- Genetic testing: sequencing of NPHS1, NPHS2, WT1 and other genes.

A prospective study was conducted by Alam *et al.* (Alam *et al.,* 2016) from January 2004 to December 2015 among children who were suffering from nephrotic syndrome admitted in the Pediatric nephrology department, National Institute of Kidney Disease and Urology, Dhaka, Bangladesh. Total admitted childhood nephrotic syndrome during this period was 1512 and renal biopsy was done in 354 patients (Table 1.1). Among the 354 children, histopathological findings were mesangial proliferative glomerulonephritis was 92 (25.98%), minimal change disease was 79 (22.32%), immunoglobulin M nephropathy was 69 (19.49%), focal segmental glomerulosclerosis was 37 (10.45%), membranoproliferative glomerulonephritis was 37 (10.45%), immunoglobulin A nephropathy was 20 (5.65%), membranous nephropathy was 08 (2.27%) and others were 12 (3.39%). So, from this study, we found that the selected patient for renal biopsy in the last 12 years showed that

minimal change disease had been decreasing but the other histological types are increasing gradually such as mesangial prliferative glomerulonephritis, FSGS and IgM nephropathy.

Table 1.1: Renal biopsy according to the year (2004-2015)

Year	Nephrotic syndrome	Renal biopsy	Percentage (%)
2004	71	28	39.44
2005	97	28	28.87
2006	125	22	17.60
2007	110	14	12.73
2008	134	21	15.67
2009	84	17	20.24
2010	127	11	08.66
2011	115	22	17.39
2012	160	27	16.87
2013	127	53	41.73
2014	126	58	46.03
2015	236	53	22.46
Total	**1512**	**354**	**23.41**

1.1.8 Management of Steroid-Resistant Childhood Nephrotic Syndrome

Children who are suffering from steroid-resistance nephrotic syndrome need an aggressive treatment plan to achieve remission. Calcineurin inhibitors, like cyclosporine and tacrolimus, are used as the first line of therapy when intravenously used high-dose of methylprednisolone are failed. If remission is not achieved, a major range of patients with steroid-resistance nephrotic syndrome progress to the end-stage renal disease. It has observed that renal replacement therapy can also be problematic for these children. A significant number of proteins are lost through the peritoneal membrane during the peritoneal dialysis, and therefore transplantation of kidney

allograft may be complicated by the recurrence of steroid-resistant nephrotic syndrome. Initially, after transplantation plasmapheresis and rituximab were used for the treatment of recurrent steroid-resistance nephrotic syndrome, this area currently into account as rescue therapies for refractory steroid-resistance nephrotic syndrome. Though the prognosis of steroid-resistance nephrotic syndrome is unfavorable and complicated, in the early stages of this disease intensive treatment plan may achieve remission in more than half of patients. For that reason, timely referral of pediatric steroid-resistance nephrotic syndrome patients to pediatric nephrology specialists for histological and genetic diagnosis and treatment is highly recommended (Kang, 2011).

Table 1.2: Treatment regimens for steroid-resistant nephrotic syndrome (Rahman *et al.,* 2013)

Drug	**Dosage**	**Side effects**
Cyclophosphamide PO with prednisolone or IV with prednisolone*	2-3 mg/kg/day for 12 wk 500-700 mg/m^2/month for 6 months	Alopecia, hemorrhagic, bone marrow suppression, nausea, vomiting, cystitis (with IV therapy).
IV pulse steroids* (Methylprednisolone), PO cyclophosphamide and prednisolone	20-30 mg/kg per pulse	Hypertension, hypokalaemia, infections, arrhythmia, steroid psychosis, hyperglycaemia.
Dexamethasone, PO cyclophosphamide and prednisolone**	4-5 mg/kg per pulse	Same
Cyclosporine with prednisolone*	4-6 mg/kg/day for 2-3 yr	Nephrotoxicity, hypertrichosis, hypertension, gingival hyperplasis.

*Prednisolone administered at 1 mg/kg on alternate days; dose reduced after 2-3 months** Six alternate day pulses, then 4 fortnightly pulses and 8 monthly pulses; oral cyclophosphamide for 12 wk; tapering prednisolone over 52 wk.

1.2 Pharmacogenetics

Different patients respond in different ways to the same medication, it is well recognized. These differences are often greater among members of a population than they are within the same person at different times (or between monozygotic twins) (Vesell, 1989). It is noticed that these inter-individual variations in drug responses may be influenced by a number of variables. However, these factors do not only influence therapeutic drug responses such as the degrees of efficacy and potency, but also the propensity to develop specific adverse effects (Fenech, 2007).

Even though several non-genetic factors influence the results of medications, together with age, organ function, concomitant therapy, drug interactions and therefore the nature of the disease, there are currently several samples of cases within which inter-individual variations in drug responses are as a result of sequence variants in genes encoding drug-metabolizing enzymes, drug transporters, or drug targets (Evans *et al.,* 1999; McLeod *et al.,* 2001). It is calculable that genetic science will account for twenty to ninety-five percent of the variability in drug disposition and effects (Kalow *et al.,* 1998).

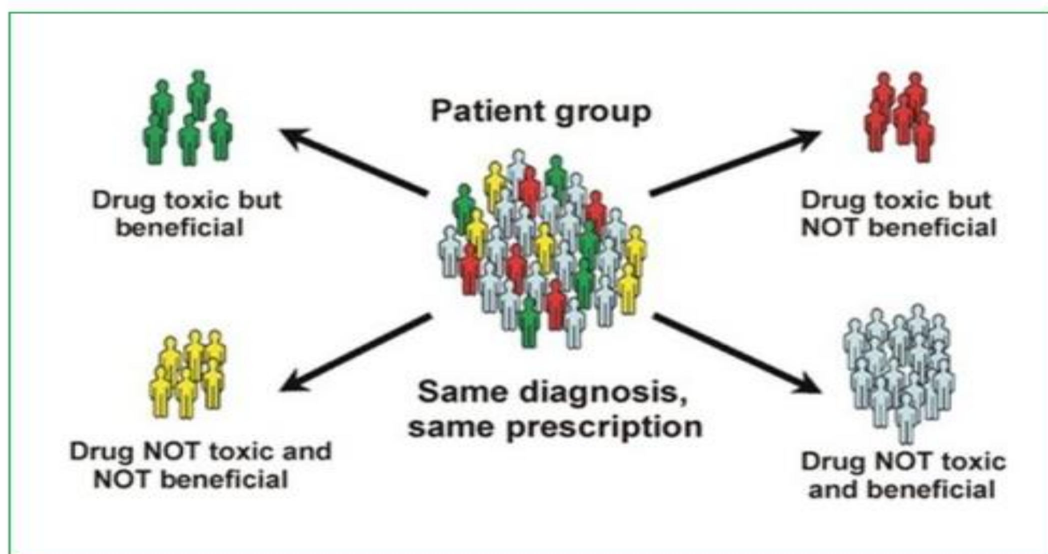


Figure 1.4: Inter-individual variations in drug responses (Kumar *et al.,* 2007)

Unlike different factors influencing drug response, these inheritable determinants usually stay stable throughout a person's life period. Clinical observations of

inheritable variations in drug effects were 1st documented within the 1950s, it is giving rise to the field of pharmacogenetics (Kalow, 1956; Carson *et al.,* 1956; Evans *et al.,* 1960).

Pharmacogenetics can be quoted as the study of individual genetic profiles in response to pharmaceuticals. It blends vital parts of the disciplines of genetic science and pharmacology and aims to explain the influence of inheritance on variable drug response. A crucial aim in pharmacogenetics is to discover genetic variants that contribute to the variable effects of medicine and to develop deoxyribonucleic acid screening devices that may detect these variants in patients. Identification of susceptibleness genes is supposed to be vital to determine who is going to be possible to respond to a given treatment and who will be likely to respond to a given treatment and who will be susceptible to serious side effects (Johnson, 2003; Hoedmaekers *et al.,* 2001).

Pharmacogenetics also constitutes the study of single-gene mutations and their results on drug response (Surendiran *et al.,* 2008). The principle behind pharmacogenetics is to seek out genetic polymorphisms within the genes encoding proteins and enzymes involved in drug transport, metabolism and action which will predict the effectiveness of a selected drug, increasing the number of responders and decreasing the number of subjects affected by adverse drug actions (Ingelman-Sundberg, 2005).

Thus the supreme goal of pharmacogenetics research is to assist prescribers to utilize a patient's genetic information or pharmacogenetic profile so as to enable the choice of the drug from the appropriate therapeutic repertoire, which would demonstrate the best efficaciousness and therefore the least adverse effects therein specific patient, and therefore the prescription of that drug at a dose appropriate for that patient. This is called genotype-guided prescribing or more universally termed as personalized medicine (Mayor, 2007). Although it is often considered by the practitioner community to still be a remote vision, the concept of personalized medicine is advancing rapidly and pharmaceutical companies are these days already incorporating pharmacogenetic aspects into the drug development method (Fenech, 2007).

1.2.1 Benefits of Pharmacogenetics

- **Effective Medication**

The discrepancy in gene expressions and proteomics are indicators that discover different patterns of gene and protein expression in tissues (Blackstock and Weir, 1999; Kozian and Kirschbaum, 1999). The disease sensitive genes identification and analysis of the function of the gene variants can be used to identify targets that will be associated with the disease in patients (Rao and Gorey, 2007).

- **Enhancing Efficacy**

Many medicines do not seem to be effective for everybody with the same disease. Only around 60% of patients are responsive to some common treatments for diabetes, depression, and asthma. Pharmacogenetics may permit physicians to prescribe medicine just for those patients most likely to respond. As an alternative, new medicines may be designed on the premise of genetic information concerning the reason behind the disease (McLeod and Evans, 2001).

- **Screening for Disease Identification**

Differential gene expression, transgenic animal models, proteomics and in-situ hybridization are used to imply relationships between a gene and a disease process (Roses, 2000). Likewise, advance knowledge of specific disease susceptibility will permit careful monitoring, and treatments can be introduced at the most appropriate stage to maximize their therapy.

- **Improvements in the Drug Discovery and Approval Process**

To discover a new drug sometimes many molecular targets are chosen on the basis of scientific hypotheses and do not lead to effective medicines because the initial hypotheses have subsequently been disproved (Curran, 1998; Marton *et al.,* 1998). However, using pharmacogenetics developed optimized molecules (leads) can be obtained, which have properties of well-tolerated and useful medicines (Rao and Gorey, 2007).

- **The Decrease in the Overall Cost of Health Care**

Decreases within the variety of adverse drug reactions, the number of unsuccessful drug trials, the time is taken to get a drug approved, the length of time patients are on medication, the amount of medications patients should go for notice an effective therapy, the consequences of an illness on the body (through early detection) and an increase in the range of possible drug targets will endorse a net decrease in the cost of health care.

- **Better Vaccines**

Vaccines made from genetic material, either deoxyribonucleic acid or ribonucleic acid; promise all the advantages of existing vaccines while not all the risks. They will trigger the immune system however are going to be unable to cause infections. Better vaccines will be inexpensive, stable, easy to store and capable of being built to hold many strains of an infective agent without delay.

- **Safer Drugs**

Instead of the standard trial-and-error methodology of matching patients with the correct medication, doctors are going to be able to study a patient's genetic profile and recommend the best obtainable drug therapy from the very beginning. Not solely can this take the shot out of finding the correct drug, it will speed the recovery process and increase safety because the chance of adverse reactions will be eliminated. Pharmacogenetics has the potential to dramatically lessen the estimated 100,000 deaths and 2 million hospitalizations that occur annually in the United States because of the results of adverse drug reaction.

- **More Accurate Methods of Determining Appropriate Drug Dosages**

The other anticipated advantages of pharmacogenetics include additional correct strategies for determining appropriate drug dosages. The ability of a person's body to process a drug that while his genetic profile will form the basis for dosage calculations rather than the weight and age of the patient (Rao and Gorey, 2007).

1.2.2 Genetic Polymorphism

A combination of the Greek words *poly* meaning multiple and *morph* meaning form, this term is employed in genetic science to explain the multiple varieties of a single gene that may exist in an individual or among a group of individuals. (Polymorphism-gene sequencing - genetics - Biotech/Biomedical Theresa Phillips, last review at Dec 2014).

The term 'Polymorphism' literally provides that means of the variability of form, shape, size, structure and composition and it has currency in a wide variety of disciplines in science and art. Genetic polymorphism is a much more specific expression describing frequent variation at an exact locus in a genome. A helpful applicable definition suggests that a locus is also polymorphic once when there is the presence of two or more allelic forms within the same population and the most common allelic form has a frequency of 0.99 or less (Harris, 1980). A genetic polymorphism happens if, among a population, a single gene liable for making a metabolizing enzyme has an altered allele with an arbitrary frequency of 1% (Meyer zu Schwabedissen and Kim, 2009). For several such genes, single nucleotide polymorphisms exist and an allelic site may have more than one single nucleotide polymorphism (SNP). The genotype is the detailed gene structure of an individual whereas the more commonly measured phenotype is that the outcome of metabolism of a drug in an individual.

There is a substantial level of variability between individuals at the genetic level, as manifested by the polymorphisms present inside their genome (Sachidanandam *et al.*, 2001; Oscarson, 2003). Over 90% of those polymorphisms are believed to be accounted for by changes in a single nucleotide, namely SNPs, with the remainder of the variation caused by insertions and deletions (indels), variable number tandem repeats and microsatellites (Quirk *et al.*, 2004; Marsh and McLeod, 2006). However, in contrast to several other previously characterized polymorphisms, such as VNTRs and microsatellites, SNPs are typically found among the coding and regulatory regions of genes and thus can have functional outcomes for gene expression and gene product functionality (Campbell *et al.*, 2000; Gray *et al.*, 2000).

Genetic polymorphisms are natural variations within the genomic deoxyribonucleic acid sequence present in a minimum of or over 1% of the population. For example, a place in the genome where 93% of people have a T and therefore the remaining 7% have an A is a polymorphism. Therefore, genetic polymorphisms for therapeutic targets and also the metabolic pathways can significantly influence both drug efficacy and drug toxicity.

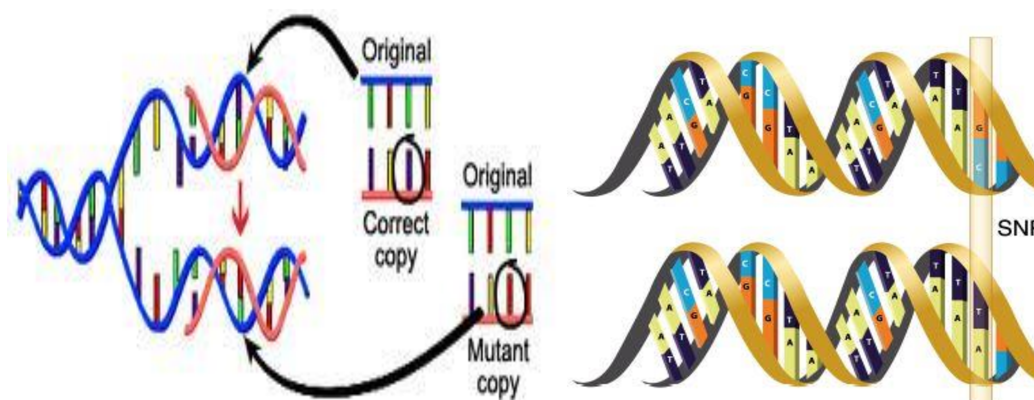


Figure 1.5: Mutation, SNP and polymorphism (Broad Institute of Harvard and MIT)

If one of the possible sequences is present in less than 1% of people (99.9%) having a G and 0.1% having a C, then the variation is called a mutation. Informally, the term mutation is often used to indicate a harmful genome variation that is associated with a specific human disease, while the word polymorphism implies a variation that is neither harmful nor beneficial. Several pharmacogenetic polymorphisms have been identified, however, the most frequent genetic changes being SNPs, almost 90% (Yang and Roden, 2003; Brumfield *et al.,* 2003).

Other types of genetic polymorphism involve variation caused by insertions and deletions (indels), variable number tandem repeats and microsatellites (Quirk *et al.,* 2004; Marsh and McLeod, 2006).

These polymorphisms could also be existing in coding and noncoding regulatory regions, possibly changing protein function or opulence of key regulatory molecules, and thereby influencing the efficacy and toxicity of therapy or leading to a predisposition to diseases (Yang and Roden, 2003).

Genetic mutation is a change in the nucleotide sequence of a deoxyribonucleic acid molecule. Genetic mutations are a kind of genetic polymorphism. The term "Mutation," as opposed to "Polymorphism", is mostly used to refer to changes in deoxyribonucleic acid sequence that do not seem to be present in most individuals of a species and either have been associated with disease or risk of disease or have resulted from damage indicted by external agents (such as viruses or radiation).

Recent studies have suggested that the presence of sequence variants, like pSNPs, within intronic regions may have an effect on basic preliminary-mRNA splicing mechanisms and thereby causing altered levels of normal transcripts (Pagani *et al.*, 2003). A pSNP within the 3-untranslated region following the coding sequence could have an effect on the intracellular stability of the mRNA gene transcript (Quirk *et al.*, 2004).

1.2.3 Single Nucleotide Polymorphism

SNPs within the coding regions of a gene which cause changes in the amino acid sequence of the encoded protein are known as coding SNPs which, because of greater selective pressures against changes at positions dictating amino acid sequence, are generally less common than SNPs or synonymous changes in coding sequence (Gray *et al.,* 2000). A single nucleotide polymorphism is a single base mutation in DNA and a source variance in a genome. SNPs are the most simple form and most common source of genetic polymorphism in the human genome (90% of all human DNA polymorphisms). There are two types of nucleotide base substitutions resulting in SNPs:

Type I- A transition substitution occurs between purines (A, G) or between pyrimidines (C, T). This type of substitution constitutes two-thirds of all SNPs (Schwartz *et al.,* 1996*).*

Type II- A transversion substitution occurs between a purine and a pyrimidine.

The different types of SNPs are thus multiple, as are their effects. Depending on their location within the genome and their patterns of co-occurrence (i.e. haplotypes), SNPs can alter expression levels of a gene as well as the functionality of the encoded

protein product or its affinity for its intended substrates. These effects of SNPs can, as is the case with many other phenotypic characteristics, greatly affect the manner in which a patient responds to drug therapy.

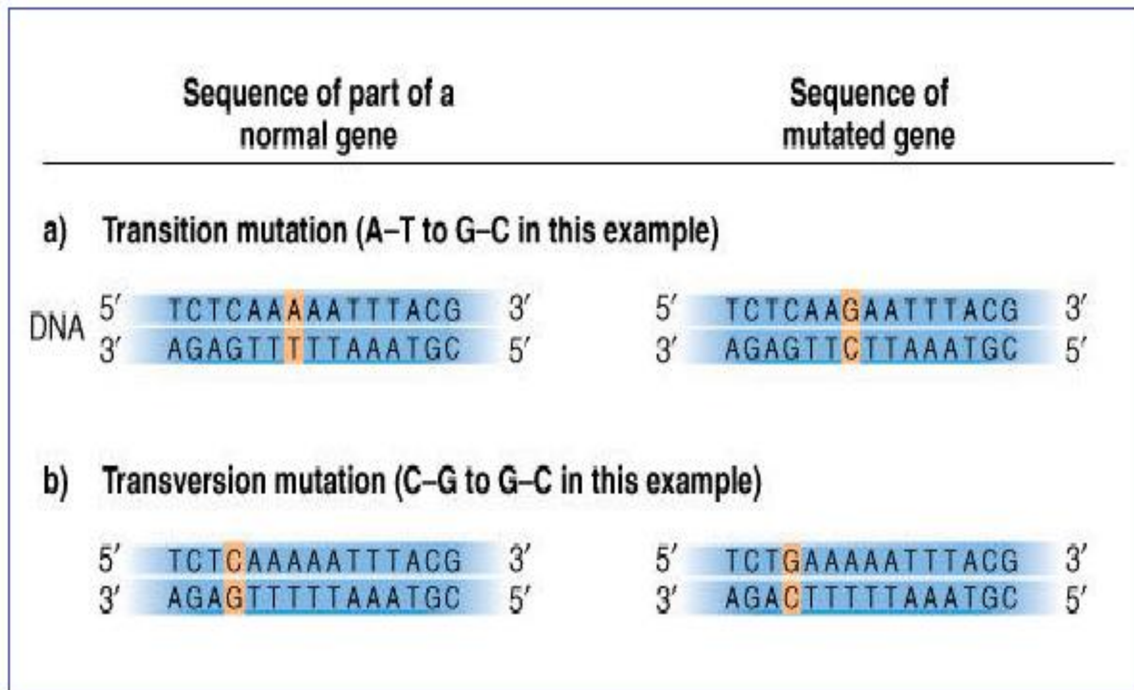


Figure 1.6: Schematic of single nucleotide polymorphism

1.2.4 Distribution of Single Nucleotide Polymorphism

Single nucleotide polymorphisms do not seem to be uniformly distributed over the whole human genome, neither overall chromosomes and nor inside a single chromosome (Guo and Jamison, 2005). There is one third as several single nucleotide polymorphisms inside coding regions as in non-coding regions. It is additionally been shown that sequence variation is much lower for the sex chromosomes. Within a single chromosome, single nucleotide polymorphisms can be concentrated about a specific region, usually implying a region of medical or research interest. For example, compared to the other regions of that chromosome the sequence that encodes proteins which present antigens to the immune system in chromosome 6 exhibit very high diversity of nucleotide.

1.2.5 Coding Region of Single Nucleotide Polymorphism

Single nucleotide polymorphism in a coding region may have two diverse effects on the resulting protein:

- Synonymous- the substitution caused no amino acid to the resulting protein. This can be additionally referred to as a silent mutation.

- Non-synonymous- the substitution results in an alteration of the encoded amino acid (Nei *et al.*, 2000). By causing a change of codon a missense mutation changes the protein. A nonsense mutation results in a misplaced termination codon. One-half of all coding sequence single nucleotide polymorphisms result in non-synonymous codon changes.

Single nucleotide polymorphisms may occur in regulatory or non-coding regions of genes. These single nucleotide polymorphisms are capable of altering the amount or timing of the production of a protein. Such single nucleotide polymorphisms are much more difficult to find and understand and gene regulation itself is not however clearly understood.

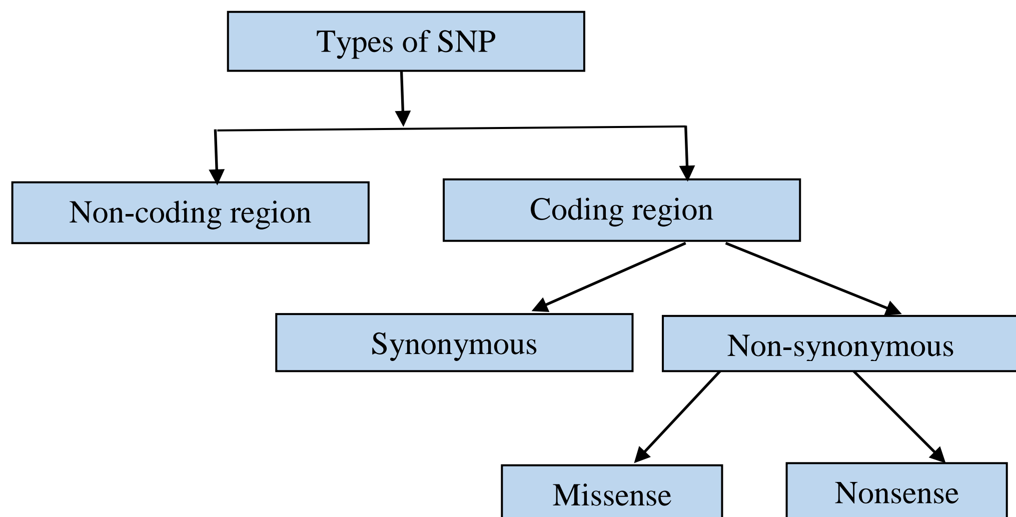


Figure 1.7: Types of single nucleotide polymorphism

1.3 Prednisolone-Drug of Interest

Over the 60 years, glucocorticoids have been the cornerstone for the treatment of childhood nephrotic syndrome, as over 80–90% of the patients have achieved a complete remission with the treatment of prednisone or prednisolone. Unfortunately, 80% of these patients will have one or several relapses and will need additional courses of glucocorticoid therapy. Furthermore, approximately 10-20% of children with nephrotic syndrome are steroid-resistant and do not respond to the standard steroid treatment regimen. Treatment guidelines for the first manifestation and relapse of steroid-sensitive nephrotic syndrome are mostly standardized and based on practice guidelines rather than clinical trials. As the optimum glucocorticoid dosing regimens for childhood nephrotic syndrome are still under debate and large-scale clinical trials are lacking, current clinical practice among physicians is variable, especially in the treatment of subsequent relapses and the choice of second-line immuno-suppressive drugs (Schijvens *et al.,* 2019).

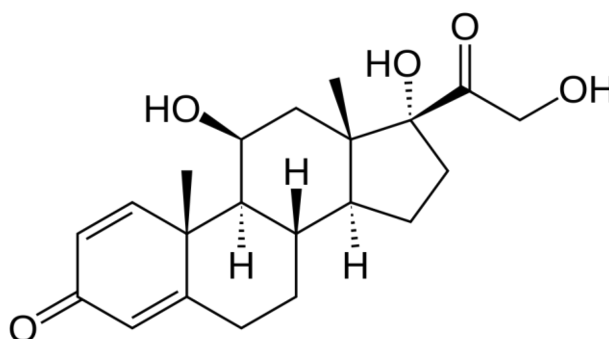


Figure 1.8: Chemical structure of prednisolone

1.3.1 Role of Prednisolone in Childhood Nephrotic Syndrome

Corticosteroids have been used to cure childhood nephrotic syndrome since 1950 when large doses of adrenocorticotrophic hormone and cortisone is given for two to three weeks were found to induce diuresis with loss of edema and proteinuria (Arneil, 1971). Corticosteroid usage has reduced the mortality rate in childhood nephrotic

syndrome to around 3%, with infection remaining the most important cause of death (ISKDC, 1981). Of children who present with their first episode of nephrotic syndrome, approximately 95% will achieve remission with corticosteroid therapy (Koskimies, 1982). Due to this dramatic before-after evidence that corticosteroids improve the outcome of nephrotic syndrome, for the treatment of childhood idiopathic nephrotic syndrome the oral corticosteroids are the first-line treatment. However, corticosteroids have known adverse effects such as poor growth, obesity, hypertension, diabetes mellitus, osteoporosis and adrenal suppression. Adverse effects are particularly prevalent in those children who relapse frequently and thus require multiple courses of corticosteroids (Hodson *et al.,* 2002).

1.4 Pharmacogenetics of Steroid-Resistant Nephrotic Syndrome

The genetics of SRNS has focused on genes playing a role in cell-cell signaling at the podocyte slit membrane (NPHS1, NPHS2, CD2AP and PTPRO/GLEPP1), regulation of foot process actin network (ACTN4 and INF2) or foot process glomerular basement membrane interaction (LAMB2 and ITGA3) (Wiggins, 2007). More recently, podocyte cell migration has been connected to the pathogenesis of SRNS (Gee *et al.,* 2013). In terms of the other histopathologic variants of MCD, which foretell a steroid-resistant course, genes involved in the pathogenesis of diffuse mesangial sclerosis have been identified (Hinkes, *et al.,* 2006). Truncating mutations in phospholipase C epsilon 1 prevent normal glomerular development and lead to diffuse mesangial sclerosis.

In summary, the role of podocyte is vital for the understanding of the pathogenesis of the nephrotic syndrome. For the maintenance of foot process integrity, the development, migration, basement membrane interaction and regeneration of the podocyte are all critical processes. Therefore, the genetic mutations in any one of these processes that can lead to the phenotype of nephrotic syndrome and more specifically, mutations within genes coding for key podocyte proteins (NPHS2, PLCE1, ACTN4 and TRPC6) cause FSGS, the histopathologic finding most commonly associated with SRNS (Wiggins, 2007). In all, 24 genes are presently known to be associated with SRNS (McCarthy *et al.,* 2013) (Table 1.3).

Table 1.3: Genes associated with SRNS

Gene	Disease
Alpha-actinin 4	Familial and sporadic SRNS
Asparagine-linked glycosylation 1	Congenital disorder of glycosylation
Apolipoprotein L1	Increase susceptibility to FSGS
CD2-associated protein	FSGS/SRNS
Collagen, type IV, alpha 3	Alport's disease
Collagen, type IV, alpha 4	Alport's disease
Collagen, type IV, alpha 3	Alport's disease
Coenzyme Q2	Mitochondrial disease/isolated nephropathy
Inverted formin-2	Familial and sporadic SRNS
Laminin, B2	Pierson syndrome
LIM homeobox transcription factor 1B	Nail patella syndrome
Nonmuscle myosin heavy chain 9	MYH9-related disease
Myosin 1E	Familial SRNS
Nephrin (NPHS1)	Congenital NS/SRNS
Podocin (NPHS2)	Congenital NS/SRNS
Prenyl diphosphate synthase, subunit 2	Leigh syndrome
Phospholipase C, e1	Congenital NS/SRNS
Phosphomannomutase 2	Congenital disorder of glycosylation
Protein tyrosine phosphatase, receptor type, O	Familial SRNS
Scavenger receptor class B member 2	Action myoclonus renal failure syndrome
SMARCALI	Schimke immune-osseous dysplasia
Transient receptor potential cation channel, subfamily C, member 6	Familial and sporadic SRNS
Wilm's tumor	Sporadic SRNS
Zinc metallopeptidase STE24 homolog	Mandibuloacral dysplasia

1.5 The Gene of Interest: ABCB1, NR3C1 and CYP3A5

1.5.1 The Gene: ABCB1

Permeability glycoprotein abbreviated as P-gp or Pgp was discovered in 1971 by Victor Ling. P-gp is a glycoprotein that in humans is encoded by the ABCB1 or MDR1 gene (Ueda *et al.,* 1987). P-gpI also have known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 is an important protein of the cell membrane that pumps many foreign substances out of cells. More formally, it is also an ATP-dependent efflux pump with broad substrate specificity. It is a well-characterized ABC-transporter (which transports a wide variety of substrates across extra and intracellular membranes) of the MDR/TAP subfamily (Dean *et al.,* 2001*).* Multidrug resistance can arise from several cellular processes, of which excessive drug expulsion by the transmembrane efflux pump P-glycoprotein has been described extensively by Germann *et al*., in 1993 (Germann *et al*., 1993). Active expulsion from drugs out of the cell is considered one of the major mechanisms underlying drug-resistant disease. It also exists in animals, fungi and bacteria, and it likely evolved as a defense mechanism against harmful substances. Glucocorticoids are known substrates for P-glycoprotein and may also induce P-glycoprotein expression (Shimada *et al.,* 2002; Karssen *et al.,* 2002). In humans, it is highly expressed in the small intestine and kidneys. In the kidney, P-glycoprotein is expressed in the brush border membrane of proximal tubular epithelial cells. Increased expression of P-glycoprotein results in decreased intracellular drug concentrations and may consequently decrease treatment response. Previous research has shown higher expression of MDR1 and increased P-glycoprotein activity in children with steroid-resistant nephrotic syndrome (Wasilewska *et al.,* 2006; Stachowski *et al.,* 2000). In addition, increased expression of the multidrug resistance gene/ABCB1 has been associated with diminished GC sensitivity *in vitro* studies of malignant cells (Bourgeois *et al.,* 1993) and colonic mucosa cells from patients with inflammatory bowel disease *(*Sambuelli *et al.,* 2006), although clinical studies yielded contradictory results (Den Boer *et al.,* 1998; Farrell *et al.,* 2000; Beck *et al.,* 1995; Brophy *et al.,* 1994; Palmieri *et al.,* 2005). The interpretation of the influence of the genetic polymorphisms on P-glycoprotein expression, however, is vague and may vary depending on tissue type, pathological

status and ethnicity (Sakaeda *et al.,* 2003). A recent systematic review on pharmacogenetics and adverse drug reactions in pediatric oncology patients indicated protective effects from two genetic polymorphisms of the MDR1 gene in methotrexate and vincristine related neurotoxicity in pediatric acute lymphoblastic leukemia patients (Conyers *et al.,* 2018). Several studies have been conducted to evaluate the association of P-glycoprotein polymorphisms with the receptiveness to glucocorticoids in patients with nephrotic syndrome. The results of these studies on the significance of the genetic polymorphisms are contradictory (Suvanto *et al.,* 2016; Wasilewska *et al.,* 2007; Chiou *et al.,* 2012; Youssef *et al.,* 2013; Safan *et al.,* 2017; Choi *et al.,* 2011; Dhandapani *et al.,* 2015; Cizmarikova *et al.,* 2015). A recent meta-analysis concluded that there is evidence of an association between ABCB1 1236T>C (rs1128503) and increased risk of steroid-resistance in children with nephrotic syndrome (Han *et al.,* 2017). The normal excretion of xenobiotics back into the gut lumen by P-gp pharmacokinetically reduces the efficacy of some pharmaceutical drugs (which are said to be P-gp substrates). Many drugs inhibit P-gp, some foods do as well, typically incidentally rather than as their main mechanism of action; (Yu *et al.,* 2017). Any such substance can sometimes be called a P-gp inhibitor. In addition, *in vitro* data suggested that the prednisolone is also a substrate of P-glycoprotein. Expression of P-glycoprotein or ATP-dependent efflux membrane transporter in the intestinal epithelium limits the absorption of drug substrates from the gastrointestinal tract. Therefore, theoretically, co-administration of P-glycoprotein inhibitors could increase glucocorticoid absorption and oral bioavailability and might affect glucocorticoid distribution (Czock *et al.,* 2005). A previous study that has been conducted in adult renal patients, however, showed a normal metabolism and renal clearance of prednisolone in patients treated with cyclosporine, which is a P-glycoprotein inhibitor (Frey *et al.,* 1987).

1.5.2 The Gene: NR3C1

The glucocorticoid receptor (GR) also known as NR3C1 (nuclear receptor subfamily 3, group C, member 1) is the receptor to which cortisol and other glucocorticoids bind. The GR is expressed in almost every cell in the body and regulates genes controlling the development, metabolism and immune response. Since the receptor

gene is expressed in various forms, it has diverse (pleiotropic) effects in different parts of the body. Glucocorticoid (GC) acts via its cytoplasmic glucocorticoid receptor. When the GR binds to glucocorticoids, its primary mechanism of action is the regulation of gene transcription (Lu *et al.,* 2006; Rhen and Cidlowski, 2005).

Sensitivity to glucocorticoids thus depends on both functionality and expression of the glucocorticoid receptor (Gross *et al.,* 2009). Glucocorticoid receptors are expressed in virtually every tissue of the body (Pujols *et al.,* 2002), including cells of the distal convoluted tubules, collecting ducts and all subsets of human glomerular cells (Yan *et al.,* 1999). The GR is an intracellular receptor located in the cytoplasm as a homodimer and belongs to the nuclear hormone receptor family. The GR gene or NR3C1 gene is located on chromosome 5 (5q31-q32). It consists of nine exons encoding for three functional domains: 1) The N-terminal harbors a transactivation domain, which accounts for transcriptional activation of GC sensitive (target) genes. 2) The C-terminal contains a ligand-binding domain (AF2) and is required for binding of GC, nuclear localization, receptor dimerization and binding of hsp90. 3) The central DNA-binding domain is essential for binding to the glucocorticoid response elements (Heitzer *et al.,* 2007). The primary, generalized GC resistance syndrome is rare (Chrousos *et al.,* 1993) and established mutations of the GR gene associated with GC resistance are not found in all patients presenting with endogenous cushing's syndrome (Huizenga *et al.,* 2000). Haack *et al.* (Haack *et al.,* 1999) assessed GR expression in mononuclear leukocytes in children with NS and found alike expression of the receptor during active NS and remission. The expression of GR was also similar in steroid-responsive children compared to children with steroid-resistant NS. This was confirmed in a study by De Carvalho *et al.* (De Carvalho *et al.,* 2004). Wasilewska *et al.* (Wasilewska *et al.,* 2003) found a temporary decrease of GR number in lymphocytes and monocytes during glucocorticoid treatment in children with steroid-sensitive NS, which resolved spontaneously. They did not investigate a relationship between GR expression and clinical consequence.

In children with NS, expression of GR has not been assessed in glomerular cells. In Korean adults with minimal change NS, a correlation was found between GR mRNA expression in glomerular cells and time to achieve remission during GC treatment (Han *et al.,* 2007). From these results, it was speculated that GR expression in

glomerular cells rather than immune cells may serve as an indicator for therapeutic response in nephrotic syndrome (Han *et al.,* 2007).

1.5.3 The Gene: CYP3A5

The human cytochrome P450 system comprises a number of CYP isoforms that have important functions in the oxidative and reductive metabolism of a variety of endogenous and exogenous compounds. Many CYP families, such as CYP1, CYP2, CYP3, CYP4, CYP5, CYP7, CYP8, CYP11 and CYP17 are found in humans (Nelson *et al.,* 1996; Hakkola *et al.,* 1998). The human cytochrome P450 3A (CYP3A) subfamily of enzymes plays an important role in drug metabolism. Four CYP3A genes, i.e., CYP3A5, CYP3A7, CYP3A4 and CYP3A43, are found in this sequential order within a 218-kb region of chromosome 7q22.1. CYP3A enzymes, primarily CYP3A4 and CYP3A5, catalyze the metabolism of a multitude of exogenous and endogenous compounds. Although the nucleotide sequences of CYP3A cDNAs resemble one another, their expression patterns are different. CYP3A4 is predominantly expressed in the human adult liver and intestines, whereas CYP3A5 is variably expressed in adult tissues and is the major CYP3A enzyme found in the human fetal liver. The expression of human CYP3A genes is regulated through different mechanisms. CYP3A5*3 is an A to G transition (A6986G) within intron 3 of CYP3A5. This transition creates an alternative splice site in the pre-mRNA, leading to the production of aberrant mRNA with a premature stop codon. This SNP results in a polymorphic expression of CYP3A5. CYP3A5*3 homozygotes (GG genotype) lack CYP3A5 expression, while individuals with at least one CYP3A5*1 wild-type allele (AA and AG genotypes) express CYP3A5. Polymorphic expression of CYP3A5 may account for some of the interindividual variations in the clearance of CYP3A substrates. Indeed, the CYP3A5 genotype is predictive of tacrolimus doses for lung and kidney transplant recipients (Chiou *et al.,* 2012). The CYP3A5*3 polymorphism has also been included in a study to identify pharmacogenomic predictors of outcome in 70 pediatric heart transplant patients followed for at least 1-year post-transplantation but in this population, no correlation of the CYP3A5*3 SNP with steroid-response after transplantation was observed (Zheng *et al.,* 2004). In a study estimating the influence of genetic polymorphisms of CYP3A5 (A6986G, CYP3A5*3), ABCB1 (C1236T, G2677T/A, C3435T) and PXR/NR1I2 (A7635G) on

pharmacokinetics of prednisolone in renal transplant recipients, NR1I2 (A7635G) rather than CYP3A5 or ABCB1 allelic variants affected patient variability of plasma prednisolone concentration. Patients carrying the NR1I2 7635G allele seemed to have higher metabolic activity for prednisolone in the intestine (Miura *et al.,* 2008). The distribution of CYP3A5*1 wild type allele is different among ethnic groups and varies in frequency ranges from 5%-15% in Caucasians, 15%-35% in Asians, 25% in Mexicans and 45%-73% in African-Americans (Provenzani *et al.,* 2013).

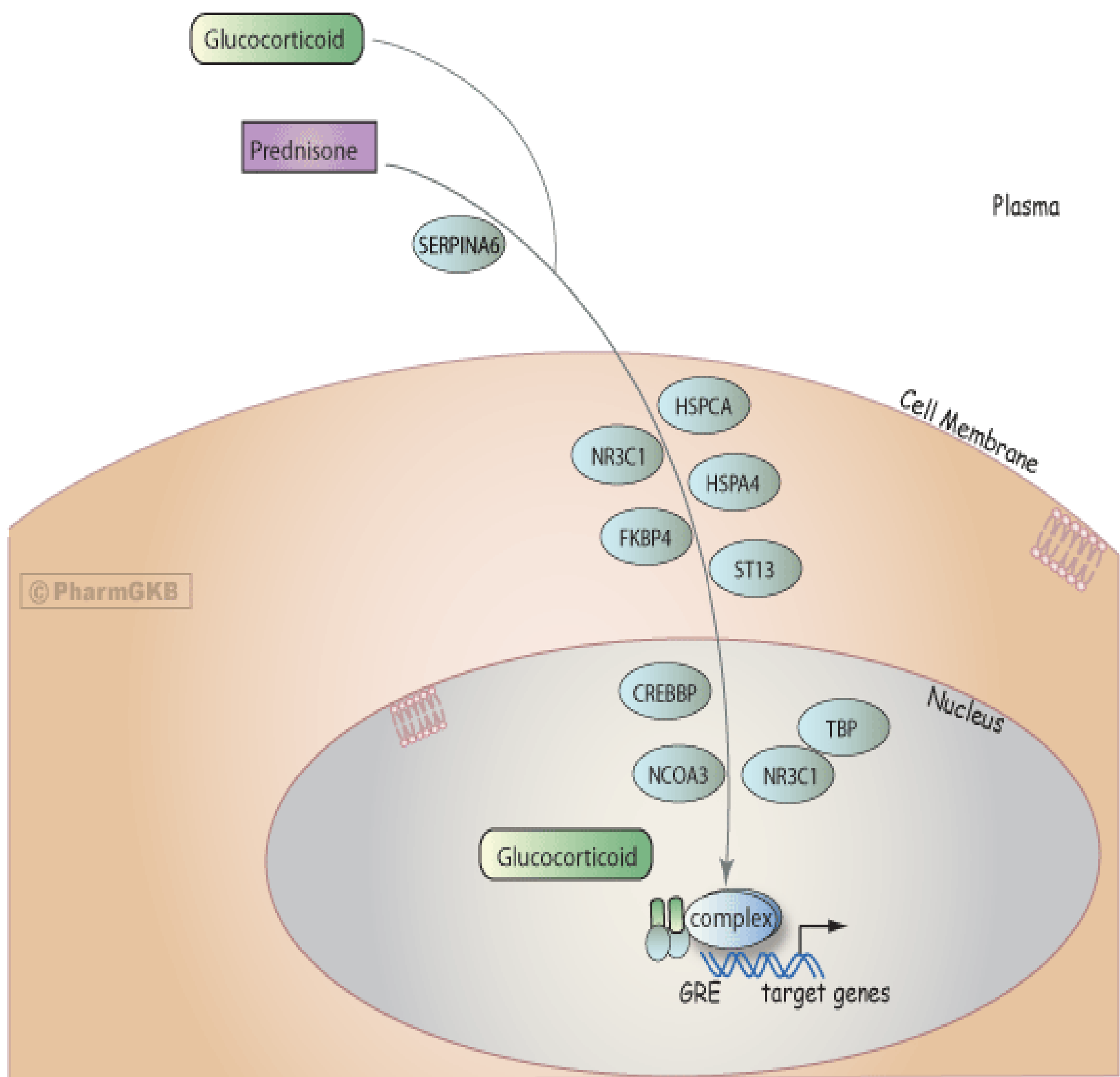


Figure 1.9: Glucocorticoids pathway

1.6 Justification of the Study

Diagnosis and treatment of steroid-resistant nephrotic syndrome remain a difficult challenge in pediatric nephrology. At present, there is no diagnostic marker for children displaying the nephrotic syndrome that can be used as a predictor of steroid responsiveness or resistance. An important prognostic marker is that children with nephrotic syndrome show a response to steroid treatment initially. The children with a family history of steroid-resistant nephrotic syndrome or in those who have a known gene mutation, initially steroid treatment can be avoided from their treatment plan. Currently, it should be an urgent need to distinguish those patients as soon as possible who may be benefitted from prolonged immunosuppressive treatment from those who will not get benefitted from these treatments and who will just suffer from its main adverse effects. There is emerging evidence that the majority of genetic forms of SRNS should receive symptomatic treatment only (Hoyer *et al.,* 2008).

In all, 24 genes are presently known to be associated with hereditary SRNS (Table 1.3) (McCarthy *et al.,* 2013) such as NPHS1, NPHS2, ADCK4, COQ6, CRB2, WDR73, EMP2, WT1, LAMB2, CD2AP, PLCE1, ACTN4, TRPC6, INF2, etc. have been implicated in different forms of SRNS. Nephrin, podocin and CD2AP, encoded by NPHS1, NPHS2 and CD2AP gene, respectively, are the main structural elements of the slit diaphragm. Besides these genes, several researchers have found in their study that ABCB1, NR3C1, CYP3A5 genes are responsible for developing steroid-resistant in childhood nephrotic syndrome in different ethnic groups.

Oral GC treatment regimens may influence the natural history of NS (Lande *et al.,* 2003). Interindividual and intraindividual variability in response to exogenous GC may be partially due to differences in the activity of factors responsible for its systemic bioavailability, tissue distribution and elimination. It is becoming increasingly evident that genetic polymorphisms in the genes for membrane transporters e.g. P-gp and metabolizing enzymes may determine the consequence of drug therapies (Sakaeda *et al.,* 2004).

P-gpI also have known as multidrug resistance protein 1 or ATP-binding cassette sub-family B member 1 or cluster of differentiation 243 (Dean *et al.,* 2001). Multidrug resistance can arise from several cellular processes, of which excessive drug expulsion by the transmembrane efflux pump P-glycoprotein (P-gp) has been

described extensively. Increased expression of P-gp results in reduced intracellular drug concentrations and may consequently weaken treatment response. Funaki *et al.* (Funaki *et al.,* 2008) found highly variable expression levels of ABCB1 mRNA during the active phase of NS in 15 patients. These expression levels decreased after remission. Patients from Northern India showed that NS patients carrying homozygous mutations in G2677T/A are more prone to developing steroid-resistance. The synergistic effect of the presence of the mutant genotype of the G2677T/A and C3435T MDR1 gene in different combinations may increase the risk of developing steroid-resistance in NS patients (Jafar *et al.,* 2011). A study conducted by Chiou *et al.* (Chiou *et al.,* 2012) demonstrated that C1236T polymorphism in the ABCB1 gene was associated with steroid-resistance in Chinese pediatric patients with INS. Several studies have been conducted to evaluate the association of P-gp expression with the responsiveness to steroids in patients with idiopathic NS. Wasilewska *et al.* (Wasilewska *et al.,* 2006) measured P-gp expression on CD3-positive lymphocytes and found that CD3/P-gp expression was significantly higher in patients with idiopathic NS than in the controls and that the difference was higher in steroid-dependent and frequent relapsing groups than in the non-frequent relapsing group. In another study by Stachowski *et al.* (Stachowski *et al.,* 2000) reported that MDR1 activity and mRNA expression in peripheral lymphocytes was higher in patients with steroid, cyclophosphamide or cyclosporine-resistant NS than in patients who were sensitive to those drugs. Moreover, in a recent work, Prasad *et al.* (Prasad *et al.,* 2015) found that steroid therapy in INS decreased P-gp expression in peripheral blood lymphocytes and several studies have demonstrated that genetic polymorphisms in this gene lead to functional alterations and are associated with altered drug disposition (Hoffmeyer *et al.,* 2000; Cascorbi *et al.,* 2001). Numerous genetic polymorphisms exist within the MDR1 gene and some are in strong linkage disequilibrium (Soranzo *et al.,* 2004). Novel indicators of the course of the disease have the potential to lead to improvements in the established therapeutic regimens. In our case-control study, we assessed the distribution of the three most frequent exonic polymorphisms like 1236T>C, 2677G>T and 3435T>C SNPs of the ABCB1/MDR1 gene in both prednisolone resistance nephrotic syndrome and prednisolone sensitive nephrotic syndrome patients and to investigate their functional polymorphism responsible for developing prednisolone resistance in childhood nephrotic syndrome patients.

Furthermore, glucocorticoid, the first line of drug for childhood NS, exerts its effects by its binding to the glucocorticoid receptor, a ligand-dependent transcription factor, which belongs to the superfamily of nuclear receptors (Baxter and Rousseau, 1979). Different studies suggest that the genetic variations of NR3C1 are associated with GC sensitivity. Huizenga *et al.* (Huizenga *et al.,* 1998) have demonstrated that the common NR3C1 polymorphism, GR N363S variant, may be associated with an increased sensitivity to GCs and recently Stevens *et al.* (Stevens *et al.,* 2004) have demonstrated that GC sensitivity is determined by a specific GR haplotype. Furthermore, different investigators have demonstrated that mutations in the NR3C1 gene are associated with both familial steroid-resistance (Ye *et al.,* 2006; Bray and Cotton, 2003) and acquired steroid-resistance in some diseases, such as cushing's disease (Ye *et al.,* 2006; Karl *et al.,* 1996), leukemia (Ye *et al.,* 2006; Hillmann *et al.,* 2000), lupus nephritis (Ye *et al.,* 2006; Jiang *et al.,* 2001) and female pseudohermaphroditism (Ye *et al.,* 2006; Mendonca *et al.,* 2002). Therefore, we hypothesized that NR3C1 variants are associated with steroid-resistance in sporadic NS. Mutations of the NR3C1 gene have been found in familial steroid-resistance. These mutations are associated with decreased functional GR expression levels and obstruction of GC binding and transactivational capacity (Karl *et al.,* 1993; Malchoff *et al.,* 1993). So, in our current study, we have tried to find out the association between rs10482634 and rs6877893 polymorphisms of the NR3C1 gene and prednisolone resistant in childhood nephrotic syndrome patients.

In addition, the human CYP3A5 gene encodes a member of the cytochrome P450 3A subfamily of enzymes, which plays an important role in drug metabolism, including that of prednisolone (Chiou *et al.,* 2012). The G allele of CYP3A5*3 causes alternative splicing and produces a truncated protein with a loss of enzyme activity (Kuehl *et al.,* 2001). The reductive and oxidative metabolism of GC and other endogenous and exogenous compounds is mainly mediated by the human cytochrome P450 system that comprises numerous isoforms, including CYP3A5 (Lamba *et al.,* 2002). This CYP3A5 is variably expressed in adult tissues and is the major CYP3A enzyme found in the human fetal liver. CYP3A5*3 is an A to G transition (A6986G) that generates alternative splicing leading to a truncated protein with a loss of enzyme activity (Hustert *et al.,* 2001). Several studies (Thervet *et al.,* 2003; Zheng *et al.,* 2003) have demonstrated the predictive role of the CYP3A5 genotype of tacrolimus

doses for lung and kidney transplant recipients. Indeed, genetic polymorphisms of CYP3A5 and MDR1 could have a role in the pharmacokinetics of prednisolone; in particular, intestinal CYP3A5 and P-gp are important in the absorption, systemic drug distribution and cellular accumulation of GC (Moussa *et al.,* 2017). While, in different studies, Chiou *et al.* (Chiou *et al.,* 2012) and Moussa *et al.* found that the CYP3A5*3 polymorphism in the CYP3A5 gene showed a trend of association to GC resistance in pediatric NS patients but these association did not reach the statistical significance ($p > 0.05$). This might be due to the small number of sample size. So, from the studies, we found that CYP3A5*3 polymorphism has an influence on prednisolone resistance in childhood NS. Therefore, in our current study, we have tried to find out the role of CYP3A5*3 polymorphism of the CYP3A5 gene for developing prednisolone resistance nephrotic syndrome in Bangladeshi pediatric patients.

Therefore, the purpose of this study was to investigate the association of ABCB1 gene polymorphisms 1236T>C, 2677G>T and 3435T>C, NR3C1 gene polymorphisms rs10482634 and rs6877893 and CYP3A5 gene polymorphism CYP3A5*3 and risk of developing prednisolone resistance in nephrotic syndrome in Bangladeshi population by polymerase chain reaction-restriction fragment length polymorphism methods.

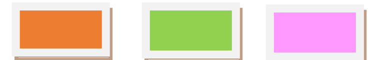
In Bangladesh, there were no previous pharmacogenetics studies on prednisolone resistance in childhood nephrotic syndrome patients. Because of the poor prognosis in the past in prednisolone resistance nephrotic syndrome, the majority of the children have received intensive treatment regimens and many of them have been over treated. So, to develop genotype base prediction for the safety and efficacy of the treatment, it is essential to find out candidate genes and their polymorphisms contributing to this clinical feature. As a result, the prognosis of the disease will be faster and the mortality rate will decrease. Besides this, the study will help us to develop a safe, efficient and cost-effective treatment plan for the patients. Furthermore, the current data available on pharmacogenetics of prednisolone resistance in patients with nephrotic syndrome are summarized and areas for future research to improve individualization of prednisolone resistance therapy in children with nephrotic syndrome are identified. Furthermore, we did not find any citations regarding the relationship between rs10482634 and rs6877893 SNPs of the NR3C1 gene and prednisolone resistant in childhood nephrotic syndrome till now which gave us the

motivational force to go through the study. Besides, no pharmacogenetic study regarding ABCB1, NR3C1, CYP3A5 genes and their role in developing prednisolone resistance in nephrotic syndrome have yet done on the Bangladeshi population.

Genetic polymorphisms that we are studying in this research are potentially related to the prednisolone resistance in childhood NS patients. So, the findings of this research will assist the doctor in making the most optimal treatment decision possible for both prednisolone resistance patients and sensitive patients based on an individual's genetic make-up. The doctor can select the best drug for Bangladeshi nephrotic syndrome patients with the least adverse effects and maximum efficacy. If a particular genotype is found to be significantly associated with severe toxic effects or lower response rate to prednisolone, then prednisolone based treatment can be avoided for individuals having this particular genetic polymorphism. Thus this present study will help to improve nephrotic syndrome treatment, decreasing the kidney disease-associated mortality rate.

CHAPTER TWO

MATERIALS AND METHODS



2. MATERIALS AND METHODS

2.1 Selection of Volunteer

2.1.1 Volunteer

A case-control study was carried out on 180 pediatric nephrotic syndrome patients in Bangladesh. Among them, 30 cases with clinically proven prednisolone resistance in childhood nephrotic syndrome patients and 150 controls with prednisolone sensitive in childhood nephrotic syndrome patients were recruited from different hospitals of Bangladesh namely, Bangladesh Institute of Child Health, Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University from the mid of June 2014 to the end of February 2018. By the ethical committees of the Bangladesh Institute of Child Health or Dhaka Shishu hospital, the study protocol and consent form of the clinical investigation was approved. Each patient's guardian signed an informed consent document before entering the study and was free to withdraw from the study at any time without any obligation. All the patients were treated with prednisolone, some patients may show response and some are shown resistance to prednisolone. Genotyping study was conducted in the "Laboratory of Pharmacogenetics and Pharmacokinetics" of the Departments of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh. The study blood samples were collected from the patient's routine blood testing and follow up at the hematology department's blood sampling area of the different hospitals. The study was conducted in accordance with the International Conference of Harmonization for Good Clinical Practice and in compliance with the Declaration of Helsinki and its further amendments (adopted by the 18th WMA general assembly, Helsinki, Finland, June 1964 and last amendment in Seoul, South Korea on October 2008). The clinical data were collected retrospectively from the patients' hospital records.

All NS patients fulfilled the criteria for the diagnosis of NS of the International Study of Kidney Disease in Children. They were diagnosed if they had massive proteinuria of $\geq$40 mg/m^2/h with hypoalbuminemia of $\leq$2.5 g/dl without knowing the causes (ISKDC, 1981). NS patients were classified according to their initial response to steroids as steroid-sensitive or steroid-resistant. Steroid-sensitive “responders” NS patients fulfilled the following criteria; disappearance of proteinuria (negative to trace in a urine for 3 consecutive days, or a urine protein/creatinine level of $<$0.2 g/g) within the first 4 weeks of full dose prednisolone therapy (2 mg/kg/day or 60 mg/m^2/day). While steroid non-responder “resistant” NS patients had nephrotic range proteinuria $>$40 mg/m^2/h, serum albumin level $<$2.5 g/dl, age $>$1 year, the secondary causes of NS are absence, the follow-up duration was $\geq$6 months from the period of diagnosis and resistant to the steroid treatment of 60 mg/m^2/day for 4 months followed by 3 pulse methylprednisolone dose at 1 g/ 1.73 m^2/ 48 h (Kidgo, 2012).

All hospitals were in Dhaka city and all subjects sampled came from different villages and cities all over Bangladesh since these institutions are specialized health care centers each with the capacity to treat a large number of nephrotic syndrome patients at any given point of time.

2.2 Patient Consent Form

A form regarding the approval of the patients is included in the appendix-1 portion**.**

2.3 Data Collection Form

The data collection form needed to encompass all the information about the patients is included in the appendix-2 portion.

2.4. Materials and Methods

2.4.1. Instruments

Instruments	Sources
UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge	Hettich GmbH & Co., Germany
MJ Mini Gradient Thermal Cycler	Bio-Rad Laboratories, USA
Alpha Imager HP (Gel Doc. System)	Alpha Innotech Corporation, USA
Gel Electrophoresis Machine (Elite)	Wealtec, Germany
UV Probe V.2.1 Spectrophotometer	Shimadzu, USA
P^H Meter (Cyber Scan 500)	Eutech, Singapore
Distillation Plant (Distinction D4000)	Bibby Sterlin Ltd., UK
Micropipette	Bio-Rad Laboratories, USA
Freeze (-80°C)	DAIREI, Sweden
Ultrapure Water System (Arium 611)	Sartorius, Germany
Microcentrifuge Machine (Mikro 20)	Hettich GmbH & Co., Germany
Freeze (-20°C)	Siemens, USA
Vortex Mixer Machine (Rotamixer-9590)	Hook & Tucker Instruments Ltd., UK
Autoclave Machine	Yongfeng Enterprise Co., UK
Heidolph Unimax-2010 Incubator	Wolf Laboratories Ltd., UK
Electronic Balance	Ohaus Co. Ltd., USA
Incubator	Labtech, Daihan Co. Ltd., USA
Incubator	Cleaver Scientific Ltd., USA

1.4.2 Consumable Materials

Materials	Sources
Reagent Bottle (250, 500, 1000 ml)	Schott GL-45, German
Conical Flasks	Schott GL-45, Germany
Pipettes (Precicolor)	HBG, Germany
Eppendorf Tube (1.5 ml)	Hamburg, Germany
Pipette Tips	ALA, USA
PCR Tubes (0.2/0.5 ml)	Bio-Rad Laboratories, USA
Falcon Tubes (50 ml)	Hamburg, Germany
Polypropylene Tubes (15 ml)	Hamburg, Germany

2.4.3 Chemicals and Reagents

2.4.3.1 Agarose

Type	DNA size (kbp)	Gel strength (gm/cm^2)
HS	0.5-30	>2000 (1.5%)
H	1-200	>2800 (1.5%)
X	0.01-1.0	>1000 (3.0%)
1600	0.01-1.0	>1400 (1.5%)

2.4.3.2 Other Reagents

Reagents	Sources
Triton-X 100	Sigma Chemical Company, USA
Sodium Lauryl Sulphate	Sigma Chemical Company, USA
Ethanol	Sigma Chemical Company, USA
Chloroform	Sigma Chemical Company, USA
Sodium Perchlorate	Sigma Chemical Company, USA
Glacial Acetic Acid	Sigma Chemical Company, USA
Sodium Chloride	Sigma Chemical Company, USA
Sucrose	Sigma Chemical Company, USA
Magnesium Chloride	Sigma Chemical Company, USA
Tris-HCl	Sigma Chemical Company, USA
EDTA-Na_2	Sigma Chemical Company, USA
Nuclease Free Water	Promega Corporation, USA
Ethidium Bromide	BDH, UK
Sample Loading Dye, 6X	Promega Corporation, USA
Taq DNA Polymerase	NEB, USA
Standard Reaction Buffer	NEB, USA
$MgCl_2$ Solution	NEB, USA
Deoxyribonucleotide Solution Mix (dNTP)	NEB, USA
Quick-Load® 50 bp DNA Ladder	NEB, USA
100 bp DNA Ladder	NEB, USA
50 bp DNA Ladder	NEB, USA

2.4.4 Restriction Enzymes

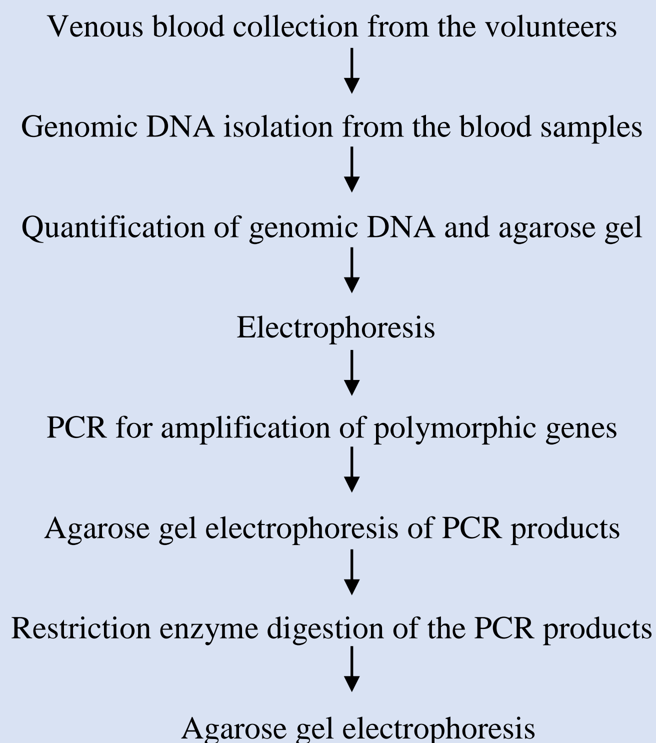
Genes and SNPs	Restriction enzymes	Recognition sites	Source
ABCB1 1236 T>C	*HaeIII*	5′ …..GG CC.....3′ 3′ …..CC GG.....5′	New England BioLabs Inc., USA
ABCB1 2677G>T	*Hpy1881*	5′ …..TCN GA.....3′ 3′ …..AG NCT.....5′	New England BioLabs Inc., USA
ABCB1 3435 T>C	*MboI*	5′ ….. GATC.....3′ 3′ …….CTAG .....5′	New England BioLabs Inc., USA
NR3C1 rs10482634	*BstUI*	5′ ….. CG CG.....3′ 3′ ….. GC GC.....5′	New England BioLabs Inc., USA
NR3C1 rs6877893	*HaeIII*	5′ …..GG CC.....3′ 3′ …..CC GG.....5′	New England BioLabs Inc., USA
CYP3A5 CYP3A5*3	*RsaI*	5′ ….. GT AC.....3′ 3′ ….. CA TG.....5′	New England BioLabs Inc., USA

→ **Cutting site**

2.4.5 Solutions

Name	Composition
TAE Buffer (10x)	0.4 M Tris-Base (Tris (Hydroxymethyl)-aminomethane), 11.4% (v/v) /0.2 M Glacial acetic acid, 0.01 M EDTA-Na_2, p^H to 7.6 with Glacial acetic acid or Tris.
TBE Buffer (10x)	0.89 M Tris-Base Boric acid, 20 mM EDTA-Na_2, P^H to 8.0 with Boric acid or Tris.
TE Buffer (1x)	10 mM (Tris-HCl (Tris (Hydroxymethyl)-aminomethane hydrochloride), 0.001M EDTA-Na_2, p^H to 8.0 with Tris.

2.5 Methods of Genotyping



2.5.1 Venous Blood Collection

After explanation and counseling about the study, approximately 3 ml of venous blood was collected from each patient in a sterile eppendorf tube containing ethylenediaminetetraacetic acid disodium. Then samples were stored at -80ºC until DNA extraction.

2.5.2 Genomic DNA Isolation

There are many different protocols and a large number of commercially available kits used for the extraction of genomic DNA from whole blood. In this study, we isolated DNA by using Daly's Method (Daly *et al.*, 1998). This procedure is routinely used in both research and clinical service provision in our laboratory and is cheap and robust.

2.6 Preparation of DNA Isolation Reagents

Reagent name	Composition and preparation procedure	Storage condition
Cell Lysis Buffer (1L)	To prepare 1L buffer, 10 mM Tris-(hydroxymethyl)-aminomethane, 320 mM sucrose and 5 mM $MgCl_2$ were added to 850 ml of distilled water. Add dropwise HCl to it for the adjustment of pH 8.0. Then it was autoclaved. After that, 1% Triton X-100 was added to it and the total solution was prepared up to 1L by adding distilled water.	4°C

Nuclear Lysis Buffer (1L)	To prepare the buffer, 400 mM Tris-(hydroxymethyl)-aminomethane, 60 mM $EDTANa_2$ and 150 mM sodium chloride were added to 850 ml of distilled water. Then the pH was adjusted to 8.0 by adding dropwise HCl. After autoclaving, 1% sodium lauryl sulphate was added to it and the total solution was made up to 1L by adding distilled water.	Room Temperature
5 M Sodium Perchlorate (100 ml)	61.22 gm of sodium perchlorate was dissolved in 100 ml of distilled water. Then shake to make the solution.	4°C
5 mM Tris HCl Buffer (250 ml)	Weight 0.197 gm of Tris HCl, then added in 150 ml of distilled water. Shake the mixture, then pH was adjusted to 8.0 by adding NaOH. The total solution was made up to the volume of 250 ml by adding distilled water. Then it was autoclaved.	4°C

2.7 Genomic DNA Isolation Procedure

From the blood, DNA was isolated by the previously published method (Daly *et al.,* 1998).

Briefly, it is given below,

1. First of all, we were taken 3 ml of blood in a 50 ml Falcon centrifuge tube which contains 2 mg of EDTA.
2. Then Lysis buffer of 20 ml was added to it and it was mixed smoothly for 2 minutes by inversion. By using UNIVERSAL 240V 50-60Hz Refrigerated Bench-Top Centrifuge Machine (Hettich GmbH & Co., Germany), it was then centrifuged for 10 minutes at 3000 rpm at 4°C.
3. The supernatant was discarded into a bottle that contains enough savlon. The pellet was collected.
4. Nuclear lysis buffer of 2 ml and 0.5 ml of 5 M sodium perchlorate were added to it.
5. Then at room temperature, the tube was mixed in a rotary mixture for about 15 min so that the pellet was dissolved completely with the mixtures.
6. Then in an incubator, the sample tube was incubated at the temperature of 65°C for 30 min. (Heidolph Unimax- 2010 Incubator, Wolf Laboratories Limited, UK).
7. Then chilled chloroform of 2.5 ml was added to it.
8. At room temperature, it was mixed in a rotary mixture for 10 min.
9. Then 1500 rpm for 5 min. at 37°C, the tube was centrifuged.
10. The uppermost phase which is known as DNA containing phase was transferred to a fresh autoclaved 15 ml polypropylene tube using a disposable Pasteur pipette.
11. After that, two volumes of ethanol which was double that of the DNA phase was added to it.

12. By slow gentle inversion, it was mixed immediately until all the cloudiness was disappeared.

13. It was seen that the DNA was come out of the solution as a white ‘cotton-wool’ pellet.

14. With the help of a disposable microbiology loop, the white ‘cotton-wool pellet’ was collected.

15. The loop was kept for air- dried.

16. 5 mM Tris-HCl buffer was taken in a 1.5 ml screw cap tube. Then the DNA was dissolved in it.

17. Then in an incubator, the tube was kept at 65°C overnight.

18. Then it was taken back and was stored in the Freezer at -20°C for further analysis.

2.8 Quantification of Genomic DNA

The quantity and purity of DNA which was isolated from the blood samples were assessed by using a UV spectrophotometer (UV Prove v2.1) at a wavelength of 260 nm. In order to ensure complete homogeneity of the sample, which is critical when measuring the concentration of the genomic DNA and the purity with this instrument, samples were very gently shaken with the help of the vortex shaker for approximately 30 minutes before measurements were taken. A sample volume of 1.5 to 2 μl was pipetted onto the fiber optic measurement surface. By using nuclear-free water genomic DNA of standard concentration of 50 ng/μl was made up which was known as working solution of genomic DNA, except in cases where the sample was prepared that had an initial concentration of less than 50 ng/μl, in which case an undiluted aliquot was taken as a working solution.

For calculation of DNA concentration of samples free of RNA, the following conversion factor is used: 1 OD260 = 50 mg of DNA/ml. The DNA concentration in µg/µl was calculated as follows:

$$\text{DNA Concentration } (\mu g/\mu ml) = \frac{\text{OD } 260\text{X}50 \text{ (dilution factor)X } 50 \text{ } \mu g/ml}{1000}$$

Where, OD260/OD280; should be = 1.7-1.9 (OD= Optical Density)

A value out of this range is not acceptable. It may, therefore, indicate that the DNA sample is not in solution or that there are contaminants (i.e., protein) within the sample that may inhibit subsequent reactions. All working solutions of genomic DNA were stored at -40ºC until genotype analysis. By using agarose gel electrophoresis, the purity and integrity of isolated genomic DNA were also evaluated 5 µl (50-70 ng/µl) of sample volume was resolved on a 2% (w/v) agarose gel.

2.9 Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphism analysis is one of the earliest and most widely used genotyping methods that work on the principle of allele-specific enzymatic cleavage. A restriction fragment length polymorphisms (RFLP) is generated when a single nucleotide polymorphism occurs at a restriction endonuclease recognition sequence, and one allele preserves the sequence whereas the other destroys it. If we have a tendency to consider any deoxyribonucleic acid fragment with three adjacent restriction sites, with the middle one containing a single nucleotide polymorphism, with the help of appropriate restriction enzyme the digestion of amplified DNA was done that will produce either a large single fragment (if the central restriction site is absent) or two smaller fragments (if the central restriction site is present and therefore cleavage occurs). By gel electrophoresis, the fragments are then separated which can be compared to others to detect the differences. These differences based on the length of the digested DNA fragments are known as restriction fragment length polymorphisms (Saiki *et al*., 1985; Osborn *et al*., 2000).

2.10 Genotyping of Single Nucleotide Polymorphisms of ABCB1 (1236T>C, 2677G>T, 3435T>C), NR3C1 (rs10482634, rs6877893) and CYP3A5 (CYP3A5*3)

To facilitate the accurate genotyping of the volunteer's DNA samples for the selected single nucleotide polymorphisms, PCR-RFLP was employed due to its affordability, efficiency, ease of use and reliability. This genotyping method produces the restriction enzyme digestion of the polymerase chain reaction amplification product. For the PCR amplification product, the subsequent digestion or lack of digestion has occurred on the basis of the presence or absence of an SNP within the restriction endonuclease recognition site that allows for reliable and accurate genotyping and the subsequent determination of the frequencies of SNP within a sample cohort. The SNP genotype was classified as 'wild-type' or 'variant' which was done according to an accepted nomenclature and on the basis of the relevant reference sequences that were available from the National Centre for Biotechnological Information Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

2.10.1 DNA Amplification by Polymerase Chain Reaction

The relevant genomic target regions, containing the SNPs of interest, were amplified by means of primer-directed polymerase chain reaction (PCR) using thermostable DNA polymerase, as originally described by (Saiki *et al.,* 1985; Saiki *et al.,* 1988). This primer-directed PCR method facilitates the *in vitro* amplification of single-copy genomic DNA sequences by a factor of more than ten million with extremely high sequence specificity.

2.10.2 Primer Design

For primer design, we should follow the following guidelines:

1. Generally, PCR primers should be consists of 15-30 nucleotides.

2. 40-60% is the optimal GC content of the primer. Preferably, C and G nucleotides should be distributed evenly along with the primer.

3. During primer design, we should avoid placing more than three G or C nucleotides at the 3'-end to decrease the risk of non-specific priming.

4. It should avoid primer self-complementarities or complementarities between the primers to prevent the hairpin formation and primer dimerization.

5. We should check for possible sites of non-desirable complementarities between primers and the template DNA.

6. The differences in the melting temperatures of the two primers should not exceed 5°C.

The primers for the current study were designed by considering all the above factors. In the present study, the sequences of the primers used and their sizes are presented in Table 2.1.

Table 2.1: Name of the allele, sequence of the designed primer with their size and melting temperature

No.	Genes and SNPs	Primer type	Primer sequence	M. T (°C)	Size (bp)
1.	ABCB1 1236T>C	FP	5’-TTACCCATCTCGAAAAGAAGTTAAGGT-3′	65.5	27
		RP	5’-TGCCCACTCTGCACCTTCATGTTC-3′	73.3	24
2.	ABCB1 2677G>T	FP	5’-TTACCCAGAATATAGCAAATCTTGG-3′	62.9	25
		RP	5’-CATATTTAGTTTGACTCACCTTCTCAG-3′	62.0	27
3.	ABCB1 3435T>C	FP	5′-TGCTGGTCCTGAAGTTGATCTGTGAAC -3′	60.5	27
		RP	5′-ACATTAGGCAGTGACTCGATGAAGGCA -3′	61.6	27
4.	NR3C1 rs10482634	FP	5’-CACAGATACTTGACTTGGCTATGG-3′	55.3	24
		RP	5’-AACACCTACTTATTTGAGCAGCTT-3′	53.2	24
5.	NR3C1 rs6877893	FP	5′-AGAACTGGAGATTGCCAAGG-3′	54.5	20
		RP	5′-AAAGCTGCATTTTAGCAGCA-3′	52.2	20
6.	CYP3A5 CYP3A5*3	FP	5′-CCTGCCTTCAATTTTTCACT -3′	51.2	20
		RP	5′-GGTCCAAACAGGGAAGAGGT-3′	56.9	20

FP= Forward Primer; RP= Reverse Primer; M.T= Melting Temperature;

Primers were procured from Bio Basic Inc, USA.

2.11 Required Conditions for PCR

GoTaq® DNA polymerase, reaction buffer, dNTPs and $MgCl_2$ were used for the PCR amplification of the relevant genomic target regions, containing the SNPs of interest. A 25 µl reaction volume was used containing 1.0 µl of genomic DNA (50-70 ng/µl), 5.0 µl of 5X GoTaq® reaction buffer, 4.0 µl of $MgCl_2$ (25 mM), 2.0 µl of dNTPs (2.5 mM), 1.0 µl of each primer (10 µM), 0.1 µl of GoTaq® DNA polymerase (5 U/µl) and 10.9 µl of Nuclease free water. PCR conditions to synthesize various alleles with their respective lengths are given in Table-2.2.

Table 2.2: PCR conditions to synthesize various alleles and their respective lengths

Genes and SNPs	PCR conditions (35 cycles)	Size of PCR products (bp)
ABCB1 1236T>C	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	234
ABCB1 2677G>T	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	198
ABCB1 3435T>C	94°C for 1 min 55°C for 1 min 72°C for 2 min	248
NR3C1 rs10482634	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	300
NR3C1 rs6877893	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	225
CYP3A5 CYP3A5*3	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	196

2.12 Restriction Enzyme Digestion

After PCR amplification, 10 µl of the PCR products were digested with approximately 2 units of respective restriction enzymes that were obtained from New England BioLabs Inc., USA. Incubation conditions are listed in Table 2.3. Electrophoresis was done for the digested products using 2% agarose gel.

Table 2.3: The restriction enzymes, digestion condition and length of the expected fragments on digestion to diagnose genes

Genes and SNPs	Restriction enzymes (REs)	Digestion condition	Expected fragments (bp)
ABCB1 1236 T>C	*HaeIII*	Incubation at 37°C overnight	NH: 234 HE: 234, 207, 27 MH: 207, 27
ABCB1 2677 G>T	*Hpy1881*	Incubation at 37°C overnight	NH: 198 HE: 198, 173, 25 MH: 173, 25
ABCB1 3435 T>C	*MboI*	Incubation at 37°C overnight	NH: 248 HE: 248, 192, 56 MH: 192, 56
NR3C1 rs10482634	*BstU1*	Incubation at 60°C overnight	NH: 300 HE: 300, 118, 182 MH: 118, 182
NR3C1 rs6877893	*HaeIII*	Incubation at 37°C overnight	NH: 225 HE: 225, 60, 165 MH: 60, 165
CYP3A5 CYP3A5*3	*RsaI*	Incubation at 37°C overnight	NH: 102, 94 HE: 102, 94, 74, 20 MH: 102, 74, 20

NH: Normal Homozygote; HE: Heterozygote; MH: Mutant Homozygote

2.13 Visualization of PCR Products and Restriction Endonuclease Digestion Fragments

PCR amplification products were visualized by means of agarose gel electrophoresis in order to allow for size estimation and thus confirmation of amplification of the desired genomic target region. Restriction endonuclease (REase) digestion fragments that were of sufficient size (>100 bp) and size differential between fragments (>30 bp) were also visualized on the agarose gel. EZ Load™ Molecular ruler (100 bp) was used for size estimation of PCR amplification products, which served as confirmation that amplification of the desired genomic target region had occurred, as well as for quantification of the PCR product prior to restriction endonuclease digestion reactions. EZ Load™ 100 bp DNA ladder was also used for size estimation of all REase digestion fragments, allowing for accurate and reliable genotyping of samples. EZ Load™ 50 bp or 100 bp DNA ladder is thus evident in lane 1 or any other marked position of all agarose gel photos. All agarose gels were visualized under ultraviolet light and photographed with a Gel Documentation and Analysis System.

2.14 Gel Electrophoresis

The electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose gel electrophoresis of DNA is employed to work out the presence and distinguish the sort of nucleic acids found after extraction and to analyze digestion products. The required DNA fragments will be physically isolated for numerous functions like sequencing, probe preparation, or for cloning fragments into other vectors. For DNA analysis, both agarose and polyacrylamide gels are used. In case of analysis of size larger fragments (greater than 200 bp), agarose gels are usually run and polyacrylamide gels are run to less than 200 bp size fragments. Usually, agarose gels are used for most of the purposes and polyacrylamide gels are used when fragments are small, like digests of 16S rRNA genes, are being distinguished. The regular agarose gels may range in concentration from 0.6 to 3.0%.

Agarose is a polysaccharide purified from seaweed. The agarose gel is formed by suspending dry agarose in a buffer solution. It was boiling until the solution becomes

clear, and then it was pouring into a casting tray and allowing it to cool. After that, it forms a flexible gelatine-like slab. In the electrophoresis process, the gel is submerged in a chamber that contains a buffer solution and a positive and negative electrode. By using the electrical current, the DNA that is analyzed is forced through the pores of the gel. Then the DNA will move to the positive electrode (red) and away from the negative electrode (black) under an electrical field. Many factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose within the gel and most significantly, the size of the DNA molecules. Smaller DNA molecules passage through the pores of the agarose quicker than larger molecules. By adding the ethidium bromide, DNA in the gel will be visualized. When exposed to ultraviolet light, DNA illuminates with the binding of ethidium bromide, causing the DNA to 'glow'.

By electrophoresis in 2% (w/v) agarose gel at 80 volts, all PCR products were resolved. The restriction endonuclease digestion fragments were also observed in 2% (w/v) agarose gel electrophoresis. All restriction endonuclease digestion fragments were resolved at 80 V, thus ensure enough resolution to allow for accurate genotyping.

2.15 Agarose Gel Electrophoresis Procedure

All agarose gels were made with 1X Tris-acetate- EDTA buffer, which was made and stored as a 10X stock solution and diluted to the required working concentration as was needed. In order to facilitate the visualization of DNA within the agarose gel under UV light, 1 µg of ethidium bromide per ml agarose solution was added -i.e. 0.01% (v/v) ethidium bromide stock solution (10 mg/ml).

- **Procedure**

A. Casting a Gel

1. In a conical flask, appropriate volume of 1X Tris-acetate-EDTA buffer and an appropriate amount of agarose (these values are determined on the basis of the gel dimensions and the desired percentage of agarose) was taken. Then mixed the ingredients. For even distribution of the agarose, the flask was swirled.

2. The solution was then heated for 1 minute in the microwave oven. Protective gloves were worn and the flask was removed from the microwave oven (before it boiled over), swirled, and reheated while keeping constant watch to be sure it did not boil over. When it started to boil, boiling was stopped and swirled again repeating the process until all of the agaroses went into solution.

3. The flask was allowed to cool. The gel was poured when the temperature of the solution was 55-65°C.

4. While the agarose was cooling, the gel apparatus was prepared for casting the gel.

5. Before pouring the gel into a casting tray, ethidium bromide was added with the gel to the dissolved agarose and swirled to mix.

6. Then the gel was poured into the casting tray and also the comb was adjusted carefully to keep the wells perpendicular. Before use, the gel was allowed to cool and was hardened which took 20-30 minutes.

7. No bubble or black particles were allowed to settle in the tray.

B. Preparing the Gel for Electrophoresis

8. A few ml of 1X TAE buffer was added to the good area of the gel and the comb was carefully removed by pulling straight up.

9. The electrophoresis tank was filled with buffer solution (1X TAE) and the gel was placed (in the casting tray) on the tank platform.

C. Preparing Samples for Loading/Running the Gel

10. An appropriate volume of loading dye (6X) was added to the sample (1 µl of 6X sample dye for every 5 µl of the sample).

11. The sample was loaded using a 1-10 µl micropipette. The marker was also loaded.

12. After loading the gel, the cover was smoothly placed on the apparatus and also the Power leads were connected. Then, the power was adjusted to 80 volts (constant

voltage). The gel was run till the first dye front (bromophenol blue) had migrated almost two-thirds the length of the gel and also the second dye front (xylene cyanol) had migrated just about one-third of the length of the gel.

13. Before removing the gel for photographing, the power was switched off.

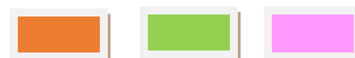
14. To visualize the DNA, the gel was placed on the UV transilluminator.

2.16 Statistical Analysis

Distributions of demographic and clinicopathological variables were compared between cases and controls using $\chi 2$-test, two-sided unpaired *t*-test and their correlations were established by using Pearson's correlation test. Genotype and allelic frequencies were reported as a percentage. The distribution of genotype frequency was also compared by $\chi 2$-test. $\chi 2$-test was also used to estimate the odds ratios (ORs) and their 95% confidence intervals (CIs), using the statistical software package SPSS version 25.0 (SPSS, Inc., Chicago, IL, USA). Deviation of genotype frequencies in the controls group from cases group under Hardy-Weinberg equilibrium (HWE) was measured by the chi-square test ($\chi 2$). In all of the analyses, $p < 0.05$ (two-tailed) was considered as statistical significant.

CHAPTER THREE

RESULTS AND DISCUSSION



3. RESULTS

3.1 Cases and Controls Characteristics

The distributions of demographic and clinicopathological characteristics of all the recruited patients are summarized in Table 3.1. This case-control study consisted of 30 prednisolone resistance nephrotic syndrome (PRNS) patients as cases whereas 150 prednisolone sensitive nephrotic syndrome (PSNS) patients were recruited as controls. Besides the genetic study, other related factors such as age, BMI, sex, socioeconomic status, biochemical parameters related to serum albumin, serum cholesterol, urine protein/creatinine ratio and clinical features of cases and controls were compared to find out whether these factors are involved or not for developing prednisolone resistance in childhood nephrotic syndrome. We were also analyzed these recorded values to confirm the observed effects were solely due to the genetic variation.

Table-3.1: Distribution of demographic data and clinicopathological characteristics of prednisolone resistance and prednisolone sensitive nephrotic syndrome patients

Characteristics	**Cases (n=30)**	**Controls (n=150)**	**p value**
Age	9.07 ± 3.27	7.69 ± 3.40	0.061
Body max index (kg/m^2)	18.62 ± 2.49	17.74 ± 2.58	**0.044**
Gender			
Male	18 (60%)	95 (63.33%)	-
Female	12 (40%)	55 (36.67%)	-
Socioeconomic status			
Upper class	2 (6.67%)	9 (6%)	-
Middle class	10 (33.33%)	54 (36%)	-
Lower class	18 (60%)	87 (58%)	-

Residence			
Urban	10 (33.33%)	44 (29.33%)	-
Rural	20 (66.67%)	106 (70.67%)	-
Histological findings of the renal biopsy			
Mesangioproliferative glomerulonephritis	13 (43.33%)	-	-
Membranoproliferative glomerulonephritis	6 (20%)	4 (2.67%)	-
Focal segmental glomerulosclerosis	4 (13.33%)	-	-
Minimal change nephrotic syndrome	5 (16.67%)	6 (4%)	-
Immunoglobulin A nephropathy	1 (3.33%)	-	-
Inadequate tissue	1 (3.33%)	-	-
Not done	-	140 (93.33%)	-
Biochemical findings			
Serum albumin (gm/l)	10.23 ± 0.65	9.79 ± 0.79	**0.003**
Serum cholesterol (mg/dl)	435.32 ± 66.15	404.65 ± 88.21	**0.037**
Urine protein/creatinine ratio	3.14 ± 0.75	3.47 ± 0.80	**0.011**
Others parameters			
Edema at the presentation moment	30 (100%)	146 (97.33%)	-
Massive proteinuria	30 (100%)	150 (100%)	-
Microhematuria	18 (60%)	30 (20%)	-
Persistent hypertension	11 (36.67%)	13 (8.67%)	-
Urinary tract infection	2 (6.67%)	7 (4.67%)	-
Respiratory tract infection	1 (3.33%)	3 (2%)	-
Cellulitis	3 (10.00%)	12 (8%)	-

Peritonitis	5 (16.67%)	21 (14%)	-
Obesity	0 (0.00%)	4 (2.67%)	-
Malnutrition	2 (6.67%)	6 (4%)	-
Growth and development retardation	2 (6.67%)	0 (0%)	-

Table-3.2: Correlation study among different demographic data and clinicopathological characteristics of prednisolone resistance and prednisolone sensitive nephrotic syndrome patients

Correlation parameters	**r-value**	**p-value**
BMI vs. Age	0.985	**<0.005**
BMI vs. Albumin	0.315	**0.045**
BMI vs. Cholesterol	0.361	**0.025**
BMI vs. Urine protein/creatinine ratio	-0.175	0.178
Albumin vs. Cholesterol	-0.337	**0.034**
Albumin vs. Urine protein/creatinine ratio	-0.309	**0.048**
Cholesterol vs. Urine protein/creatinine ratio	-0.609	0.358
Age vs. Albumin	0.284	0.064
Age vs. Cholesterol	0.361	**0.025**
Age vs. Urine protein/creatinine ratio	-0.170	0.185

r = Correlation co-efficient; p = Significance; Negative values specify opposite correlation

3.2 Genomic DNA Extraction

For the extraction of genomic DNA from whole blood, there are many different types of protocols and a large number of commercially available kits used. In this study, the genomic DNA of 30 prednisolone resistance nephrotic syndrome patients and 150 prednisolone sensitive nephrotic syndrome patients were successfully isolated by Daly's method (Daly *et al.,* 1998). The purity of the DNA and their concentrations were measured by SHIMADZU UV-Spectrophotometer at 260 nm. The purity (OD 260/OD 280) of all the genomic DNA samples was found to be in the range of 1.7 to 1.9 and the average concentration was found to be 50 to 70 µg/ml of whole blood. Agarose gel electrophoresis was done for all the DNA samples of the 30 prednisolone resistance and 150 prednisolone sensitive nephrotic syndrome patients.

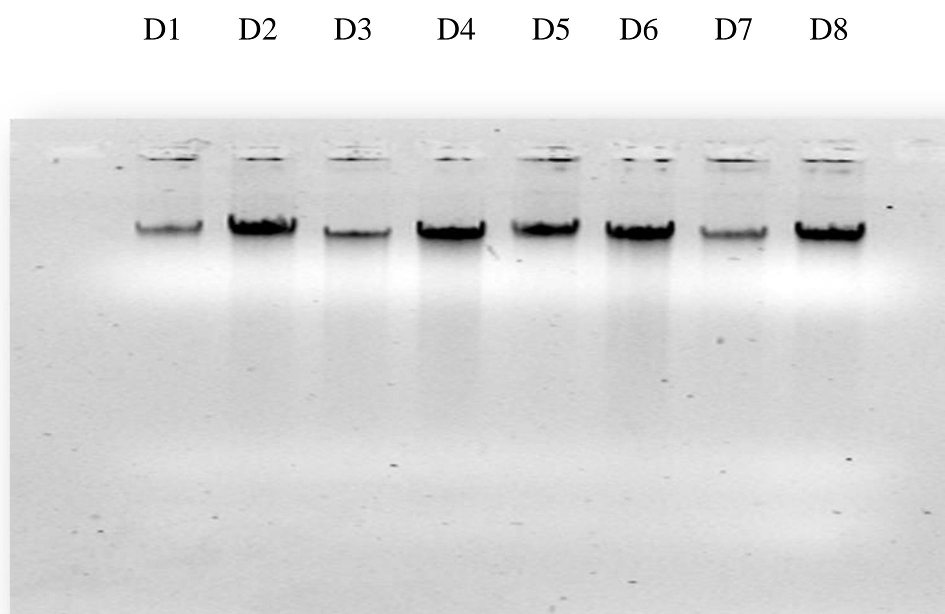


Figure 3.1: Agarose gel electrophoresis (2% w/v agarose) of genomic DNA (DNA no. 1 to 8)

3.3 Genotyping of Genes

The genotyping and allelic distribution of selected genes in the Bangladeshi population was done by using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The genomic target regions, which contain the SNPs of interest, were amplified using primer-directed PCR using DNA polymerase originally described by Saiki *et al.* (Saiki *et al.*, 1988). This primer-directed PCR method facilitates the *in vitro* amplification of single-copy genomic DNA sequences by a factor of more than ten million with extremely high sequence specificity.

3.4 PCR-RFLP of ABCB1 1236T>C (rs1128503) SNP

The size of PCR product is 234 bp. The PCR product was visualized in 2% (w/v) agarose gel. Later on, after digestion of the PCR product, two sequences will be obtained containing two fragments of 207 and 27 bp.

TTACCCATCTCGAAAAGAAGTTAAGGTACAGTGATAAATGATTAATCAACA
ATTAATCTATTGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTATATTTAAACCTAGTGAACAGTCAGTTCCTATATCCTGTGTCT
GTGAATTGCCTTGAAGTTTTTTTCTCACTCGTCCTGGTAGATCTTGAAGGGYC
TGAACCTGAAGGTGCAGAGTGGGCA (PCR Product = 234 bp)

YELLOW → Forward primer sequence

BLUE → Antisense of reverse primer sequence

PINK → Restriction enzyme recognition site

GREEN → SNP of interest

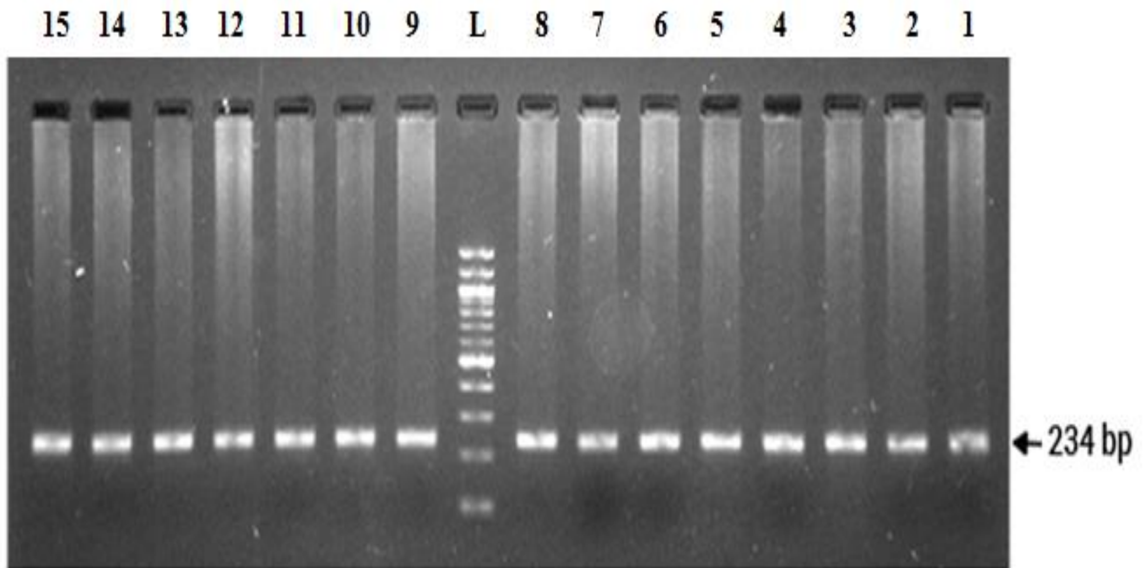


Figure 3.2: PCR product of ABCB1 1236T>C polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-15 show the PCR products (234 bp)

3.4.1 Fragmentation Pattern

The PCR product was digested with *HaeIII*. The fragments were visualized in agarose gel (2%). After digestion of the PCR product of 234 bp, two sequences were obtained containing two fragments of 207 and 27 bp.

Table 3.3: Name of the restriction enzyme with its sites of digestion in case of ABCB1 1236T>C polymorphism

Restriction enzyme	Sites of digestion
HaeIII	**5′ …..GG│CC…..3′** **3′ …..CC│GG…..5′**

→ **Cutting site**

TTACCCATCTCGAAAAGAAGTTAAGGTACWGTGATAAATGATTARTCAACA
AYWAAYCTATYGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTRTATTTAAACCTAGTGAACAGWCAGTTCCTATAYCCTGWGT
CTRTGAATYGCCTTGAAGTTTTTTYCTCACTYRTCCTGRTAGATCTTGAARGG
(Fragment = 207 bp)

and

YCTGAACATGAAGGTGCAGAGTGGGCA (Fragment = 27 bp)

Table 3.4: Type of nucleotide changes, cutting sites and fragments of the allele in case of ABCB1 1236T>C polymorphism

Changes	Fragments	Types	Reference
When Y = T in both chromosome (T/T)	234	Glycine/Glycine	
When Y = T in one chromosome and Y = C in another chromosome (T/C)	234, 207, 27	Glycine/Glycine (nonsense)	Cizmarikova *et al.,* 2015
When Y = C in both chromosome (C/C)	207, 27	Glycine/Glycine (nonsense)	

<u>When Y = T in both of the sister chromosomes: (Normal Homozygote) (T/T)</u>

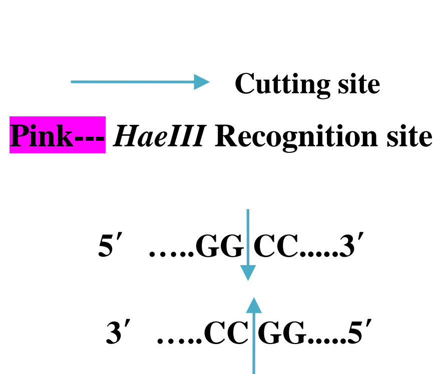
There will be no cutting site for both the chromosomes. So there will be one fragment for each chromosome.

TTACCCATCTCGAAAAGAAGTTAAGGTACWGTGATAAATGATTARTCAACA
AYWAAYCTATYGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTRTATTTAAACCTAGTGAACAGWCAGTTCCTATAYCCTGWGT
CTRTGAATYGCCTTGAAGTTTTTTYCTCACTYRTCCTGRTAGATCTTGAARGG
TCTGAACATGAAGGTGCAGAGTGGGCA (one uncut Fragment = 234 bp)

When Y = T in one and Y = C in another sister chromosomes: (Heterozygote) (T/C)

There is no cutting site for the polymorphic chromosome (Y = T), but for the other chromosome (Y = C) there will be one cutting site (207). So there will be three fragments for the two sister chromosomes.

TTACCCATCTCGAAAAGAAGTTAAGGTACWGTGATAAATGATTARTCAACA
AYWAAYCTATYGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTRTATTTAAACCTAGTGAACAGWCAGTTCCTATAYCCTGWGT
CTRTGAATYGCCTTGAAGTTTTTTYCTCACTYRTCCTGRTAGATCTTGAARGG
YCTGAACATGAAGGTGCAGAGTGGGCA (Fragment = 234 bp)



Fragment: 1= 234 bp (For uncut sister chromosome),

TTACCCATCTCGAAAAGAAGTTAAGGTACWGTGATAAATGATTARTCAACA
AYWAAYCTATYGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTRTATTTAAACCTAGTGAACAGWCAGTTCCTATAYCCTGWGT
CTRTGAATYGCCTTGAAGTTTTTTYCTCACTYRTCCTGRTAGATCTTGAARGG
(Fragment: 2 = 207 bp)

and

CCTGAACATGAAGGTGCAGAGTGGGCA (Fragment: 3 = 27 bp)

When Y = C in both of the sister chromosomes: (Mutant Homozygote) (C/C)

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

TTACCCATCTCGAAAAGAAGTTAAGGTACWGTGATAAATGATTARTCAACA
AYWAAYCTATYGAATGAAGAGTTTCTGATGTTTTCTTGTAGAGATTATAAAA
AAGTGCATGTRTATTTAAACCTAGTGAACAGWCAGTTCCTATAYCCTGWGT
CTRTGAATYGCCTTGAAGTTTTTTYCTCACTYRTCCTGRTAGATCTTGAARGG
(Fragment: 1 = 207 bp)

and

CCTGAACATGAAGGTGCAGAGTGGGCA (Fragment: 2 = 27 bp)

3.4.2 Observed Results of ABCB1 1236T>C Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.5: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of ABCB1 1236T>C polymorphism

Allele	PCR product size (bp)	Restriction enzyme	Digestion condition	Expected fragments (bp)	Observed fragments (bp)
ABCB1 1236T>C	234	*HaeIII*	Incubation at 37°C overnight	**TT:** 234	**234**
				TC: 234, 207, 27	**234, 207, 27**
				CC: 207, 27	**207, 27**

TT: Normal Homozygote; TC: Heterozygote; CC: Mutant Homozygote

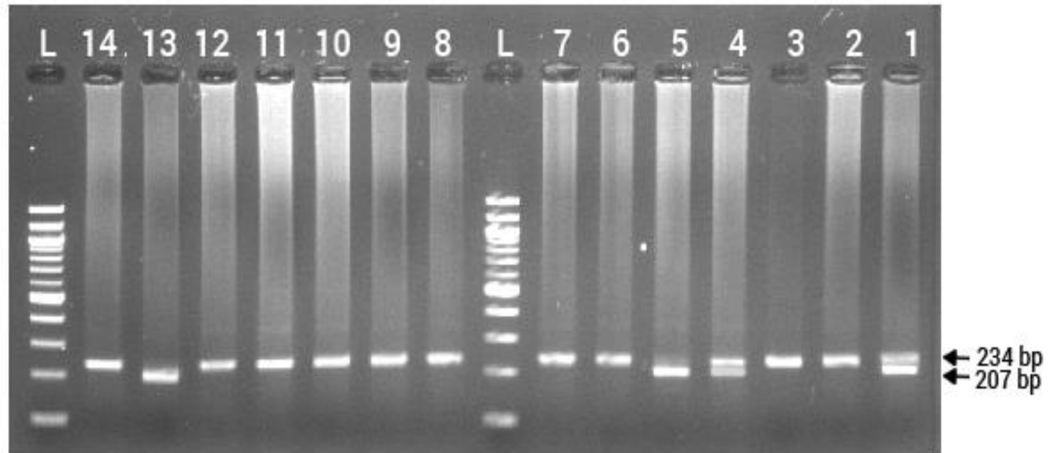


Figure 3.3: Restriction endonuclease *(HaeIII)* digestion fragment of ABCB1 1236T>C polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14: Restriction digestion products; Lanes 2, 3, 6, 7, 8, 9, 10, 11, 12 and 14 show the wild (TT) form (234 bp); Lanes 1 and 4 show the heterozygous (TC) form (234, 207 and 27 bp); and Lanes 5 and 13 show the mutant (CC) form (207 and 27 bp)

3.5 PCR-RFLP of ABCB1 2677G>T (rs2032582) SNP

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA
TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA
GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG
AAAGAACTAGAAGGTDCTGAGAAGGTGAGTCAAACTAAATATG

(PCR Product = 198 bp)

YELLOW ⟶ Forward primer sequence

BLUE ⟶ Antisense of reverse primer sequence

PINK ⟶ Restriction enzyme recognition site

GREEN ⟶ SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of ABCB1 2677G>T polymorphism was obtained. The PCR product size was 198 bp and this was visualized in 2% (w/v) agarose gel.

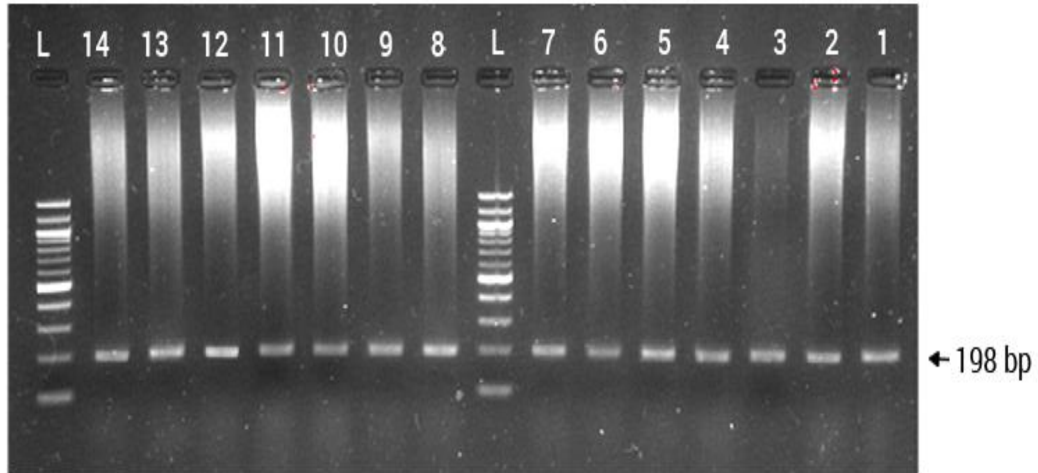


Figure 3.4: PCR product of ABCB1 2677G>T polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14 show the PCR products (198 bp)

3.5.1 Fragmentation Pattern

The fragments were visualized in agarose gel (2%) after digestion of the PCR product with the restriction enzyme *Hpy1881*. After digestion of the PCR product of 198 bp, two sequences were obtained containing two fragments of 173 and 25 bp.

Table 3.6: Name of the restriction enzyme with its sites of digestion in case of ABCB1 2677G>T polymorphism

Restriction enzyme	Sites of digestion
Hpy1881	**5′ .....TCN|GA.....3′** **3′ .....AG|NCT.....5′**

→ **Cutting site**

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA
TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA
GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG
AAAGAACTAGAAGGTDCT (Fragment = 173 bp)

and

GAGAAGGTGAGTCAAACTAAATATG (Fragment = 25 bp)

Table 3.7: Type of nucleotide changes, cutting sites and fragments of the allele in case of ABCB1 2677G>T polymorphism

Changes	Fragments	Types	Reference
When D = G in both chromosome (G/G)	198	Alanine/Alanine	
When D = G in one chromosome and D = T in another chromosome (G/T)	198, 173, 25	Alanine/Serine	Cizmarikova *et al.*, 2015
When D = T in both chromosome (T/T)	173, 25	Serine/Serine	

<u>When D = G in both of the sister chromosomes: (Normal Homozygote) (G/G)</u>

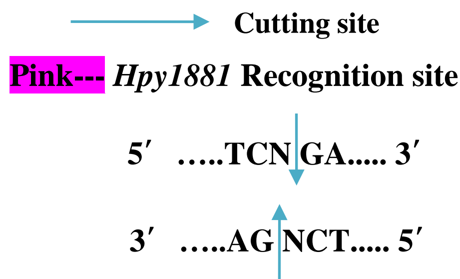
There will be no cutting site for both the chromosomes. So there will be one fragment for each chromosome.

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG AAAGAACTAGAAGGT**GCTGA**GAAGGTGAGTCAAACTAAATATG (one uncut Fragment = 198 bp)

When D = G in one and D = T in another sister chromosomes: (Heterozygote) (G/T)

There is no cutting site for the polymorphic chromosome (D = G), but for the other chromosome (D = T) there will be one cutting site (173). So there will be three fragments for the two sister chromosomes.

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG AAAGAACTAGAAGGT**DCTGA**GAAGGTGAGTCAAACTAAATATG (Fragment = 198 bp)



Fragment: 1 = 198 bp (For uncut sister chromosome),

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG AAAGAACTAGAAGGT**TCT** (Fragment: 2 = 173 bp)

and

GAGAAGGTGAGTCAAACTAAATATG (Fragment: 3 = 25 bp)

When D = T in both of the sister chromosomes: (Mutant Homozygote) (T/T)

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

TTACCCAGAATATAGCAAATCTTGGGACAGGAATAATTATATCCTTCATCTA
TGGTTGGCAACTAACACTGTTACTCTTAGCAATTGTACCCATCATTGCAATA
GCAGGAGTTGTTGAAATGAAAATGTTGTCTGGACAAGCACTGAAAGATAAG
AAAGAACTAGAAGGTTCT (Fragment: 1 = 173 bp)

and

GAGAAGGTGAGTCAAACTAAATATG (Fragment: 2 = 25 bp)

3.5.2 Observed Results of ABCB1 2677G>T Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.8: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of ABCB1 2677G>T polymorphism

Allele	**PCR product size (bp)**	**Restriction enzyme**	**Digestion condition**	**Expected fragments (bp)**	**Observed fragments (bp)**
ABCB1 2677G>T	198	*Hpy1881*	Incubation at 37°C overnight	**GG:** 198	**198**
				GT: 198, 173, 25	**198, 173, 25**
				TT: 173, 25	**173, 25**

GG: Normal Homozygote; GT: Heterozygote; TT: Mutant Homozygote

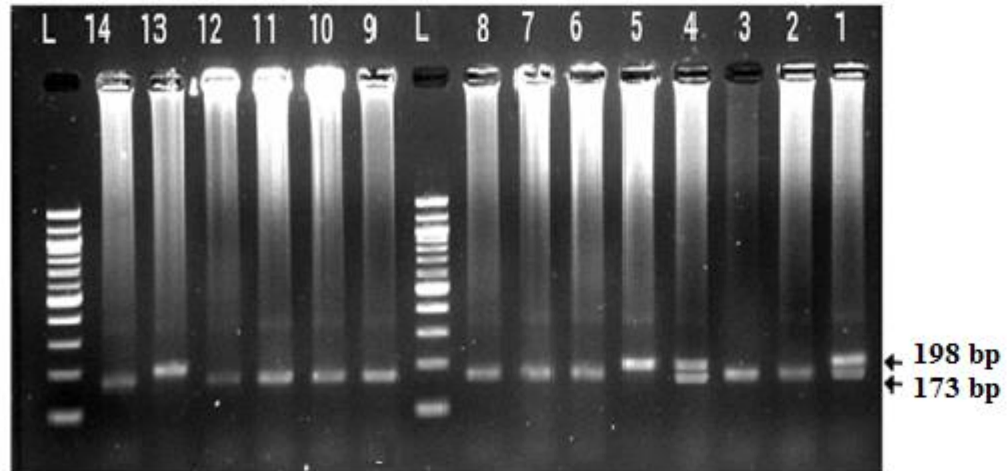


Figure 3.5: Restriction endonuclease *(Hpy1881)* digestion fragment of ABCB1 2677G>T polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14: Restriction digestion products; Lanes 5 and 13 show the wild (GG) form (198 bp); Lanes 1 and 4 show the heterozygous (GT) form (198, 173 and 25 bp); and Lanes 2, 3, 6, 7, 8, 9, 10, 11, 12 and 14 show the mutant (TT) form (173 and 25 bp)

3.6 PCR-RFLP of ABCB1 3435T>C (rs1045642) SNP

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATHGTGAGGGCAGCAAA GGAGGCCAACATACATGCCTTCATCGAGTCACTGCCTAATGT (PCR Product = 248 bp)

YELLOW ⟶ Forward primer sequence

BLUE ⟶ Antisense of reverse primer sequence

PINK ⟶ Restriction enzyme recognition site

GREEN ⟶ SNP of interest

After completing PCR amplification with appropriate reagents, a PCR product of ABCB1 3435T>C polymorphism was obtained. The PCR product size was 248 bp and this was visualized in 2% (w/v) agarose gel.

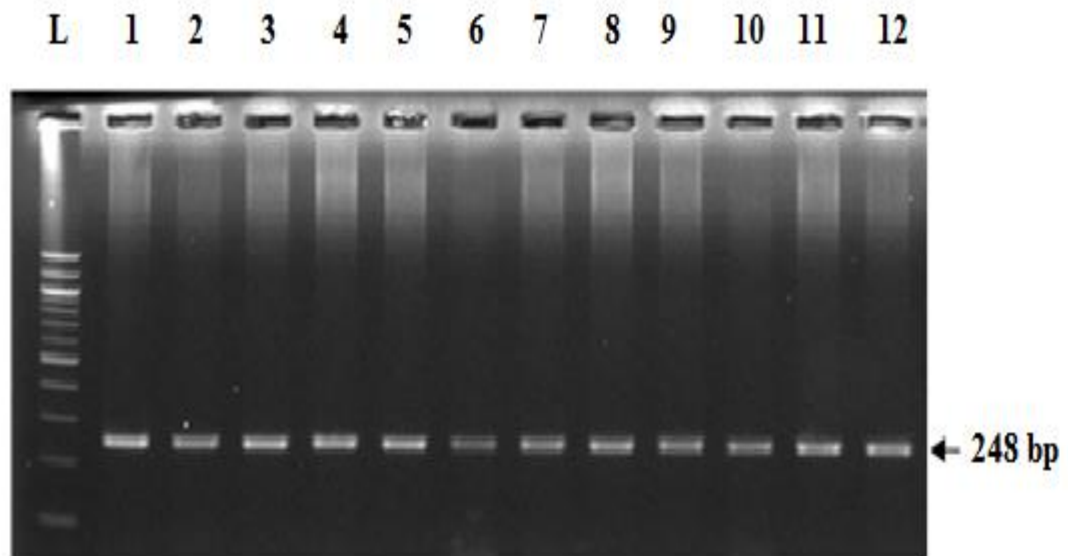


Figure 3.6: PCR product of ABCB1 3435T>C polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-12 show the PCR products (248 bp)

3.6.1 Fragmentation Pattern

The PCR product was digested with *MboI*. The fragments were visualized in agarose gel (2%). After digestion of the PCR product of 248 bp, two sequences will be obtained containing two fragments of 192 and 56 bp.

Table 3.9: Name of the restriction enzyme with its sites of digestion in case of ABCB1 3435T>C polymorphism

Restriction enzyme	Sites of digestion
MboI	**5′ ….. ↓GATC…..3′** **3′ …..CTAG↑…..5′**

⟶ Cutting site

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA
AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT
GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT
GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATH (Fragment = 192 bp)

and

GTGAGGGCAGCAAAGGAGGCCAACATACATGCCTTCATCGAGTCACTGCCT
AATGT (Fragment = 56 bp)

Table 3.10: Type of nucleotide changes, cutting sites and fragments of the allele in case of ABCB1 3435T>C polymorphism

Changes	Fragments	Types	Reference
When H = T in both chromosome (T/T)	248	Isoleucine/Isoleucine	Enabah *et al.,* 2014
When H = T in one chromosome and H = C in another chromosome (T/C)	248, 192, 56	Isoleucine/Isoleucine (nonsense)	
When H = C in both chromosome (C/C)	192, 56	Isoleucine/Isoleucine (nonsense)	

When H = T in both of the sister chromosomes: (Normal Homozygote) (T/T)

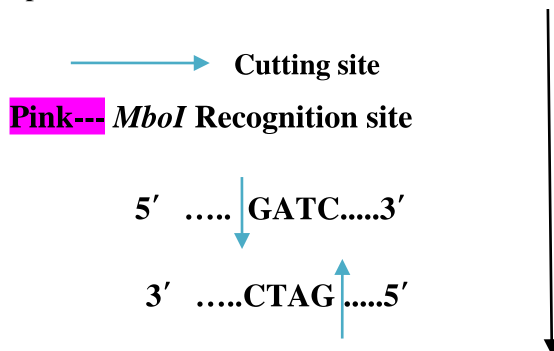
There will be no cutting site for both the chromosomes. So there will be one fragment for each chromosome.

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA
AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT
GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT
GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATTGTGAGGGCAGCAAA
GGAGGCCAACATACATGCCTTCATCGAGTCACTGCCTAATGT (one uncut Fragment = 248 bp)

When H = T in one and H = C in another sister chromosomes: (Heterozygote) (T/C)

There is no cutting site for the polymorphic chromosome (H = T), but for the other chromosome (H = C) there will be one cutting site (192). So there will be three fragments for the two sister chromosomes.

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA
AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT
GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT
GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATHGTGAGGGCAGCAAA
GGAGGCCAACATACATGCCTTCATCGAGTCACTGCCTAATGT (Fragment = 248 bp)



Fragment: 1 = 248 bp (For uncut sister chromosome),

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA
AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT
GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT
GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATC (Fragment: 2 =192 bp)

and

GTGAGGGCAGCAAAGGAGGCCAACATACATGCCTTCATCGAGTCACTGCCT
AATGT (Fragment: 3 = 56 bp)

When H = C in both of the sister chromosomes: (Mutant Homozygote) (C/C)

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

TGCTGGTCCTGAAGTTGATCTGTGAACTCTTGTTTTCAGCTGCTTGATGGCAA
AGAAATAAAGCGACTGAATGTTCAGTGGCTCCGAGCACACCTGGGCATCGT
GTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAACATTGCCTAT
GGAGACAACAGCCGGGTGGTGTCACAGGAAGAGAT (Fragment: 1 = 192 bp)

and

GTGAGGGCAGCAAAGGAGGCCAACATACATGCCTTCATCGAGTCACTGCCT
AATGT (Fragment: 2 = 56 bp)

3.6.2 Observed Results of ABCB1 3435T>C Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.11: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of ABCB1 3435T>C polymorphism

Allele	PCR product size (bp)	Restriction enzyme	Digestion condition	Expected fragments (bp)	Observed fragments (bp)
ABCB1 3435T>C	248	*MboI*	Incubation at 37°C overnight	**TT:** 248	**248**
				TC: 248, 192, 56	**248, 192, 56**
				CC: 192, 56	**192, 56**

TT: Normal Homozygote; TC: Heterozygote; CC: Mutant Homozygote

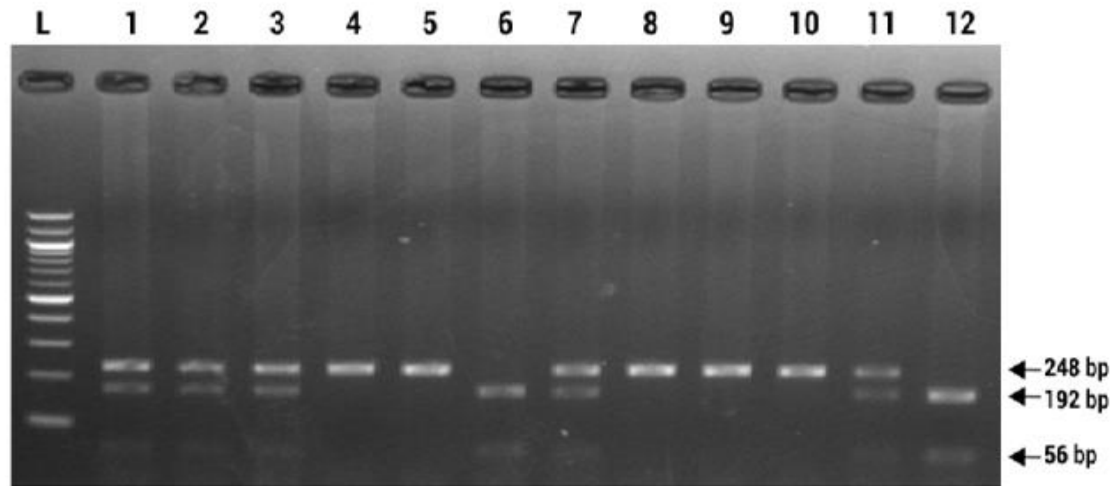


Figure 3.7: Restriction endonuclease *(MboI)* digestion fragment of ABCB1 3435T>C polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-12: Restriction digestion products; Lanes 4, 5, 8, 9 and 10 show the wild (TT) form (248 bp); Lanes 1, 2, 3, 7 and 11 show the heterozygous (TC) form (248, 192 and 56 bp); and Lanes 6 and 12 show the mutant (CC) form (192 and 56 bp)

3.7 PCR-RFLP of NR3C1 (rs10482634) SNP

After completing PCR amplification with appropriate reagents a PCR product of NR3C1 (rs10482634) polymorphism was obtained. The PCR product size was 300 bp and this was visualized in 2% (w/v) agarose gel.

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGAYGCGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGC
TTTTATACAGACCTAATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTAT
TTGATTGAAATATTGAGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTC
TTCATCTATCATGAAGCTGCTCAAATAAGTAGGTGTT (PCR Product = 300 bp)

YELLOW ⟶ Forward primer sequence

BLUE ⟶ Antisense of reverse primer sequence

PINK ⟶ Restriction enzyme recognition site

GREEN ⟶ SNP of interest

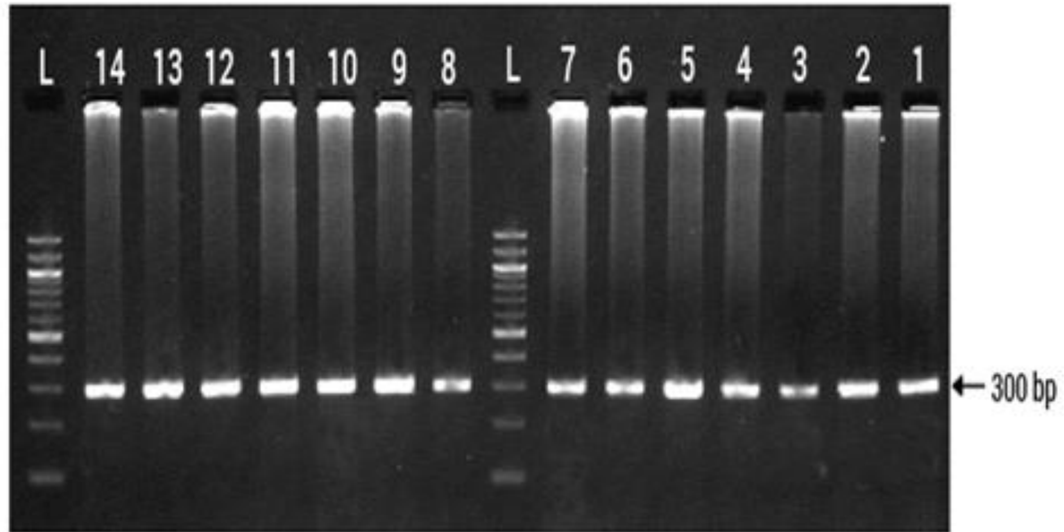


Figure 3.8: PCR product of NR3C1 (rs10482634) polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14 show the PCR products (300 bp)

3.7.1 Fragmentation Pattern

The PCR product was digested with *BstUI*. The fragments were visualized in agarose gel (2%). After digestion of the PCR product of 300 bp, two sequences will be obtained containing two fragments of 118 and 182 bp.

Table 3.12: Name of the restriction enzyme with its sites of digestion in case of NR3C1 (rs10482634) polymorphism

Restriction enzyme	Sites of digestion
BstUI	**5′ ….. CG↓CG…..3′** **3′ ….. GC↑GC…..5′**

→ **Cutting site**

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGAYG (Fragment = 118 bp)

and

CGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGCTTTTATACAGACCTA
ATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTATTTGATTGAAATATTG
AGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTCTTCATCTATCATGAA
GCTGCTCAAATAAGTAGGTGTT (Fragment = 182 bp)

Table 3.13: Type of nucleotide changes, cutting sites and fragments of the allele in case of NR3C1 (rs10482634) polymorphism

Changes	Fragments	Type	Reference
When Y = T in both chromosome (T/T)	300	Intronic	Designed with primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)
When Y = T in one chromosome and Y = C in another chromosome (T/C)	300, 118, 182		
When Y = C in both chromosome (C/C)	118, 182		

<u>When Y = T in both of the sister chromosomes: (Normal Homozygote) (T/T)</u>

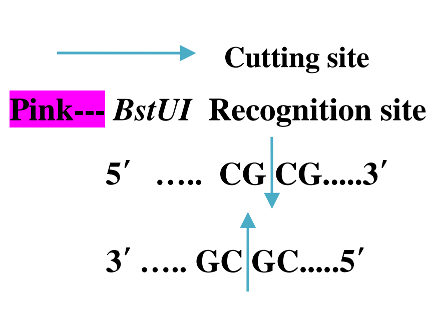
There will be no cutting site for both the chromosomes. So there will be one fragment for each chromosome.

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGATGCGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGCT
TTTATACAGACCTAATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTATT
TGATTGAAATATTGAGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTCT
TCATCTATCATGAAGCTGCTCAAATAAGTAGGTGTT (one uncut Fragment = 300 bp)

When Y = T in one and Y = C in another sister chromosomes: (Heterozygote) (T/C)

There is no cutting site for the polymorphic chromosome (Y = T), but for the other chromosome (Y = C) there will be one cutting site (118). So there will be three fragments for the two sister chromosomes.

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGAYGCGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGC
TTTTATACAGACCTAATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTAT
TTGATTGAAATATTGAGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTC
TTCATCTATCATGAAGCTGCTCAAATAAGTAGGTGTT (Fragment = 300 bp)



Fragment: 1 = 300 bp (For uncut sister chromosome),

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGACG (Fragment: 2 = 118 bp) and

CGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGCTTTTATACAGACCTA
ATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTATTTGATTGAAATATTG
AGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTCTTCATCTATCATGAA
GCTGCTCAAATAAGTAGGTGTT (Fragment: 3 = 182 bp)

When Y = C in both of the sister chromosomes: (Mutant Homozygote) (C/C))

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

CACAGATACTTGACTTGGCTATGGTCTAGATAATCCATGAAAATTTAAAGGA
CAGATTTTAACAACTTTATGCTAAATTGATAGATCTCTAGGATCAGATTGCC
ATCACTCTCAGACG (Fragment: 1 = 118 bp)

and

CGAAGCTTCCAACCACTTATAGGTTCCTGATATCTTGCTTTTATACAGACCTA
ATTTCTCTTCCTTTAAACTTTCTTTTCCTCAGTTGCTATTTGATTGAAATATTG
AGTCATTAAAAATTTCCAAGTGGGAATTTTTGTGTTTCTTCATCTATCATGAA
GCTGCTCAAATAAGTAGGTGTT (Fragment: 2 = 182 bp)

3.7.2 Observed Results of NR3C1 (rs10482634) Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.14: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of NR3C1 (rs10482634) polymorphism

Allele	PCR product size (bp)	Restriction enzyme	Digestion condition	Expected fragments (bp)	Observed fragments (bp)
NR3C1 rs10482634	300	*BstUI*	Incubation at 60°C overnight	**TT:** 300	**300**
				TC: 300, 118, 182	**300, 118, 182**
				CC: 118, 182	**118, 182**

TT: Normal Homozygote; TC: Heterozygote; CC: Mutant Homozygote

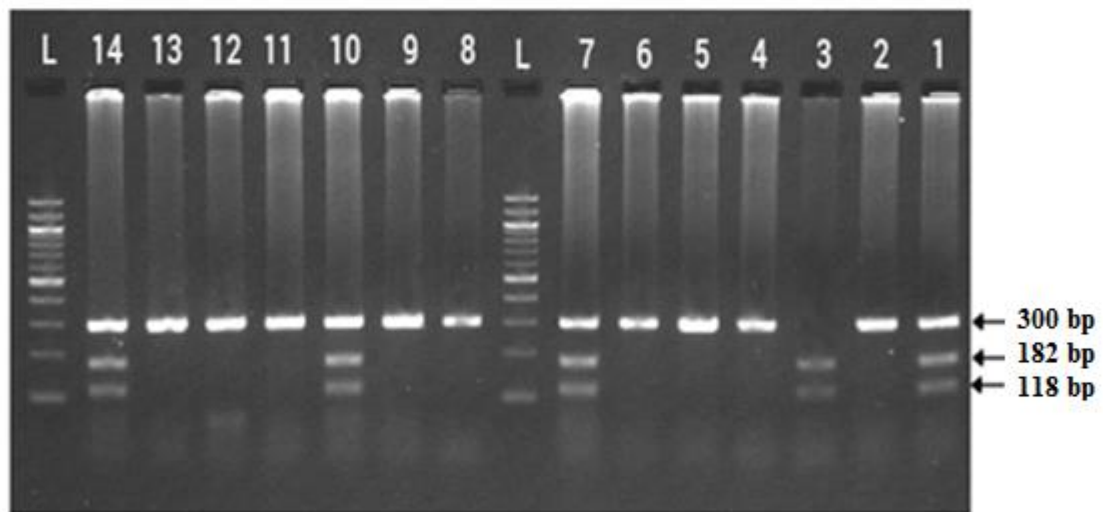


Figure 3.9: Restriction endonuclease *(BstUI)* digestion fragment of NR3C1 (rs10482634) polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14: Restriction digestion products; Lanes 2, 4, 5, 6, 8, 9, 11, 12 and 13 show the wild (TT) form (300 bp); Lanes 1, 7, 10 and 14 show the heterozygous (TC) form (300, 118 and 182 bp); and Lane 3 shows the mutant (CC) form (118 and 182 bp)

3.8 PCR-RFLP of NR3C1 (rs6877893) SNP

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTTGCTACACAAG
AATTTCRGCCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCAC
ATTCTCATTGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTG
TGAAATGACATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGC
TGCTAAAATGCAGCTTT (PCR Product = 225 bp)

YELLOW ⟶ Forward primer sequence

BLUE ⟶ Antisense of reverse primer sequence

PINK ⟶ Restriction enzyme recognition site

GREEN ⟶ SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of NR3C1 (rs6877893) polymorphism was obtained. The PCR product size was 225 bp and this was visualized in 2% (w/v) agarose gel.

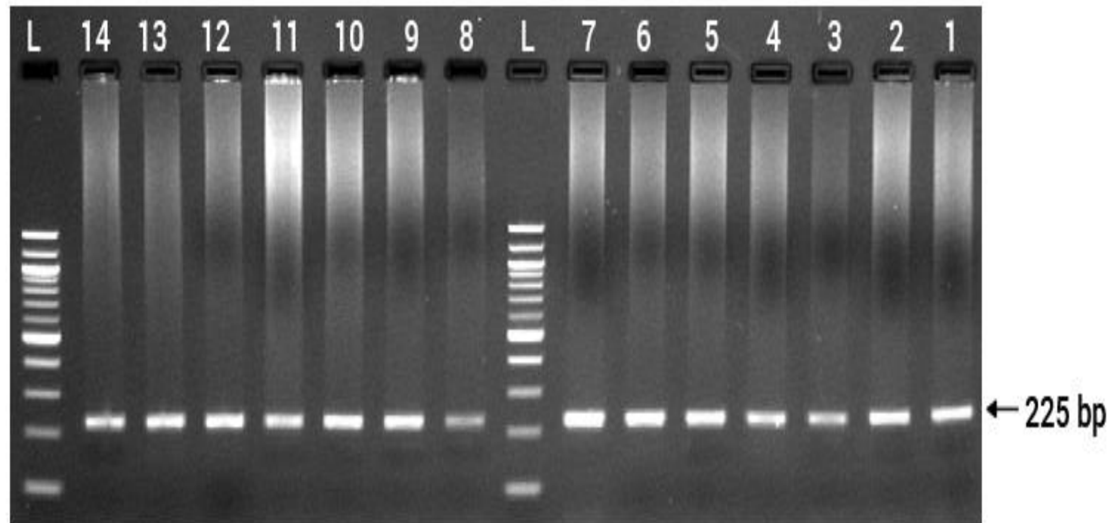


Figure 3.10: PCR product of NR3C1 (rs6877893) (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-14 show the PCR products (225 bp)

3.8.1 Fragmentation Pattern

The PCR product was digested with *HaeIII*. The fragments were visualized in agarose gel (2%). After digestion of the PCR product of 225 bp, two sequences will be obtained containing two fragments of 60 and 165 bp.

Table 3.15: Name of the restriction enzyme with its sites of digestion in case of NR3C1 (rs6877893) polymorphism

Restriction enzyme	Sites of digestion
HaeIII	5′ …..GG↓CC…..3′ 3′ …..CC↑GG…..5′

→ **Cutting site**

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTTGCTACACAAG AATTTCRG (Fragment = 60 bp)

and

CCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCACATTCTCAT TGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTGTGAAATGA CATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGCTGCTAAAA TGCAGCTTT (Fragment = 165 bp)

Table 3.16: Type of nucleotide changes, cutting sites and fragments of the allele in case of NR3C1 (rs6877893) polymorphism

Changes	Fragments	Type	Reference
When R = A in both chromosome (A/A)	225	Intronic	Designed with primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)
When R = A in one chromosome and R = G in another chromosome (A/G)	225, 60,165		
When R = G in both chromosome (G/G)	60, 165		

When R = A in both of the sister chromosomes: (Normal Homozygote) (A/A)

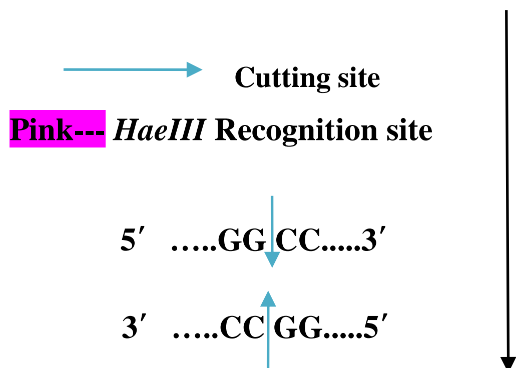
There will be no cutting site for both the chromosomes. So there will be one fragment for each chromosome.

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTTGCTACACAAG
AATTTCAGCCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCAC
ATTCTCATTGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTG
TGAAATGACATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGC
TGCTAAAATGCAGCTTT (One uncut Fragment: = 225 bp)

When R = A in one and R = G in another sister chromosomes: (Heterozygote) (A/G)

There is no cutting site for the polymorphic chromosome (R = A), but for the other chromosome (R = G) there will be one cutting site (60). So there will be three fragments for the two sister chromosomes.

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTTGCTACACAAG
AATTTCRGCCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCAC
ATTCTCATTGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTG
TGAAATGACATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGC
TGCTAAAATGCAGCTTT (Fragment = 225 bp)



Fragment: 1 = 225 bp (For uncut sister chromosome),

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTTGCTACACAAG
AATTTCGG (Fragment: 2 = 60 bp)

and

CCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCACATTCTCAT
TGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTGTGAAATGA
CATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGCTGCTAAAA
TGCAGCTTT (Fragment: 3 = 165 bp)

When R = G in both of the sister chromosomes: (Mutant Homozygote) (G/G)

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

AGAACTGGAGATTGCCAAGGGCTAACTTAAGTGTTAATTTTGCTACACAAG AATTTCGG (Fragment: 1 = 60 bp)

and

CCTGAAAAACCTACTTCCTTCCCCAGTAAAGACTATAAAATCACATTCTCAT TGTAGAAATTTAGATCAAACACCAAGGGATAAGTATCATTTGTGTGAAATGA CATTGATGTTTTCAGGCAGGTAACAGCTCTACCAAAATATATGCTGCTAAAA TGCAGCTTT (Fragment: 2 = 165 bp)

3.8.2 Observed Results of NR3C1 (rs6877893) Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.17: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of NR3C1 (rs6877893) polymorphism

Allele	PCR product size (bp)	Restriction enzyme	Digestion condition	Expected fragments (bp)	Observed fragments (bp)
NR3C1 rs6877893	225	*HaeIII*	Incubation at 37°C overnight	**AA:** 225	**225**
				AG: 225, 60, 165	**225, 60, 165**
				GG: 60, 165	**60, 165**

AA: Normal Homozygote; AG: Heterozygote; GG: Mutant Homozygote

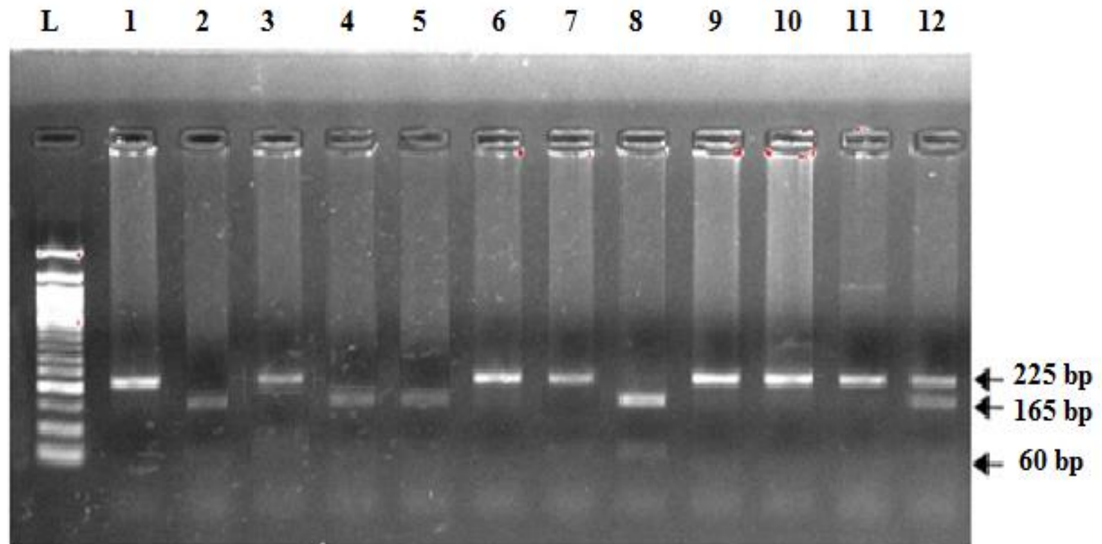


Figure 3.11: Restriction endonuclease *(HaeIII)* digestion fragment of NR3C1 (rs6877893) polymorphism (2% agarose gel). Lane L: 50 bp molecular marker; Lanes 1-12: Restriction digestion products; Lanes 1, 3, 6, 7, 9, 10 and 11 show the wild (AA) form (225 bp); Lane 12 shows the heterozygous (AG) form (225, 60 and 165 bp); and Lanes 2, 4, 5 and 8 show the mutant (GG) form (60 and 165 bp)

3.9 PCR-RFLP of CYP3A5 [CYP3A5*3 (rs776746)] SNP

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTT
AAACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGTAC
CACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAG
AGCTCTTTTGTCTTTCARTACCTCTTCCCTGTTTGGACC (PCR Product = 196 bp)

YELLOW → Forward primer sequence

BLUE → Antisense of reverse primer sequence

PINK → Restriction enzyme recognition site

GREEN → SNP of interest

After completing PCR amplification with appropriate reagents a PCR product of CYP3A5*3 polymorphism was obtained. The PCR product size was 196 bp and this was visualized in 2% (w/v) agarose gel.

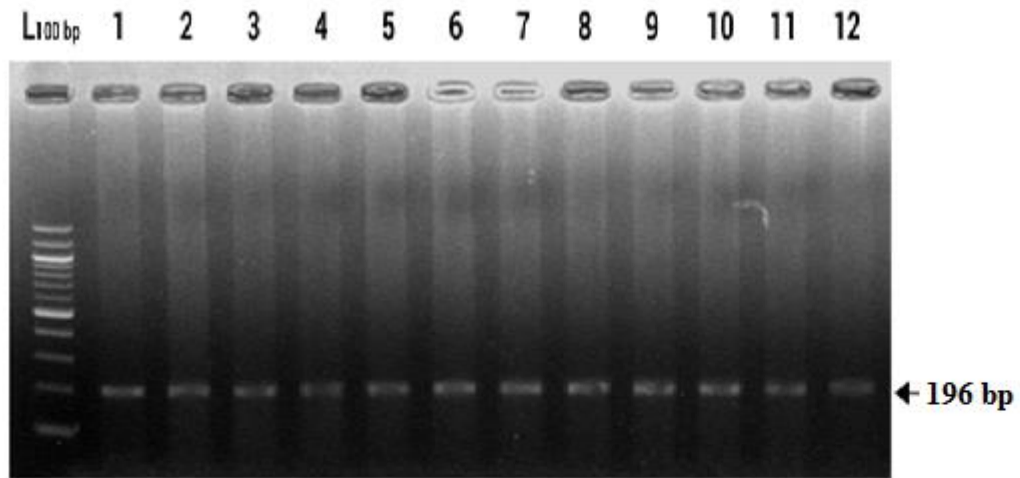


Figure 3.12: PCR product of CYP3A5*3 polymorphism (2% agarose gel). Lane L: 100 bp molecular marker; Lanes 1-12 show the PCR products (196 bp)

3.9.1 Fragmentation Pattern

The PCR product was digested with *RsaI*. The fragments were visualized in agarose gel (2%). After digestion of the PCR product of 196 bp, two sequences will be obtained containing four fragments of 102, 94, 74 and 20 bp.

Table 3.18: Name of the restriction enzyme with its sites of digestion in case of CYP3A5*3 polymorphism

Restriction enzyme	Sites of digestion
RsaI	**5′ ….. GT↓AC…..3′** **3′ ….. CA↑TG…..5′**

→ **Cutting site**

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTA AACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGT

(Fragment = 102 bp),

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA AGAGCTCTTTTGTCTTTCARTACCTCTTCCCTGTTTGGACC (Fragment = 94 bp),

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA AGAGCTCTTTTGTCTTTCART (Fragment = 74 bp)

and

ACCTCTTCCCTGTTTGGACC (Fragment = 20 bp)

Table 3.19: Type of nucleotide changes, cutting sites and fragments of the allele in case of CYP3A5*3 polymorphism

Changes	**Fragments**	**Types**	**Reference**
When R = A in both chromosome (A/A)	102, 94	Proline/Proline	Bellah *et al.*, 2015
When R = A in one chromosome and R = G in another chromosome (A/G)	102, 94, 74, 20	Proline/Threonine	
When R = G in both chromosome (G/G)	102, 74, 20	Threonine/Threonine	

When R = A in both of the sister chromosomes: (Normal Homozygote) (A/A))

There is one cutting site for both the chromosomes. So there will be two fragments for each chromosome.

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTGATAATGAAGTATTTT
AAACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGT

(Fragment: 1 = 102 bp)

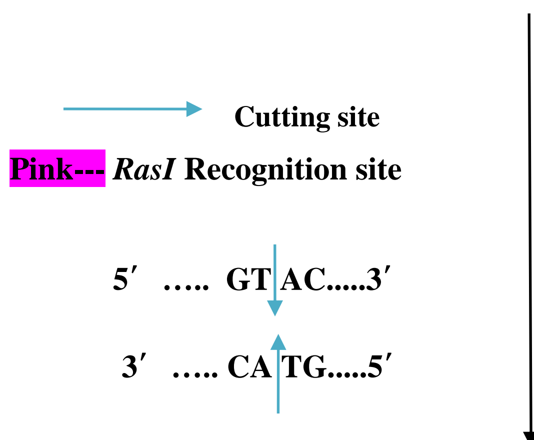
and

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA
AGAGCTCTTTTGTCTTTCAATACCTCTTCCCTGTTTGGACC (Fragment: 2 = 94 bp)

When R = A in one and R = G in another sister chromosome: (Heterozygote) (A/G)

There is one cutting site for one chromosome (R = A). So there will be two fragments for the chromosome. In another chromosome (R = G), there are two cutting sites, so there will be three fragments for another chromosome. So there will be a total of four fragments for the two sister chromosomes.

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTGATAATGAAGTATTTT
AAACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGTAC
CACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAG
AGCTCTTTTGTCTTTCARTACCTCTTCCCTGTTTGGACC (Fragment = 196 bp)



CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTT AAACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGT

(Fragment: 1 = 102 bp),

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA AGAGCTCTTTTGTCTTTCAATACCTCTTCCCTGTTTGGACC (Fragment: 2 = 94 bp),

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA AGAGCTCTTTTGTCTTTCAGT (Fragment: 3 = 74 bp)

and

ACCTCTTCCCTGTTTGGACC (Fragment: 4 = 20 bp)

<u>When R = G in both of the sister chromosomes: (Mutant Homozygote) (G/G))</u>

There are two cutting sites for both the chromosomes. So there will be three fragments for each chromosome.

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTT AAACATATAAAACATTATGGAGAGTGGCATAGGAGATACCCACGTATGT

(Fragment: 1 = 102 bp),

ACCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAA AGAGCTCTTTTGTCTTTCAGT (Fragment: 2 = 74 bp)

and

ACCTCTTCCCTGTTTGGACC (Fragment: 3 = 20 bp)

3.9.2 Observed Results of CYP3A5*3 Polymorphism

Restriction enzyme digestion products were visualized in agarose gel (2%).

Table 3.20: Name of the allele, PCR product size, restriction enzyme, length of the expected and observed fragments of digestion in case of CYP3A5*3 polymorphism

Allele	**PCR product size (bp)**	**Restriction enzyme**	**Digestion condition**	**Expected fragments (bp)**	**Observed fragments (bp)**
CYP3A5 CYP3A5*3	196	*RasI*	Incubation at 37°C overnight	**AA:** 102, 94	**102, 94**
				AG: 102, 94, 74, 20	**102, 94, 74, 20**
				GG: 102, 74, 20	**102, 74, 20**

AA: Normal Homozygote; AG: Heterozygote; GG: Mutant Homozygote

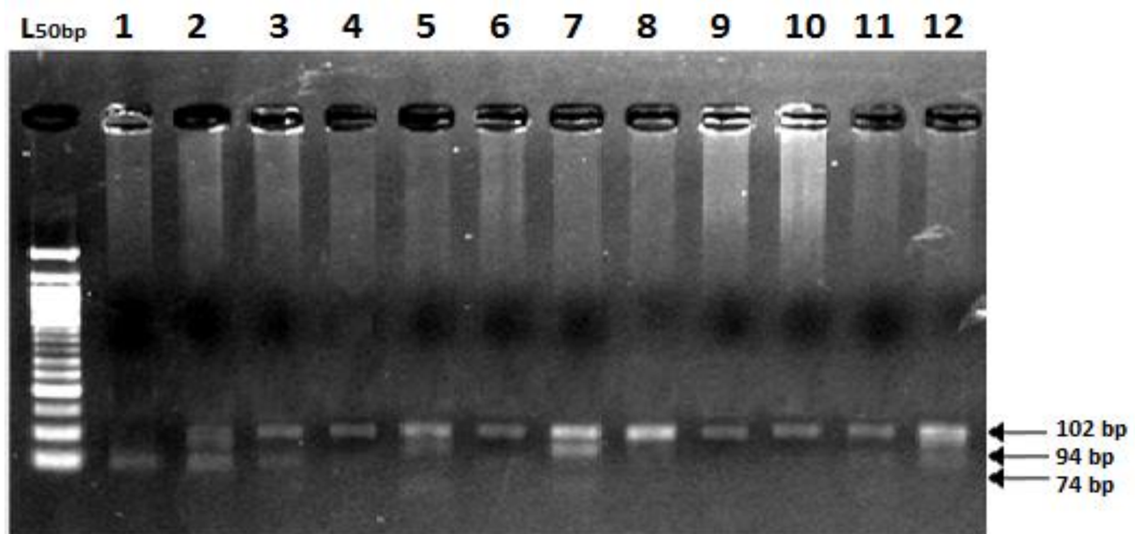


Figure 3.13: Restriction endonuclease *(RsaI)* digestion fragment of CYP3A5*3 polymorphism (2% agarose gel). Lane L: 50 bp molecular marker; Lane 1: blank; Lanes 2-12: Restriction digestion products; Lanes 4, 6, 8, 9, 10 and 11 show the wild (AA) form (102 and 94 bp); Lane 2 and 12 show the heterozygous (AG) form (102, 94, 74 and 20 bp); and Lanes 3, 5 and 7 show the mutant (GG) form (102, 74 and 20 bp)

3.10 Results of Individual SNP

3.10.1 Genotyping Results

This population-based case-control study was conducted to examine the prevalence of three polymorphisms of ABCB1 gene namely 1236T>C (rs1128503), 2677G>T (rs2032582) and 3435T>C (rs1045642), two polymorphisms of NR3C1 gene namely rs10482634 and rs6877893 and one CYP3A5*3 (rs776746) polymorphism of CYP3A5 gene in prednisolone resistance and sensitive childhood nephrotic syndrome patients of Bangladesh. Here, we genotyped 180 children diagnosed with nephrotic syndrome among them 30 cases had prednisolone resistance nephrotic syndrome (PRNS) and 150 controls had prednisolone sensitive nephrotic syndrome (PSNS) disease.

3.11 Observed Genotyping Results of ABCB1 1236T>C Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.21: The frequency distribution of genotype and allelic data of ABCB1 1236T>C polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	Genotype (Total = 180)	Cases (%) (n = 30)	Controls (%) (n = 150)	OR (95% CI)	p value
ABCB1 1236T>C	TT (103)	17 (56.67%)	86 (57.33%)	Reference	Reference
	TC (9)	3 (10%)	6 (4%)	2.53 (0.58-11.12)	0.219
	CC (68)	10 (33.33%)	58 (38.67%)	0.87 (0.37-2.04)	0.753
	TC (9) + CC (68)	13 (43.33%)	64 (42.67%)	1.03 (0.47-2.27)	0.946
	T-allele	37 (61.67%)	178 (59.33%)	-	-
	C-allele	23 (38.33%)	122 (40.67%)	0.91 (0.51-1.60)	0.737

OR = Odds Ratio, Cl = Confidence Interval, $p < 0.05$ considered as statistically significant

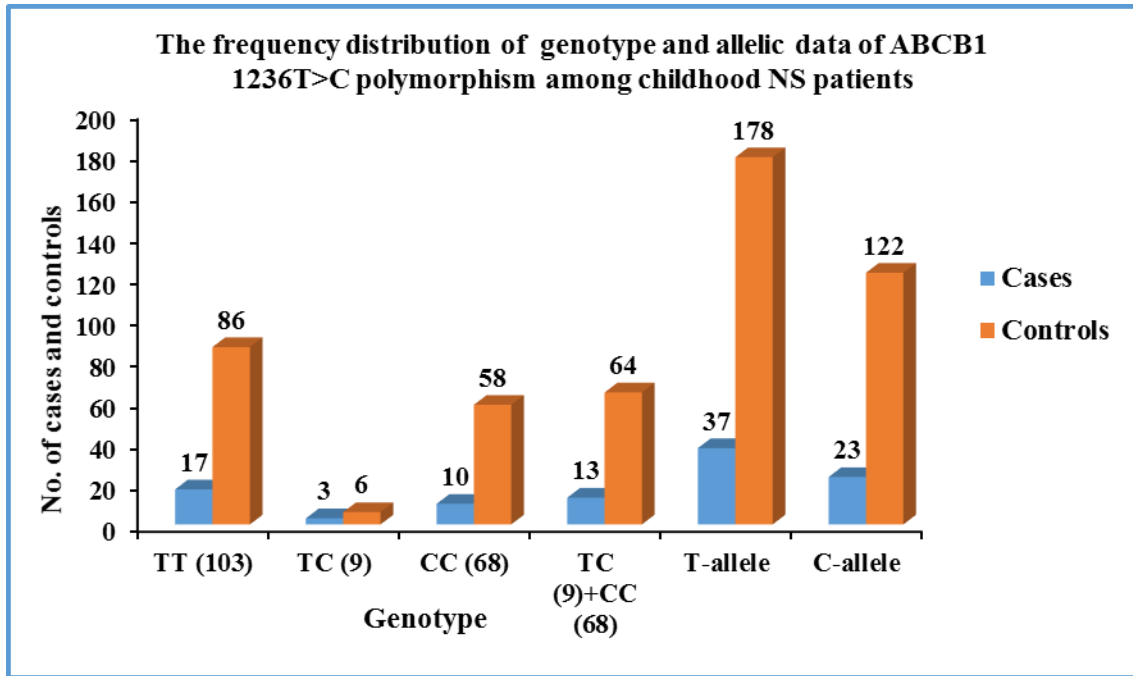


Figure 3.14: The frequency distribution of genotype and allelic data of ABCB1 1236T>C polymorphism among childhood nephrotic syndrome patients

In case of ABCB1 1236T>C polymorphism, our result revealed that the frequency of TT (Gly/Gly) normal homozygous genotype was statistically slightly lower in cases than in controls (56.67% vs. 57.33%). The frequency distribution of TC (Gly/Gly) heterozygous and CC (Gly/Gly) mutant homozygous genotypes were found to have 2.53 times more and 0.87 times lower risk of developing prednisolone resistance in childhood NS (OR = 2.53, 95% Cl = 0.58 to 11.12, p = 0.219; OR = 0.87, 95% Cl = 0.37 to 2.04, p = 0.753) compared with TT (Gly/Gly) normal homozygous genotype and the results were not statistically significant. Whereas, the combined TC (Gly/Gly) heterozygous and CC (Gly/Gly) mutant homozygous genotype TC+CC was not significantly found to be responsible for prednisolone resistance though it possesses 1.03 times higher risk of developing prednisolone resistance in NS (OR = 1.03, 95% Cl = 0.47 to 2.27, p = 0.946) (Table 3.21; Figure 3.14; 3.15; 3.16).

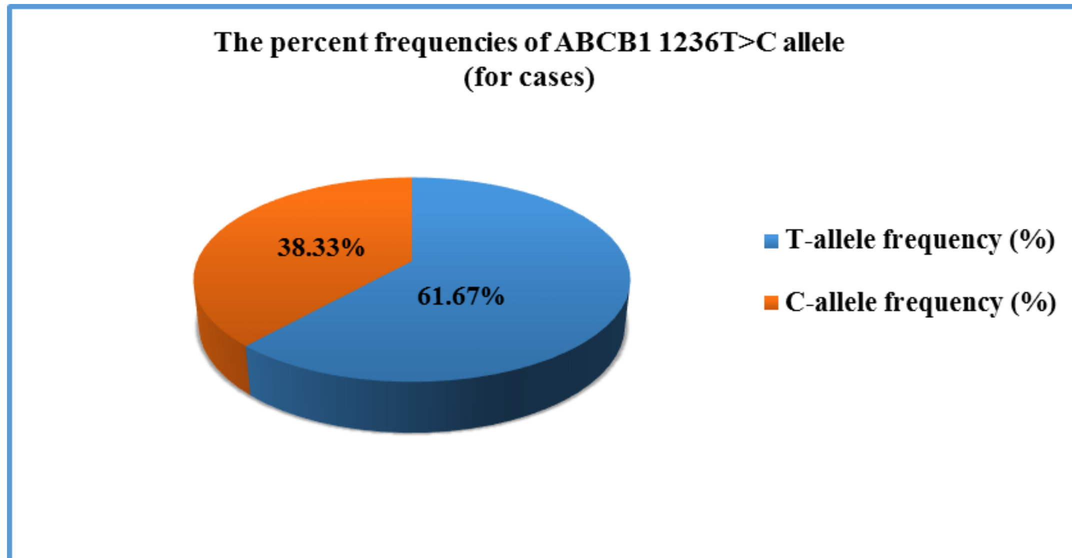


Figure 3.15: The percent frequencies of ABCB1 1236T>C allele (for cases)

For ABCB1 1236T>C polymorphism, the percent frequencies of case group were 61.67% and 38.33% for T and C allele, respectively.

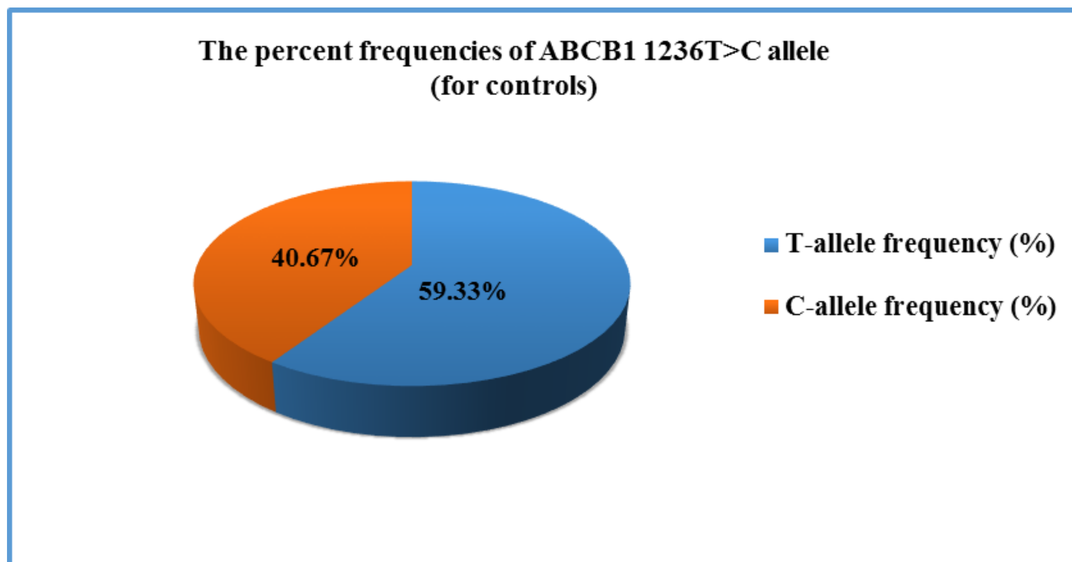


Figure 3.16: The percent frequencies of ABCB1 1236T>C allele (for controls)

Whereas, for the control group, we found that the frequency distributions were 59.33% for T allele and 40.67% for C allele.

3.12 Observed Genotyping Results of ABCB1 2677G>T Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.22: The frequency distribution of genotype and allelic data of ABCB1 2677G>T polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	**Genotype (Total = 180)**	**Cases (%) (n = 30)**	**Controls (%) (n = 150)**	**OR (95% CI)**	**p value**
ABCB1 2677G>T	GG (60)	8 (26.67%)	52 (34.67%)	Reference	Reference
	GT (16)	6 (20%)	10 (6.67%)	3.9 (1.11-13.70)	**0.034**
	TT (104)	16 (53.33%)	88 (58.67%)	1.18 (0.47-2.95)	0.721
	GT (16) + TT (104)	22 (73.33%)	98 (65.33%)	1.46 (0.61-3.50)	0.398
	G-allele	22 (36.67%)	114 (37.99%)	-	-
	T-allele	38 (63.33%)	186 (62.01%)	1.06 (0.60-1.88)	0.846

OR = Odds Ratio, Cl = Confidence Interval, p <0.05 considered as statistically significant

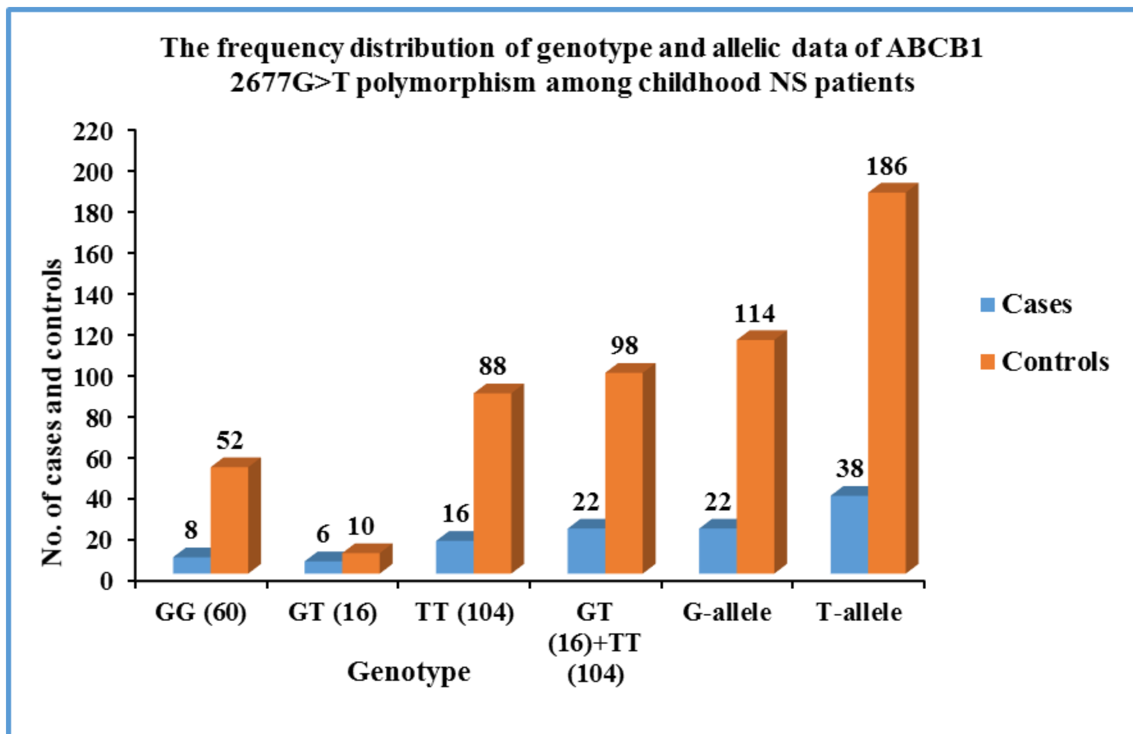


Figure 3.17: The frequency distribution of genotype and allelic data of ABCB1 2677G>T polymorphism among childhood nephrotic syndrome patients

The distribution of GG (Ala/Ala) homozygous wild-type of ABCB1 2677G>T polymorphism was statistically lower in cases than in controls (26.67% vs. 34.67%). The frequency distribution of GT (Ala/Ser) heterozygous genotype was significantly higher in cases than controls (20% vs. 6.67%). So the distribution of GT (Ala/Ser) heterozygous genotype was found to have 3.9 times elevated risk of developing prednisolone resistance in NS compared with GG (Ala/Ala) homozygous wild-type and it was statistically significant (OR = 3.9, 95% Cl = 1.11 to 13.70, p = 0.034). The TT (Ser/Ser) mutant homozygous genotype and combined GT (Ala/Ser) heterozygous and TT (Ser/Ser) mutant homozygous genotypes GT+TT have shown 1.18 and 1.46 times more risk to develop prednisolone resistance, respectively, but these results were not statistically significant (OR = 1.18, 95% Cl = 0.47 to 2.95, p = 0.721 and OR = 1.46, 95% Cl = 0.61 to 3.50, p = 0.398). The percentage of T allele was slightly higher in cases (63.33%) compared with controls (62.01%) that also increased the prednisolone resistance risk, but the result is not statistically significant (p = 0.846) (Table 3.22; Figure 3.17; 3.18; 3.19).

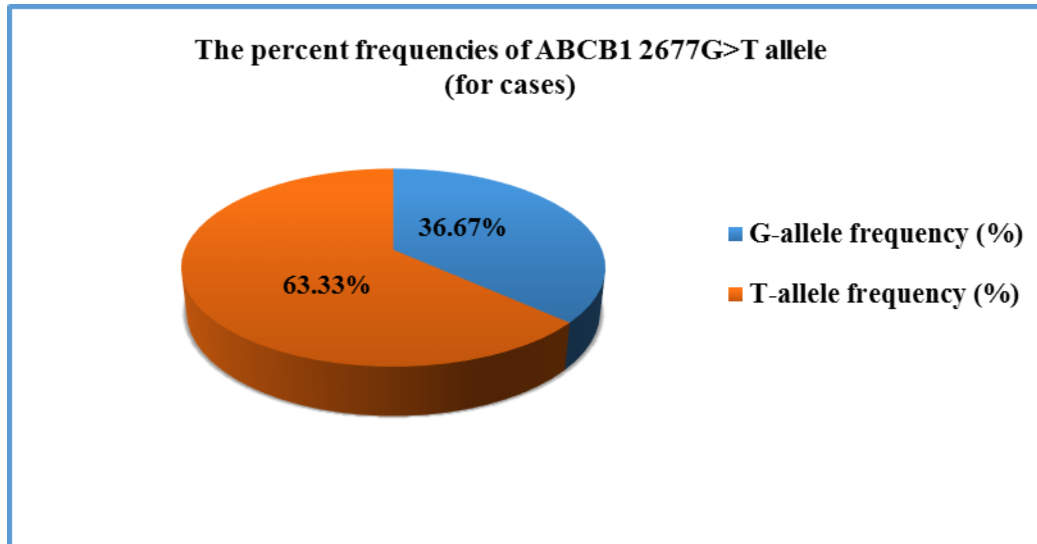


Figure 3.18: The percent frequencies of ABCB1 2677G>T allele (for cases)

The above pie chart represents the frequency distribution of cases in the ABCB1 2677G>T SNP. From the chart, we observed that G and T allele frequencies were 36.67% and 63.33%, respectively.

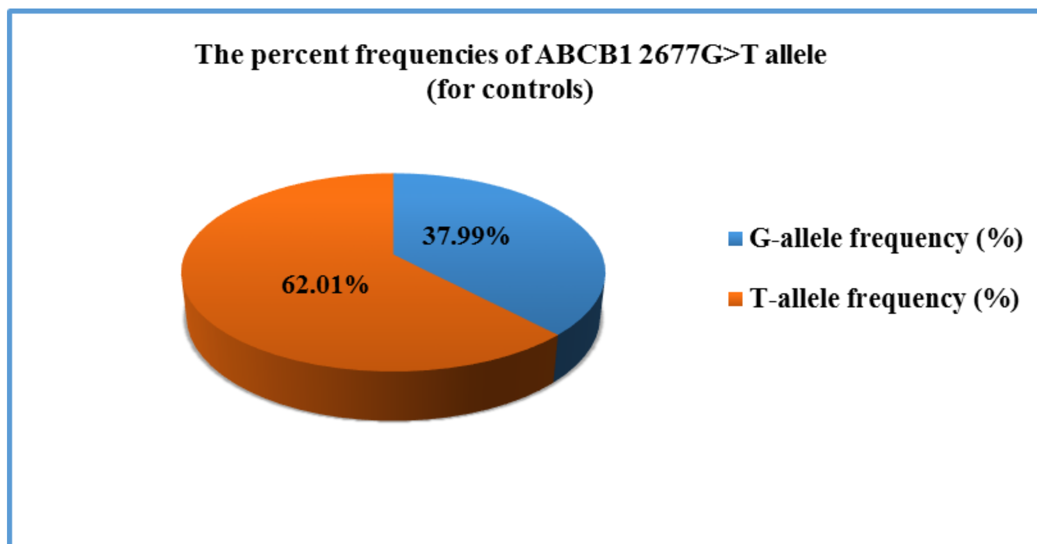


Figure 3.19: The percent frequencies of ABCB1 2677G>T allele (for controls)

In addition, the results revealed that the percentage of T allele was 62.01%, whereas, for G allele it was 37.99% in the controls in case of 2667G>T polymorphism.

3.13 Observed Genotyping Results of ABCB1 3435T>C Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.23: The frequency distribution of genotype and allelic data of ABCB1 3435T>C polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	Genotype (Total = 180)	Cases (%) (n = 30)	Controls (%) (n = 150)	OR (95% CI)	p value
ABCB1 3435T>C	TT (66)	13 (43.33%)	53 (35.33%)	Reference	Reference
	TC (93)	8 (26.67%)	85 (56.67%)	0.38 (0.15 -0.99)	**0.047**
	CC (21)	9 (30%)	12 (8%)	3.06 (1.06 -8.79)	**0.038**
	TC (93) + CC (21)	17 (56.67%)	97 (64.67%)	0.71 (0.32-1.58)	0.408
	T-allele	34 (56.67%)	191 (63.67%)	–	–
	C-allele	26 (43.33%)	109 (36.33%)	1.34 (0.76-2.35)	0.308

OR = Odds Ratio, Cl = Confidence Interval, p <0.05 considered as statistically significant

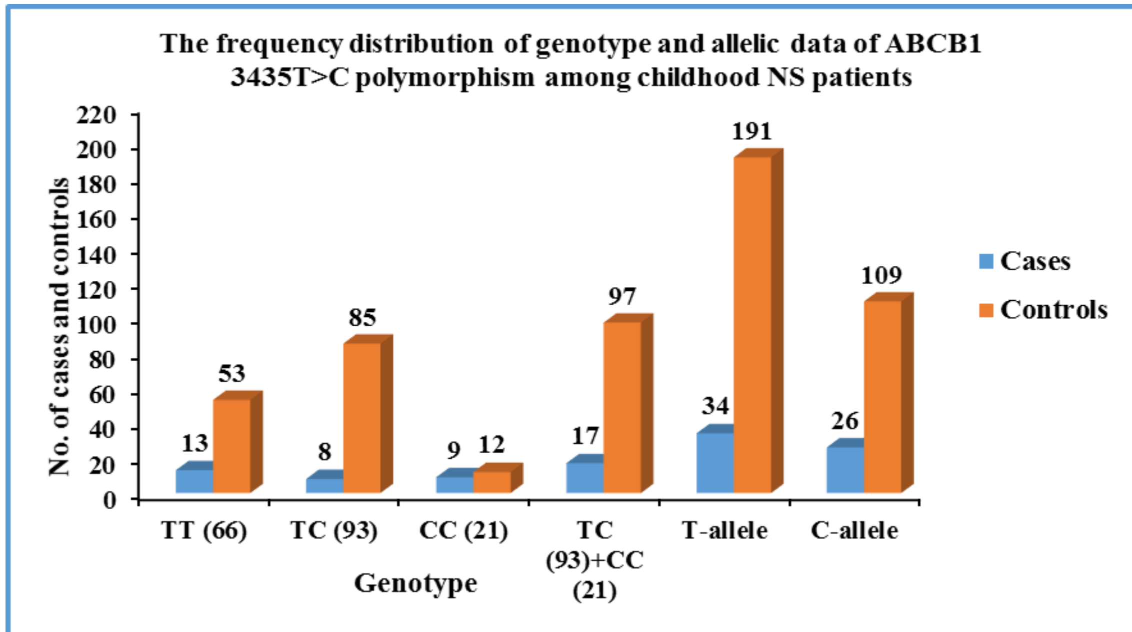


Figure 3.20: The frequency distribution of genotype and allelic data of ABCB1 3435T>C polymorphism among childhood nephrotic syndrome patients

This population-based case-control study was conducted to examine the prevalence of ABCB1 3435T>C (rs1045642, Ile1145Ile) polymorphism in the Bangladeshi population of prednisolone resistance cases and prednisolone sensitive in childhood NS controls. The TT (Ile/Ile) homozygous wild-type was statistically higher in cases than in controls (43.33% vs. 35.33%). The frequency of TC (Ile/Ile) heterozygous genotype was found to have 0.38 times lower risk of developing prednisolone resistance in childhood nephrotic syndrome compared with TT (Ile/Ile) homozygous wild-type and it was statistically significant (OR = 0.38; 95% CI = 0.15 to 0.99, p = 0.047). The CC (Ile/Ile) mutant homozygous genotype was significantly found to be responsible for developing prednisolone resistance in nephrotic syndrome (OR = 3.06; 95% CI = 1.06 to 8.79, p = 0.038) and it possesses 3.06 times more risk. TC (Ile/Ile) heterozygous genotype and CC (Ile/Ile) mutant homozygous genotype together TC+CC was not found significantly associated with PRNS and it possesses 0.71 times lower risk of developing prednisolone resistance in NS (OR = 0.71, 95% CI = 0.32 to 1.58, p = 0.408). The percentage of C allele was higher in cases (43.33%) compared with controls (36.33%) that also increased the prednisolone resistance risk non-significantly (p = 0.308) (Table 3.23; Figure 3.20; 3.21; 3.22).

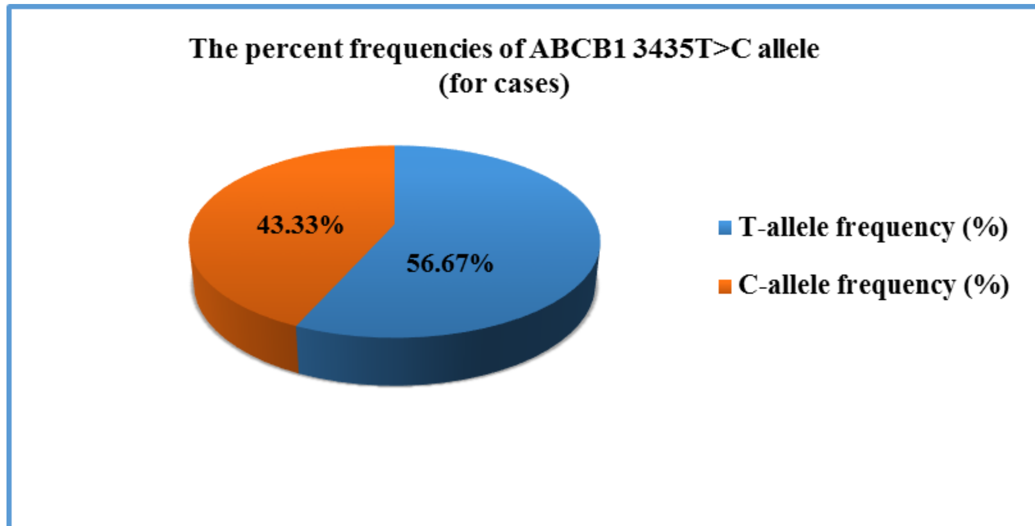


Figure 3.21: The percent frequencies of ABCB1 3435T>C allele (for cases)

In case of ABCB1 3435T>C polymorphism, the T and C allele frequencies were 56.67% and 43.33%, respectively for case group.

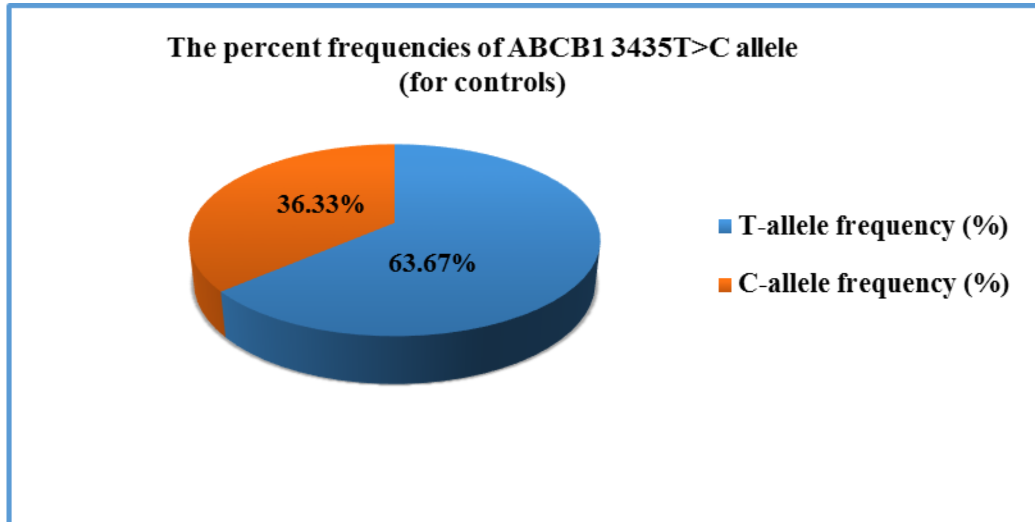


Figure 3.22: The percent frequencies of ABCB1 3435T>C allele (for controls)

For control group of 3435T>C polymorphism, the percent frequencies of T and C allele were 63.67% and 36.33%, respectively.

3.14 Observed Genotyping Results of NR3C1 (rs10482634) Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.24: The frequency distribution of genotype and allelic data of NR3C1 (rs10482634) polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	Genotype (Total = 180)	Cases (%) (n = 30)	Controls (%) (n = 150)	OR (95% CI)	p value
NR3C1 rs10482634	TT (115)	14 (46.67%)	101 (67.33%)	Reference	Reference
	TC (60)	15 (50%)	45 (30%)	2.40 (1.07-5.40)	**0.033**
	CC (5)	1(3.33%)	4 (2.67%)	1.80 (0.19-17.31)	0.609
	TC (60) + CC (5)	16 (53.33%)	49 (32.67%)	2.36 (1.06-5.21)	**0.034**
	T-allele	43 (71.67%)	247 (82.33%)	–	–
	C-allele	17 (28.33%)	53 (17.67%)	1.84 (0.98-3.48)	**0.059**

OR = Odds Ratio, Cl = Confidence Interval, p <0.05 considered as statistically significant

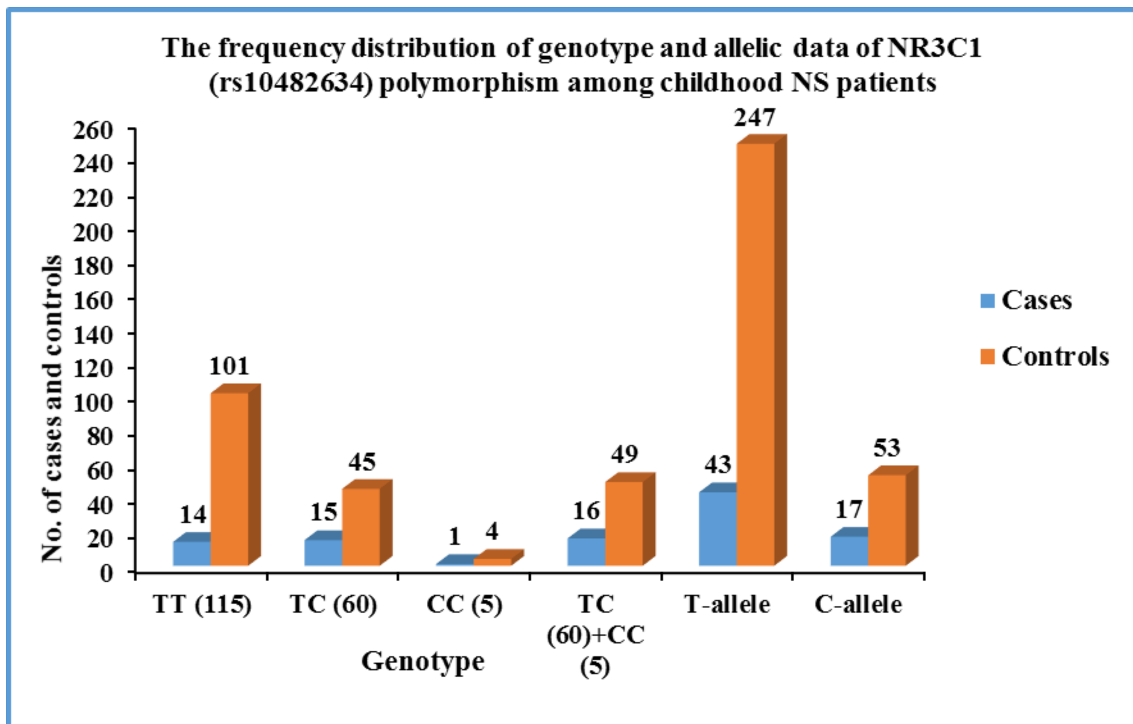


Figure 3.23: The frequency distribution of genotype and allelic data of NR3C1 (rs10482634) polymorphism among childhood nephrotic syndrome patients

For rs10482634 polymorphism of the NR3C1 gene, the TT homozygous wild-type was statistically lower in cases than in controls (46.67% vs. 67.33%). The frequency distribution of TC heterozygous genotype was found to have 2.40 times higher risk of developing prednisolone resistance compared to the TT homozygous wild-type and it was statistically significant (OR = 2.40, 95% Cl = 1.07 to 5.40, p = 0.033). The CC mutant homozygous genotypes was not significantly associated with prednisolone resistance though it possesses 1.80 times more risk to develop prednisolone resistance NS (OR = 1.80, 95% Cl = 0.19 to 17.31, p = 0.609). On the other hand, the distribution of TC+CC together was found to have 2.36 times greater risk to develop prednisolone resistance and it was statistically significant (OR = 2.36, 95% Cl = 1.06 to 5.21, p = 0.034). The percentage of C allele was higher in cases (28.33%) compared with controls (17.67%) that also increased the prednisolone resistance risk non-significantly (p = 0.059) (Table 3.24; Figure 3.23; 3.24; 3.25).

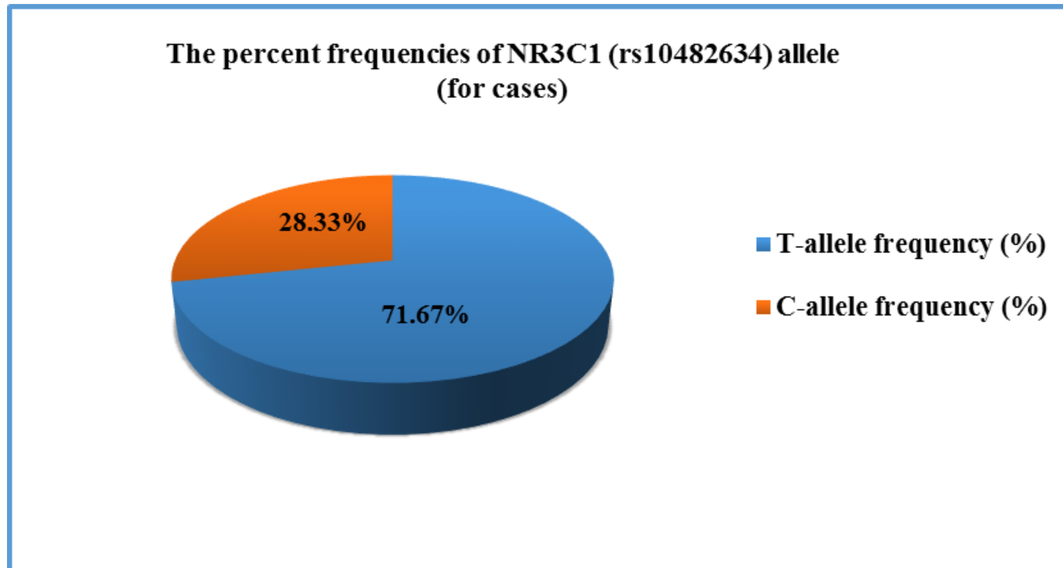


Figure 3.24: The percent frequencies of NR3C1 (rs10482634) allele (for cases)

For NR3C1 (rs10482634) polymorphism, the percent frequencies of case group were 71.67% and 28.33% for T and C allele, respectively.

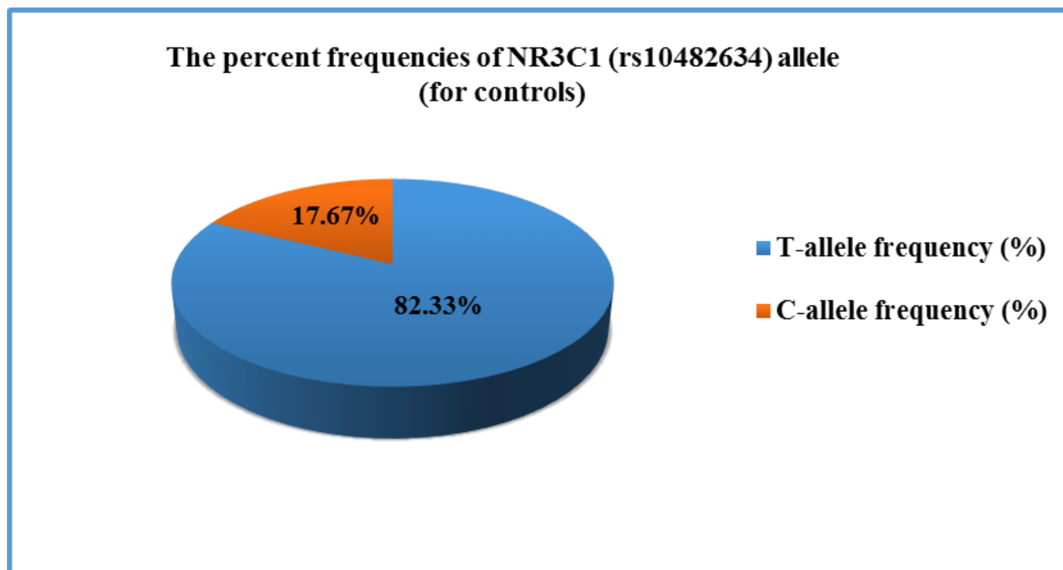


Figure 3.25: The percent frequencies of NR3C1 (rs10482634) allele (for controls)

Whereas, for the control group, we found that the percent frequencies were 82.33% for T allele and 17.67% for C allele.

3.15 Observed Genotyping Results of NR3C1 (rs6877893) Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.25: The frequency distribution of genotype and allelic data of NR3C1 (rs6877893) polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	Genotype (Total = 180)	Cases (%) (n = 30)	Controls (%) (n = 150)	OR (95% CI)	p value
NR3C1 rs6877893	AA (117)	18 (60%)	99 (66%)	Reference	Reference
	AG (5)	1 (3.33%)	4 (2.67%)	1.38 (0.15-13.02)	0.781
	GG (58)	11 (36.67%)	47 (31.33%)	1.29 (0.56-2.94)	0.549
	AG (5) + GG (58)	12 (40%)	51(34%)	1.29 (0.58-2.89)	0.530
	A-allele	37 (61.66%)	202 (67.33%)	–	–
	G-allele	23 (38.34%)	98 (32.67%)	1.28 (0.72-2.27)	0.397

OR = Odds Ratio, Cl = Confidence Interval, p <0.05 considered as statistically significant

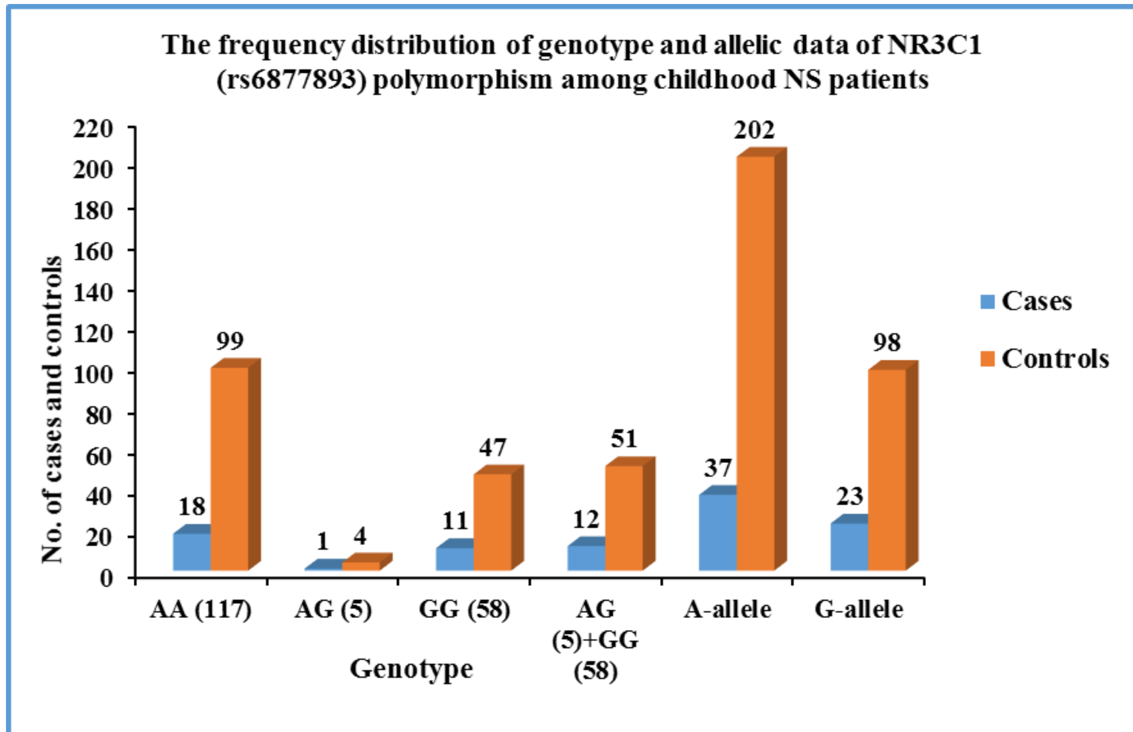


Figure 3.26: The frequency distribution of genotype and allelic data of NR3C1 (rs6877893) polymorphism among childhood nephrotic syndrome patients

Compared with the AA homozygous wild-type of rs6877893 polymorphism of NR3C1 gene, AG heterozygous, GG mutant homozygous genotype and combined AG heterozygous and GG mutant homozygous genotype AG+GG had increased risk of prednisolone resistance in childhood NS compared with AA homozygous wild-type (OR = 1.38, 95% Cl = 0.15 to 13.02, p = 0.781; OR = 1.29, 95% Cl = 0.56 to 2.94, p = 0.549 and OR = 1.29, 95% Cl = 0.58 to 2.89, p = 0.530, respectively). AG heterozygous genotype and GG mutant homozygous genotype had 1.38 and 1.29 times more risk of prednisolone resistance, whereas AG+GG combined genotype had 1.29 times more risk of prednisolone resistance compared with AA homozygous wild-type. The analysis showed no significant association between the different genotypes of rs6877893 and the risk of prednisolone resistance in NS. The percentage of G allele was higher in cases (38.34%) compared with controls (32.67%) that also increased the prednisolone resistance risk non-significantly (p = 0.397) (Table 3.25; Figure 3.26; 3.27; 3.28).

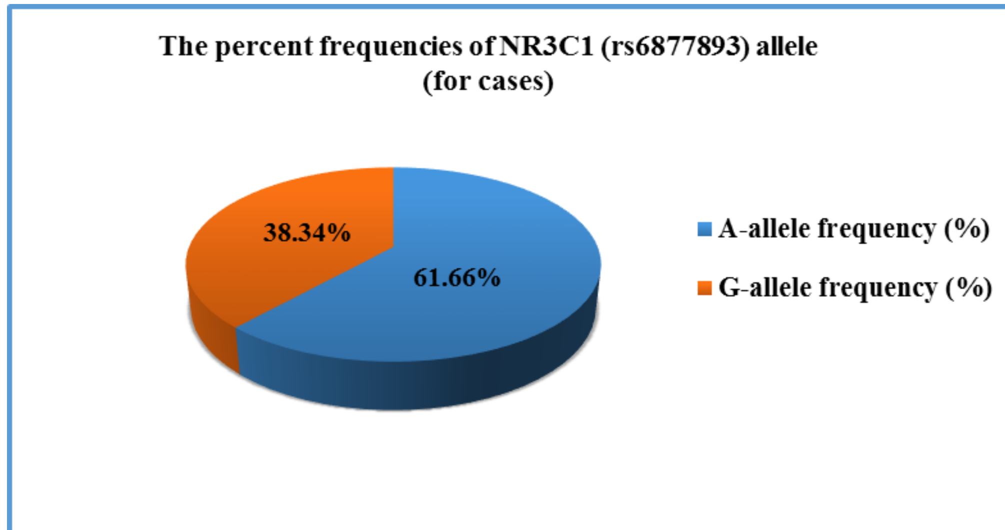


Figure 3.27: The percent frequencies of NR3C1 (rs6877893) allele (for cases)

The above pie chart represents the frequency distribution of cases in the NR3C1 (rs6877893) polymorphism. From the chart, we observed that A and G allele frequencies were 61.66% and 38.34%, respectively.

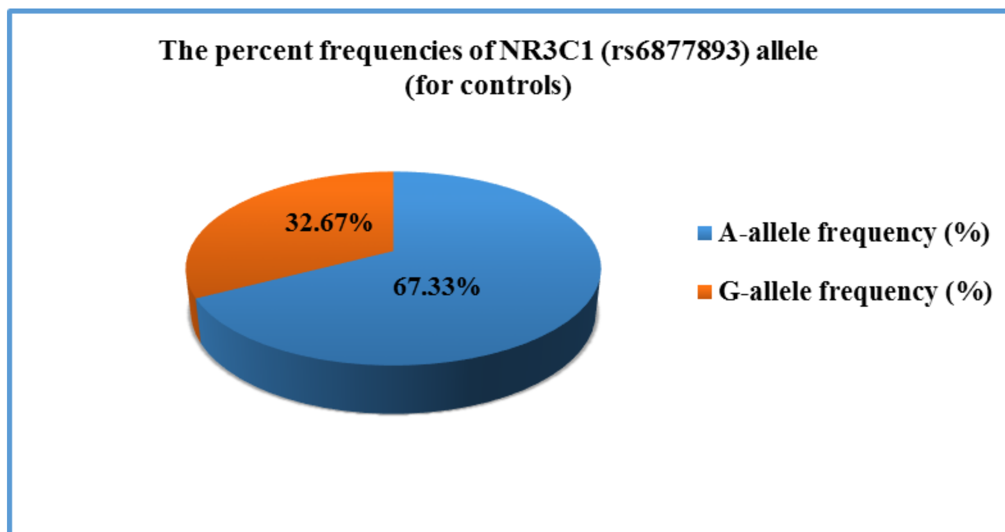


Figure 3.28: The percent frequencies of NR3C1 (rs6877893) allele (for controls)

In addition, for controls of NR3C1 (rs6877893) polymorphism, the results revealed that the percent frequencies of A allele were 67.33%, whereas, for G allele it was 32.67%.

3.16 Observed Genotyping Results of CYP3A5*3 Polymorphism for Nephrotic Syndrome Cases and Controls

Table 3.26: The frequency distribution of genotype and allelic data of CYP3A5*3 polymorphism in nephrotic syndrome cases and controls and their association with prednisolone resistance

SNP	Genotype (Total = 180)	Cases (%) (n = 30)	Controls (%) (n = 150)	OR (95% CI)	p value
CYP3A5 CYP3A5*3	AA (29)	6 (20%)	23 (15.33%)	Reference	Reference
	AG (70)	9 (30%)	61 (40.67%)	0.57 (0.18-1.77)	0.327
	GG (81)	15 (50%)	66 (44%)	0.87 (0.30-2.51)	0.799
	AG (70) + GG (81)	24 (80%)	127 (84.67%)	0.72 (0.27-1.97)	0.527
	A-allele	21 (35%)	107 (35.67%)	–	–
	G-allele	39 (65%)	193 (64.33%)	1.03 (0.58-1.84)	0.922

OR = Odds Ratio, Cl = Confidence Interval, p <0.05 considered as statistically significant

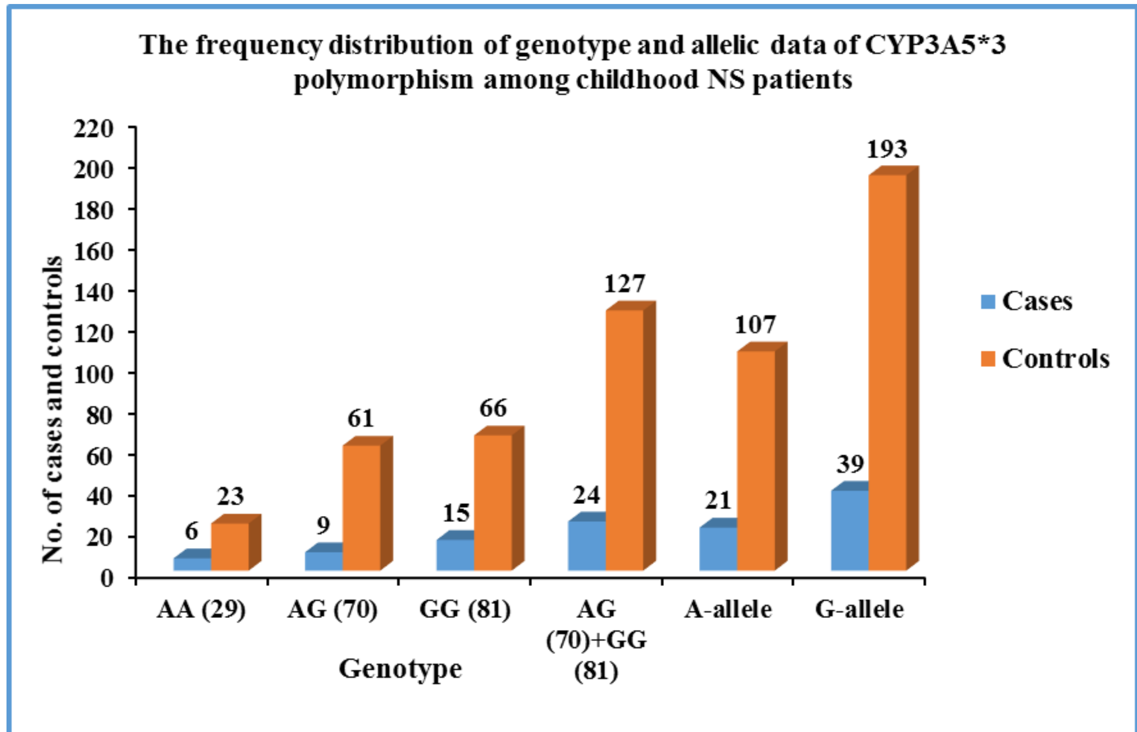


Figure 3.29: The frequency distribution of genotype and allelic data of CYP3A5*3 polymorphism among childhood nephrotic syndrome patients

In case of CYP3A5*3 polymorphism of the CYP3A5 gene, AG (Pro/Thr) heterozygous, GG (Thr/Thr) mutant homozygous genotype and combined AG (Pro/Thr) heterozygous and GG (Thr/Thr) mutant homozygous genotypes AG+GG had reduced the risk of prednisolone resistance in childhood NS compared with AA (Pro/Pro) homozygous wild-type (OR = 0.57, 95% Cl = 0.18 to 1.77, p = 0.327; OR = 0.87, 95% Cl = 0.30 to 2.51, p = 0.799 and OR = 0.72, 95% Cl = 0.27 to 1.97, p = 0.527, respectively). AG (Pro/Thr) heterozygous genotype and GG (Thr/Thr) mutant homozygous genotypes had 0.57 and 0.87 times lower risk of developing prednisolone resistance compared with AA (Pro/Pro) homozygous wild-type, whereas AG+GG combined genotype had 0.72 times lower risk of prednisolone resistance compared with AA (Pro/Pro) wild-type genotype. The obtained results were not statistically significant ($p > 0.05$). The percentage of G allele was slightly higher in cases (65%) compared with controls (64.33%) that also increased the prednisolone resistance risk but the result is not statistically significant (p = 0.922) (Table 3.26; Figure 3.29; 3.30; 3.31).

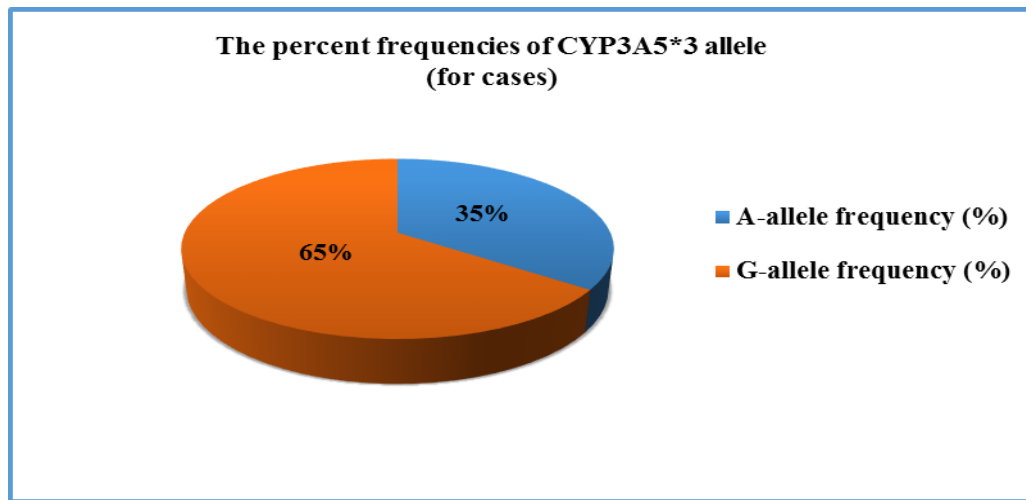


Figure 3.30: The percent frequencies of CYP3A5*3 allele (for cases)

In case of CYP3A5*3 polymorphism, the A and G allele frequencies were 35% and 65%, respectively for prednisolone resistant group.

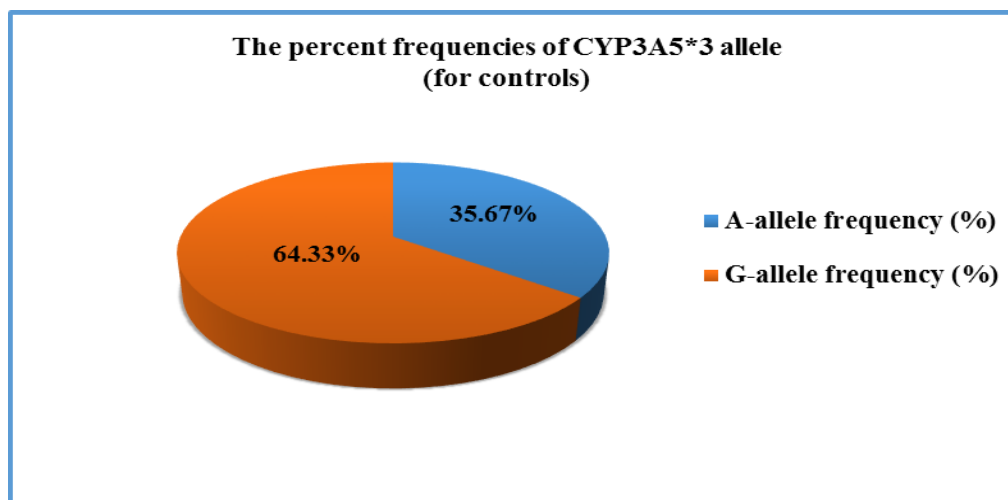


Figure 3.31: The percent frequencies of CYP3A5*3 allele (for controls)

For control group of CYP3A5*3 polymorphism, the percent frequencies of A and G allele were 35.67% and 64.33%, respectively.

The SNPs of 1236T>C, 2677G>T, 3435T>C of the ABCB1 gene and rs6877893 SNP of the NR3C1 gene showed deviations from Hardy-Weinberg equilibrium, while other SNPs like rs10482634 SNP of the NR3C1 gene and CYP3A5*3 SNP of the CYP3A5 gene were in Hardy-Weinberg equilibrium in both groups ($p \geq 0.05$).

3.17 DISCUSSION

Nephrotic syndrome is the most common form of glomerular disease in children. Glucocorticoids remain the mainstay of childhood NS treatment and response to initial oral prednisone determines disease prognosis. Patients with steroid-resistant nephrotic syndrome possess the most difficult therapeutic challenge to clinicians and scientists. Despite glucocorticoids have broad therapeutic range and effectiveness in the induction of remission; early markers that would allow optimization of their dose and duration of the therapy could improve the management of nephrotic patients (Youssef *et al.,* 2013; Wasilewska *et al.,* 2007). To the best of our knowledge, no previous studies concerning the role of ABCB1, NR3C1 and CYP3A5 polymorphisms with respect to risk of NS, and patient's response to GC have been reported in Bangladeshi population.

In this present study, we genotyped three known SNPs namely 1236T>C (rs1128503), 2677G>T (rs2032582) and 3435T>C (rs1045642) of the ABCB1 gene, two SNPs rs10482634 and rs6877893 of NR3C1 gene and one known SNP CYP3A5*3 of CYP3A5 gene in a group of pediatric Bangladeshi patients with nephrotic syndrome. We recruited 180 patients and among them, 30 cases had prednisolone resistance nephrotic syndrome and 150 controls had prednisolone sensitive nephrotic syndrome. All of the results were compared with the results of the control group. The study was done by using the PCR-RFLP assay method for the detection of candidate genetic polymorphisms responsible for developing PRNS. The PCR-RFLP method for the detection of the SNPs of NR3C1 was developed in our laboratory by using new primers. From the distribution of demographic data and clinicopathological characteristics among study population, the related factors such as age, BMI, sex, socioeconomic status, residence, histopathological finding, clinical presentation, biochemical findings and side effects of prednisolone therapy of cases and controls were compared to find out whether others factors were involved or not for developing prednisolone resistance in childhood nephrotic syndrome.

Among the enrolled patients, the mean age of onset was higher for PRNS (9.07 ± 3.27 years) than for PSNS (7.69 ± 3.40 years). There were no significant differences in the distribution of age ($p = 0.061$) between the cases and controls whereas there were

significant differences in BMI (p = 0.044) between the two groups. The mean age of onset of PRNS was comparable with the findings of Roy *et al.* (Roy *et al.*, 2014). Among the study population, the male and female were 60% and 40%, respectively, for cases (male: female -1.5:1) and 63.33% and 36.67%, respectively, for controls (male: female-1.73:1). So from the data, we found that male children were more sufferer from nephrotic syndrome than female children in both case and control group. From the studies of Alam *et al.*, Zalewski *et al.* and Siddique *et al.* (Alam *et al.,* 2016; Zalewski *et al.,* 2008, Siddique *et al.,* 2018) similar findings also observed. Regarding socioeconomic condition-for cases, 6.67% of the subjects came from the upper class followed by 33.33% from the middle class and 60% of children came from lower socioeconomic classes. In case of control group, 6%, 36% and 58% of children came from upper, middle and lower socioeconomic classes, respectively. So lower socioeconomic class children were more prone to develop PRNS than the solvent children (middle and upper class). This might be due to unhealthier lifestyles and unhealthy food habits. These findings were similar to the study of Sarker *et al.* and Roy *et al.* (Sarker *et al.,* 2012; Roy *et al.,* 2014). In case of cases, 33.33% and 66.67% of children came from urban and rural areas, respectively. On the other hand, 29.33% and 70.67% of children of the control group came from the urban and rural areas, respectively. A higher incidence of nephrotic syndrome was found in rural children than that in urban children. In our country, urban peoples have a tendency to admit to the private hospital but not the public hospital. We recruited all the patients from different public hospitals. So, this picture might not represent the actual situation. But this results are comparable with the findings of Sarker *et al.* (Sarker *et al.,* 2012).

Among the 180 pediatric patients recruited in this study, renal biopsies were performed in 40 patients (30 patients with PRNS cases and 10 patients among 150 PSNS controls). The results of renal biopsy of 30 PRNS cases were: mesangioproliferative glomerulonephritis (MesPGN) 13/30 (43.33%), membranoproliferative glomerulonephritis 6/30 (20%), focal segmental glomerulosclerosis 4/30 (13.33%), minimal change nephrotic syndrome 5/30 (16.57%), IgA nephropathy 1/30 (3.33%) and inadequate tissue 1/30 (3.33%). Biopsy results of the other ten patients were as follows: membranoproliferative glomerulonephritis 4/150 (2.67%) and minimal change nephrotic syndrome 6/150 (4%). We found that MesPGN was the cause of PRNS in the current

study NS children and its percentage was 43.33%. Our results were similar to the report by Roym*et al.* in Bangladesh, they reported that MesPGN as the underlying histopathology in 40.63% of their 32 SRNS (Roy *et al.,* 2014) cases. The prevalence of MesPGN varies from study to study. Some studies had reported higher prevalence rates also. MesPGN had been reported as a cause of SRNS as well as SSNS with a troublesome management protocol (Uszycka-Karcz *et al.,* 1982). So the MesPGN histopathology carrying children has the greatest risk to develop PRNS.

From the biochemical findings of both cases and controls, we observed that the mean serum albumin level (cases-10.23 ± 0.65; controls-9.79 ± 0.79; p = 0.003) and mean serum cholesterol level (cases-435.32 ± 66.15; controls-404.65 ± 88.21; p = 0.037) were significantly higher in patients with PRNS cases than in patients with PSNS controls. However, the mean value of the urine protein/creatinine ratio for PSNS controls (3.47 ± 0.80) was higher than in PRNS cases (3.14 ± 0.75; p = 0.011). We also observed some atypical and classical presentation in the study population. The most common symptom was edema at the presentation time, it was found 100% of cases and 97.33% of the control group. 100% of both cases and controls were presented massive proteinuria during their nephrotic syndrome disease. This might be due to edema and massive proteinuria are the most common presenting symptoms of both PRNS and PSNS patients. In our current study, 60% of cases presented with microhematuria where it was found in 20% of the control group. Among the atypical presentation, persistent hypertension was found in 36.67% of cases and 8.67% of the controls. Several types of complications were seen during the nephrotic syndrome, among them 6.67% and 4.67% of cases and controls, respectively, had urinary tract infection, whereas respiratory tract infection was found in 3.33% of cases and 2% of controls. Cellulitis and peritonitis were also presenting complications among the study population. The PRNS cases presented cellulitis and peritonitis were 10% and 16.67%, respectively and the PSNS controls presented 8% and 14%, respectively. The nephrotic syndrome patients were carrying atypical symptoms varies from patients to patients. There were no obese patients found among the PRNS cases whereas, 2.67% of obese were found in the PSNS control group. This might be due to the side effects of long-time use of prednisolone therapy. Malnutrition also noted 6.67% PRNS cases and 4% PSNS controls. In our study population, 6.67% of PRNS cases had growth and development retardation (Table 3.1).

This might be due to the prolonged use of immunosuppressive drugs and unable to strictly flow their regular diet chat.

To established inter-varible correlations among different demographic data and clinicopathological characteristics between the case and control group, we used Pearson's correlation for the investigated data. Tables 3.2 represent the correlation coefficient and the statistical significance at which the correlations were established. Pearson correlation coefficient suggested that there were a significant positive correlation between BMI and age ($r = 0.985$, $p = <0.005$), BMI and albumin ($r = 0.315$, $p = 0.045$), BMI and cholesterol ($r = 0.361$, $p = 0.025$) and age and cholesterol ($r = 0.361$, $p = 0.025$). On the other hand, age and albumin was positively correlated but not statistically significant ($r = 0.284$, $p = 0.064$). Same analysis revealed that albumin and cholesterol ($r = -0.337$, $p = 0.034$), albumin and urine protein/creatinine ratio ($r = -0.309$, $p = 0.048$) showed statistically significant ($p <0.05$) negative correlation and BMI and urine protein/creatinine ratio ($r = -0.175$, $p = 0.178$), cholesterol and urine protein/creatinine ratio ($r = -0.609$, $p = 0.358$), age and urine protein/creatinine ratio ($r = -0.170$, $p = 0.185$) were negatively correlated but not find statistically significant ($p >0.05$) (Table 3.2).

In this study, we determined and compared the genotype frequencies and allele frequencies of 1236T>C, 2677G>T and 3435T>C SNPs of the ABCB1 gene, rs10482634 and rs6877893 SNPs of the NR3C1 gene and CYP3A5*3 SNP of the CYP3A5 gene between PRNS and PSNS children. To improve the treatment of NS patients, genetic markers that predict steroid response would be useful. We found that the 2677G>T and 3435T>C polymorphisms, but not 1236T>C polymorphism, of the ABCB1 gene, rs10482634 polymorphism, but not rs6877893 polymorphism, of the NR3C1 gene was, significantly associated with prednisolone resistance in our pediatric patients with NS. However, CYP3A5*3 polymorphism of the CYP3A5 gene was not also associated with steroid-resistance. To our knowledge, no previous pharmacogenetic study has yet done on Bangladeshi prednisolone resistance in childhood nephrotic patients which promotes us to conduct this study.

The **ABCB1** gene which is also known as the **MDR1** gene encodes a membrane P-gp that functions as an ATP dependent exporter of xenobiotics from cells. P-gp is expressed in normal tissue, such as the intestine, liver and kidneys, which have excretory functions, as well as in capillary endothelial cells of the brain, placenta, testis and in peripheral blood cells (Youssef *et al.,* 2013; Thiebaut *et al.,* 1987). In the kidney, the P-gp is expressed in the brush border membrane of proximal tubular epithelial cells (Youssef *et al.,* 2013; Cordon-Cardo *et al.,* 1989; Thiebaut *et al.,* 1987). Various types of structurally unrelated drugs, including steroids, are known to be substrates for P-gp (Youssef *et al.,* 2013; Dilger *et al.,* 2004; Sakaeda, 2005). To date, approximately 50 SNPs have been reported in the ABCB1/MDR1 gene (Youssef *et al.,* 2013; Ieiri *et al.,* 2004; Kroetz *et al.,* 2003). Among these SNPs, C1236T, G2677T/A and C3435T are the most common variants in the coding region of MDR1 and they are in strong linkage disequilibrium. While C1236T and C3435T are synonymous SNPs, G2677T/A causes an amino acid substitution (Ala899Ser/Thr) (Youssef *et al.,* 2013; Siegsmund *et al.,* 2002).

Our study on **ABCB1 1236T>C (rs1128503)** polymorphism revealed that the percent frequency of TC (Gly/Gly) heterozygous genotype was 10% in cases, whereas 4% in controls and the percent frequency of CC (Gly/Gly) mutant homozygous genotype was 33.33% in cases, whereas 38.67% in controls. Thus, TC (Gly/Gly) heterozygous and combined TC+CC had increased risk but not significantly associated ($p > 0.05$) with prednisolone resistance in childhood nephrotic syndrome compared with TT (Gly/Gly) normal homozygous genotype. The risk factors for TC (Gly/Gly) and TC+CC were 2.53 and 1.03 times more compared with TT (Gly/Gly) normal homozygous genotype, respectively. Whereas CC (Gly/Gly) mutant homozygous genotype showed 0.87 times lower risk of developing prednisolone resistence NS compared to TT wild-type. The percent of the C allele was slightly lower in cases (38.33%) compared with controls (40.67%) that also lower the PRNS risk non-significantly ($p = 0.737$). So from our study, we found that 1236T>C polymorphism of the ABCB1 gene was not associated with prednisolone resistance nephrotic syndrome (Table 3.21).

Like our study, several case-control studies have demonstrated no association between the 1236T>C SNP of the ABCB1 gene and the risk of prednisolone resistance in

childhood nephrotic syndrome in different ethnic groups within consistent findings. For example, in a study by Jafar *et al.* (Jafar *et al.,* 2011) reported that there were no differences in the frequency distribution of genotypes and alleles between patients with SSNS and patients with SRNS. So, 1236T>C polymorphisms did not have any significant impact on steroid-resistance in nephrotic syndrome on their North Indian children. Furthermore, Cizmarikova *et al.* (Cizmarikova *et al.,* 2015) could not find any significant evidence of association in the distribution of the MDR1 1236T>C genotypes or alleles between steroid responders and non-responders nephrotic syndrome children of Slovak population ($p > 0.05$). Similarly, Wasilewska *et al.* (Wasilewska *et al.,* 2007) also found no significant differences in the MDR1 C1236T genotype frequencies between NS patients and healthy children. However, Choi *et al*. (Choi *et al.,* 2011) found that the frequencies of the MDR1 1236 CC or TC genotype and C allele were significantly higher in the initial steroid responders than in the non-responders in Korean children with idiopathic NS. These findings suggest that the MDR1 1236 CC genotype and C allele may be a predictor for better initial steroid responsiveness. In that study, the percent frequency of T allele and C allele of the steroid responder group (T-54.5%, C-45.5%) are almost similar to the steroid responder group in our study population (T-59.33%, C-40.67%). From the study of Choi *et al.* (Choi *et al.,* 2011), we also observed that the percent frequency of T allele and C allele of the NS patient group (T-61.2%, C-38.8%) are almost similar to the case group of our study population (T-61.67%, C-38.33%). Dhandapani *et al.* (Dhandapani *et al.,* 2015) suggested that C1236T polymorphism of the MDR1 gene showed no significantly associated with SRNS on their Indian study population. In an Egyptian study, Youssef *et al.* (Youssef *et al.,* 2013) reported that there were no significant associations of MDR1 C1236T with steroid-resistant NS. They also found no significant differences were observed in distributions of C1236T or allele between NS patients and healthy children. However, inconsistent results also exist to show an association between 1236T>C genotype and the risk of prednisolone resistance in childhood nephrotic syndrome in Chinese and Egyptian populations. Chiou *et al.* (Chiou *et al.,* 2012) also investigated the same polymorphism in China upon 74 children with idiopathic nephrotic syndrome (INS). They could find a significant association of C1236T polymorphism with steroid-resistance: the frequency of the T allele was significantly higher in steroid-resistant patients than in steroid-sensitive ones (81 vs. 62%; OR = 2.65, 95 % CI = 1.01 to 6.94,

p = 0.042) which was inconsistent with our result. Similarly, Safan *et al.* (Safan *et al.,* 2017) conducted a study on the Egyptian population that revealed a significant difference in the distribution of C1236T genotypes was observed between SS and SR patients. They also suggested that C1236T polymorphism in ABCB1 was associated with steroid-resistance in INS children (OR = 2.27, 95 % CI = 1.2 to 4.4, p = 0.012). The frequency of the T allele was significantly higher in SR than in SS patients (81.2 vs. 65.6%, respectively) which disagrees with our findings.

In 1236T>C polymorphism of the ABCB1 gene, the discrepancies of association among different studies results may be due to linkage disequilibrium with other functional polymorphisms. In addition, rs1128503 exhibits strong linkage disequilibrium with other functional polymorphisms such as rs2032582 (Sauna *et al.,* 2007). C1236T is located in exon 12, in one of the intracellular loops of the protein adjacent to the Adenosine Tri-Phosphate binding/ utilization domain. The absence of a significant effect of the C1236T may be explained by the fact that this SNP is a synonymous variant that does not result in any change in the protein sequence (Gly/Gly). However, it may affect translation regulation, RNA stability or other molecular mechanisms (Jafar *et al.,* 2011). In fact, Salama *et al.* (Salama *et al.,* 2006) have shown that TT genotypes minimize P-gp activity in a substrate-dependent manner. It may also have an indirect effect being linked to a causal variant. So, we found that 1236T>C SNP did not appear to have an association with steroid-resistance in NS patients in our present study. There are marked differences in genotype and allele frequencies between different populations.

In case of **2677G>T (rs2032582)** SNP of the ABCB1 gene, the distribution of GG (Ala/Ala) homozygous wild-type was statistically lower in cases than in controls (26.67% vs. 34.67%) and the percent frequency of GT (Ala/Ser) heterozygous genotype was 20% in cases, whereas 6.67% in controls. Patients carrying GT (Ala/Ser) heterozygous genotype was significantly found to be 3.9 times elevated risk of developing PRNS compared to the GG (Ala/Ala) wild-type (OR = 3.9, 95% Cl = 1.11 to 13.70, p = 0.034). Whereas, individuals carrying TT (Ser/Ser) mutant homozygous and combined GT+TT genotypes had 1.18 and 1.46 times more risk to develop prednisolone resistance in nephrotic syndrome compared to GG (Ala/Ala) wild-type. But these results were not statistically significant. The percent frequencies of T allele was slightly higher

in cases (63.33%) compared with controls (62.01%) that also increased the PRNS risk non-significantly ($p = 0.846$) (Table 3.22).

However, some of the earlier studies have reported the association between the 2677G>T polymorphism and prednisolone resistance risk in childhood nephrotic syndrome. Those studies are- Youssef *et al.* (Youssef *et al.,* 2013) have conducted a study consisting of 46 steroid non-responders NS patients and 92 steroid responders NS control patients in an Egyptian population and found the risks of steroid-resistance were significantly higher among NS patients carrying GT, GA and TT + AA genotypes than patients carrying GG genotype (OR = 2.3, 95% CI = 0.79 to 6.77, p = 0.04; OR = 2.8, 95% CI = 0.87 to 9.01, p = 0.026; OR = 2.9, 95% CI = 0.95 to 9.21, p = 0.016, respectively). In our study, only GT heterozygous genotype is significantly associated with prednisolone resistance compared with GG wild-type (OR = 3.9, 95% Cl = 1.11 to 13.70, p = 0.034). In another study, we also observed that this polymorphism was significantly associated with increased susceptibility of NS, early onset of NS. Moussa *et al*. (Moussa *et al.,* 2017) investigated upon 63 NS cases (10 SRNS, 53 SSNS) and 110 healthy controls in a Tunisian children and confirmed that G2677A polymorphism was significantly associated with NS susceptibility and steroid-resistance (OR = 3.5, 95% CI = 1.37 to 7.06, p = <0.001; OR = 3.07, 95% CI = 1.06 to 26.10, p = 0.048, respectively). In another study, Jafar *et al.*(Jafar *et al*., 2011) found that the frequency distribution of mutant homozygous genotype of 2677G>T was significantly higher in patients with SRNS than the patient with SSNS compared to the wild-type GG and it possessed 3.39 times more risk to develop prednisolone resistance ($p< 0.05$). In the present study, TT mutant homozygous genotype has 1.18 times increased risk but not significantly associated with prednisolone resistance (OR = 1.18, p = 0.721) but GT heterozygous genotype is significantly associated with prednisolone resistance compared with GG wild-type (OR = 3.9, 95% Cl = 1.11 to 13.70, p = 0.034). Moreover, the presence of mutant homozygous genotypes of both G2677T/A and C3435T in different combinations possess a higher risk of developing resistance to steroids in patients with NS compared to the wild and heterozygous mutants of these genes. In the same study, Jafar *et al.* also reported that the frequency distribution of mutant homozygous genotypes of SNP G2677T/A was significantly higher in children with NS compared to healthy children. Whereas, Dhandapani *et al,* (Dhandapani *et al.,* 2015) studied on

Indian population and confirmed that the SNP G2677T/A was significantly associated with the genotypes GT and GA of the MDR1 gene, indicating it may be a potential marker to detect steroid-resistance. Our findings were in harmony with that of these studies. Some of the studies disagreed with our findings. Like, upon a study on Chinese population Chiou *et al.* (Chiou *et al.,* 2012) also suggested that G2677T/A polymorphism of ABCB1 gene was not associated with steroid-resistance in children with INS. However, Cizmarikova *et al.* (Cizmarikova *et al.,* 2015) revealed that there were no significant differences in the distribution of the 2677T>G genotypes or alleles between the steroid responders and non-responders of Slovak population. However, Choi *et al.* (Choi *et al.,* 2011) conducted a study on Korean children and they reported that there was no significant difference in the distribution of genotypes and allele in 2677G>T SNP between the NS patient group and the healthy volunteer control group. In contrast, Wasilewska *et al.* (Wasilewska *et al.,* 2007) also found no significant differences in the MDR1 G2677T/A genotype frequencies between NS patients and healthy children. These finding are inconsistent with our findings.

G2677T polymorphism is located in exon 21, on the intracellular side of P-gp gene. This association with NS could be explained by the replacement of alanine at position 2677 is replaced by serine or threonine, resulting in a conversion from a lipophilic residue to a hydrophilic one. Because alanine is a structurally neutral amino acid, it is possible that substitution of serine or threonine would affect the geometric precision of the interaction site and secondary structure (Jafar *et al.,* 2011; Tanabe *et al.,* 2001). This may have led to increased efficiency of the mutated protein which was able to efflux out GC or its active metabolites resulting in steroid-resistance. In our study, G allele frequency of control group was similar to the studies of both Cizmarikova *et al.* and Choi *et al.* (Cizmarikova *et al.,* 2015; Choi *et al.,* 2011) (G-37.99%; ref values G-38.5% and G-37.0 %, respectively). On the other hand, we found the percent of T allele frequency was 62.01% and Cizmarikova *et al.* (Cizmarikova *et al.,* 2015) also found the value of 61.5% in their study. From our study, it is speculated that NS patients who carry the GT heterozygous genotype of 2677G>T polymorphism would be more resistant to steroids and more susceptible for developing PRNS. All the subjects in these different studies came from different populations; thus it is possible that the difference in results are due to genetic heterogeneity among populations.

Our experiment on **ABCB1 3435T>C (rs1045642)** polymorphism revealed that the percent frequency of TC (Ile/Ile) heterozygous genotype was 26.67% in cases, whereas 56.67% in controls and the percent frequency of CC (Ile/Ile) mutant homozygous genotype was 30% in cases, whereas 8% in controls. Patients carrying TC (Ile/Ile) heterozygous (OR = 0.38; 95% CI = 0.15 to 0.99, p = 0.047) and CC (Ile/Ile) mutant homozygous (OR = 3.06; 95% CI = 1.06 to 8.79, p = 0.038) genotypes were found to have 0.38 -fold lower and 3.06-fold higher risk of prednisolone resistance compared to TT (Ile/Ile) normal homozygote genotype and this results were statistically significant (p <0.05). Whereas, their TC +CC combined risk factor had 0.71 times lower risk in compared with TT (Ile/Ile) normal homozygote genotype and this result was not statistically significant (p >0.05). In addition, the percentage of C allele was higher in cases (43.33%) compared with controls (36.33%) that also increased the prednisolone resistance risk but the result is not significant (p = 0.308) (Table 3.23).

Many studies have revealed that some SNPs of the ABCB1 gene result in changes in P-gp expression and function among different ethnicities and subjects, with most of the attention focused on the ABCB1 C3435T SNP (Choi *et al.,* 2011). Ganesan *et al.* (Ganesan *et al.,* 2017) conducted a study comprising 173 NS cases (90 had SRNS cases and 83 had SSNS) and 198 healthy controls in a South Indian NS children and they could find an association of C3435T polymorphism with steroid-resistance in NS. They suggested that MDR1 C3435T gene polymorphisms were risk factors of increased susceptibility, earlier onset of NS as well as leads to steroid-resistance. These findings were consistent with our study. Whereas, different studies on different ethnic groups had shown results that were inconsistent with our findings. Those studies were- Jafar *et al.* (Jafar *et al.,* 2011) studied on North Indian NS children and reported that individual carrying mutant homozygous genotype of SNP C3435T was not associated with the steroid-resistant in NS but the synergistic effect of the presence of mutant genotype of the G2677T/C and C3435T MDR1 gene in different combinations may increase the risk of developing steroid-resistance in NS patients. Another study done by Cizmarikova *et al.* (Cizmarikova *et al.,* 2015) upon 46 idiopathic nephrotic syndrome patients and revealed that a significantly increased chance of therapeutic response to glucocorticoids in Slovakian children carrying the 3435TC genotype (OR = 5.13, 95% CI = 1.18 to 22.25, p = 0.022). Upon study on Egyptian NS children, Youssef *et al.* (Youssuf *et al.,*

2013) revealed that there were no significant associations of MDR1 C3435T SNP with steroid-resistant NS, but they concluded that the significant association of MDR1 C3435T with susceptibility and earlier onset of NS in Egyptian children. Wasilewska *et al.* (Wasilewska *et al.,* 2007) also detected a significant association of the ABCB1 or MDR1 C3435T polymorphism with NS. Whereas, in a study on Chinese population Chiou *et al.* (Chiou *et al.,* 2012) also reported that 3435T>C polymorphism of ABCB1 gene was not associated with steroid-resistance in children with idiopathic nephrotic syndrome and Dhandapani *et al.* (Dhandapani *et al.,* 2015) found that 3435T>C polymorphism of the MDR1 gene showed no significant association with SRNS on their Indian study population. On the other hand, Safan *et al.* (Safan *et al.,* 2017) suggested that a significant difference in the distribution of ABCB1 C3435T genotypes was observed between SS and SR patients. The odds ratio for the C3435T polymorphism in response to steroid treatment was smaller than that of the polymorphism C1236T and did not reach statistical significance (OR = 1.1, 95 % CI = 0.6 to 1.9, p = 0.77). Whereas, Choi *et al.* (Choi *et al.,* 2011) found in Korean children that there was no significant difference in the distribution of genotypes and allotypes in 3435T>C SNP between the NS patient group and the healthy volunteer control group. Those studies failed to consistent with our findings. Interestingly, Klimecki *et al.* (Klimecki *et al.,* 1994) reported that MDR1 expression in lymphocytes was negatively correlated with the response to prednisone, cyclophosphamide and cyclosporine A in children with NS. The study of Woodahl and Ho (Woodahl and Ho, 2004) also found that MDR1 SNPs were associated with altered drug disposition.

The C3435T polymorphism is located in exon 26 of the ABCB1 gene. These observations could be elucidated by the fact that the 3435T>C SNP is a silent polymorphism [no amino acid changes (Ile/Ile)] or it is a synonymous variation; it is associated with altered protein expression (Sakaeda, 2005). However it may have some effect on DNA structure, RNA stability or the mechanism by which the ABCB1 C3435T SNP affects P-gp expression may be through possible linkage disequilibrium between the C3435T SNP and other ABCB1 variants that control expression, including the nonsynonymous G2677T/A and synonymous C1236T SNPs (Jafar *et al.,* 2011; Kroetz *et al.,* 2003). In addition, the ABCB1 C3435T SNP has been known to result in functional alteration of P-gp by affecting the timing of cotranslational folding and

insertion of P-gp into the membrane, thereby altering the structure of substrate and inhibitor interaction sites (Kimchi-Sarfaty *et al.,* 2007). This susceptibility marker to NS needs to be further validated in patients with different ethnicities. So, SNP C3435T alone appears to have a significant association with steroid-resistance in NS patients in the present study and different studies on different ethnic groups reported no association between steroid-resistance and 3435T>C SNP of ABCB1 gene. The association and discrepancies described suggest that it may be in linkage disequilibrium with other functional polymorphisms.

NR3C1 gene is located on chromosome 5q31-q32 and encodes for glucocorticoid receptor that can affect the regulation of many biological functions, including responsiveness to glucocorticoid and its functional variability may play a role in the therapeutic response to GC (West *et al.,* 2006; Suvanto *et al.,* 2016). Glucocorticoid, the first line of drug for childhood idiopathic NS, exerts its effects by its binding to the glucocorticoid receptor, a ligand-dependent transcription factor, which belongs to the superfamily of nuclear receptors (Baxter and Rousseau, 1979). The actions of both endogenous and synthetic glucocorticoids are mediated by the glucocorticoid receptor (Huusko *et al.,* 2014). Approximately 20% of the genes expressed in human leukocytes are regulated positively or negatively by glucocorticoids (Galon *et al.,* 2002). Genetic mutations in NR3C1 have been found to substantially diminish the GR function (Zalewski *et al.,* 2008). The genetic variations in the NR3C1 gene are important to explain the pathogenesis of diseases. It is well known that the response to glucocorticoid treatment is variable in patients with glomerular diseases including childhood idiopathic NS, asthma or other common diseases and the association of NR3C1 polymorphisms and response to glucocorticoid treatment have been analyzed in several studies (Zalewski *et al.,* 2008; Ye *et al.,* 2006; Kuningas *et al.,* 2006; Roussel *et al.,* 2003; Lin *et al.,* 2003; Rosmond *et al.,* 2000; Ukkola *et al.,* 2001; Stevens *et al.,* 2004; Donn *et al.,* 2007).

We also investigated the correlation between the rs10482634 and rs6877893 polymorphisms of the NR3C1 gene and prednisolone resistance in children with NS.

For **rs10482634** polymorphism of the **NR3C1** gene, individual carrying the TT homozygous wild-type was statistically lower in cases than in controls (46.67% vs. 67.33%). The frequency distribution of TC heterozygous genotype was found to have 2.40 times higher risk of developing prednisolone resistance compared to the TT homozygous wild-type and it was statistically significant (OR = 2.40, 95% Cl = 1.07 to 5.40, p = 0.033). The CC mutant homozygous genotype was not significantly associated with prednisolone resistance though it possessed 1.80 times increased chance to develop prednisolone resistance NS (p = 0.609). On the other hand, the distribution of TC+CC together was found to have 2.36 times higher risk to develop prednisolone resistance and it was statistically significant (OR = 2.36, 95% Cl = 1.06 to 5.21, p = 0.034). The percentage of C allele was higher in cases (28.33%) compared with controls (17.67%) that also increased the prednisolone resistance risk non-significantly (p = 0.059) (Table 3.24).

To the best of our knowledge, no previous genetic studies or any other citation of rs10482634 polymorphism based on the NS was found. It was the first time reported genetic study in the world. So, we have found no studies like this to compare our findings. But we had tried to compare our findings with other polymorphisms of the NR3C1 gene on different ethnic groups. So far, only a few studies have evaluated the role of the NR3C1 polymorphisms on the response to exogenous GCs in patients affected by NS. *In vitro* studies by Sher *et al.* (Sher *et al.,* 1994) have demonstrated that T cells from patients with glucocorticoid-resistant asthma showed a reversible cytokine-induced reduction in GR binding affinity and an irreversible reduction in GR number. This finding suggests that NR3C1 gene polymorphisms affecting its affinity to glucocorticoids can play an important role in the response to glucocorticoids treatment. Alternatively, other cases of SRNS have an immunological basis, i.e. a disorder of T cells. T cells in these patients promote the production of an elusive circulating factor that alters the glomerular permeability of the filtration barrier (Antignac, 2002). *In vitro* studies by Russcher *et al.* (Russcher *et al.,* 2005) have demonstrated that two polymorphisms of ER22/23EK and N363S of the NR3C1 gene directly affected glucocorticoid-regulated gene expression, which was confirmed in clinical studies demonstrating that patients with the ER22/23EK allele were relatively more resistant to the effects of glucocorticoids with respect to the sensitivity of the adrenal feedback

mechanism than non-carriers, resulting in a better metabolic health profile (van Rossum *et al.,* 2002). However, the exact influence of these polymorphisms in the NR3C1 gene remains to be controversial. In addition, several GR protein isoforms are generated through alternative splicing: the most abundant and functionally active isoform is GRα, whereas GRβ is the inactive protein, unable to bind the ligand that exerts a dominant-negative effect on GRα. The GR-9β polymorphism has been associated with increased expression of the mature GR-β protein and implicated in steroid-resistance in several diseases (Honda *et al.,* 2000; Fujishima *et al.,* 2009; Lewis-Tuffin *et al.,* 2006; Zhang *et al.,* 2005; Zhang *et al.,* 2007). Liu *et al.* (Liu *et al.,* 2001) reported that in patients with INS, an increased expression of GRβ had been demonstrated in peripheral blood mononuclear cells of steroid-resistant patients, while Szilagyi *et al.* (Szilagyi *et al.,* 2009) found that the expression of the functional isoform GRα was correlated with a positive steroid-response (steroid-responders vs. partial- and non-responders, $p < 0.01$). In a study, De ludicibus *et al.* (De Iudicibus *et al.,* 2011) showed that single nucleotide polymorphisms such as TthIIII (rs10052957), ER22/23EK (rs6189/rs6190) and GR-9 (rs6198), have been related to a reduced sensitivity to endogenous and exogenous GCs. Furthermore, different investigators have demonstrated that mutations in the NR3C1 gene were associated with both familial steroid-resistance (Bray *et al.,* 2003) and acquired steroid-resistance in some diseases, such as cushing's disease (Karl *et al.,* 1996), leukemia (Hillmann *et al.,* 2000), lupus nephritis (Jiang *et al.,* 2001), and female pseudohermaphroditism (Mendonca *et al.,* 2002). Therefore, from the studies, we could hypothesize that variants of the rs10482634 SNP of the NR3C1 gene were associated with prednisolone resistance in childhood NS. Our study revealed that individuals carrying TC heterozygous genotype and combined TC heterozygous and CC mutant variant TC+CC were significantly responsible for developing prednisolone resistance in our study population by affecting its binding affinity to prednisolone and the resistance is due to mutation of the rs10482634 SNP of NR3C1 gene. NR3C1 polymorphisms could cause damage to the normal GR function. Polymorphism of NR3C1 might be involved in the development of prednisolone resistance in our study population. Although intensive efforts have been undertaken to study the associations between SRNS and renal pathology changes, pharmacokinetics of GC, densities of GC receptor and binding affinities of GR (Rostin *et al.,* 1990; Haack *et al.,* 1999; De Carvalho *et al.,* 2004), the molecular mechanisms underlying steroid-resistance in sporadic NS with a

disorder of T cells have not yet been elucidated entirely. There are also several mechanisms that may be important to resistance and susceptibility to glucocorticoids, including reduction of the number of glucocorticoid receptors and/or a reduced affinity for the ligand, polymorphisms in GR genes, and differences in other genes that modify the glucocorticoid response (transcription factors, post-translational modifications and the activity of GRb) (Buttgereit *et al.,* 2005).

In this current study, we assessed another SNP in the Bangladeshi population for the first time, since the role of **rs6877893** polymorphism of the **NR3C1** gene in relation to prednisolone resistance risk in childhood nephrotic syndrome had not yet been reported. our study revealed that AG heterozygous genotype and GG mutant homozygous genotypes had 1.38 and 1.29 times increased risk of prednisolone resistance, whereas AG+GG combined genotype had 1.29 times greater risk of prednisolone resistance in childhood nephrotic syndrome compared with AA homozygous wild-type. The analysis showed no significant association between the different genotypes of rs6877893 and the risk of prednisolone resistance in NS ($p > 0.05$). The percentage of G allele was higher in cases (38.34%) compared with controls (32.67%) that also increased the prednisolone resistance risk non-significantly ($p = 0.397$) (Table 3.25).

We have found only two studies regarding rs6877893 SNP of the NR3C1 gene on the Chinese population. Yang *et al.,* (Yang *et al.,* 2012) analyzed upon 128 cases with infantile spasms and 131 controls on Chinese children, they reported that the haplotype TG of two SNPs (rs6877893 and rs4912905) of NR3C1 gene was associated with a decreased risk of infantile spasms ($p = 0.038$, OR = 0.66, 95% CI = 0.45 to 0.98) and rs6877893 SNP was also associated with the responsiveness of adrenocorticotropic hormone. However, another study conducted by Yang *et al.* (Yang *et al.,* 2019) on Chinese population, it was a case-control study comprising 133 high-altitude pulmonary edema patients (HAPE-p) and 135 matched Han Chinese resistant to HAPE (HAPE-r) and they found 12 SNPs showed a significant difference between the HAPE-p and HAPE-r groups. In the allelic model analysis, they found that the allele "A" of rs17287745, rs17209237, rs17209251, rs6877893 and rs1866388; the allele "C" of rs6191, rs6188 and rs2918417; the allele "T" of rs33388 and rs4634384; and the allele "G" of rs41423247 and rs10052957 were associated with increased the risk of HAPE. In

the genetic model analysis, they also showed that rs17287745, rs6191, rs6188, rs33388, rs2918417, rs6877893, rs1866388, rs41423247, rs4634384 and rs10052957 were relevant to the increased HAPE risk under the dominant model. These findings provide new evidence for the association between SNPs in NR3C1 and an increased risk of HAPE in the Chinese population. NR3C1 polymorphisms were associated with the susceptibility to HAPE in Han Chinese. In this study, the A and G allele frequency of both HAPE patient and control group were 81.6%, 18.4% vs. 71.4%, 28.6%, respectively whereas, in our study, the A and G allele frequency of both cases and controls were 61.66%, 38.34% vs. 67.33%, 32.67%, respectively.

In our study, we did not find any significant association between rs6877893 SNP and prednisolone resistance in childhood nephrotic syndrome ($p > 0.05$). Recently, there have been several studies to analyze no association of different polymorphisms of the NR3C1 gene and steroid-resistance among different diseases. For example, Tissing *et al.* (Tissing *et al.,* 2005) demonstrated that ER22/ 23EK, N363S, BclI polymorphisms of the NR3C1 gene were not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. However, Ye *et al.* (Ye *et al.,* 2006) reported no significant association between NR3C1 haplotypes and steroid-resistance in Chinese children with sporadic NS in a case-control study comprising of 35 children with sporadic steroid-resistance nephrotic syndrome cases and 83 controls with sporadic steroid-sensitive nephrotic syndrome, which suggests that NR3C1 may not be a causative gene in Chinese children with sporadic steroid-resistance nephrotic syndrome. It should be noted that among the children with SRNS in this study, most had marked structural abnormalities on renal biopsy. Their impaired steroid-response might have been due to profound injury to the glomerular barrier, rather than to generalized glucocorticoid resistance on a genetic level (Ye *et al.,* 2006). In addition, the BclI polymorphism in the NR3C1 gene has been also analyzed by Cho and co-workers (Cho *et al.,* 2009) in 190 Korean children with INS and 100 controls, but no correlation with the development of INS, onset age, initial steroid responsiveness, renal pathologic findings and the progression of renal disease was found. The authors have also examined two other SNPs in the NR3C1 gene, namely ER22/23EK and N363S, but no variant allele was found in any of the patients or control subjects. While among these studies, the distribution of intron B polymorphisms in children in Poland is compatible with that in adults in the UK (Zalewski *et al.,* 2008;

Stevens *et al.,* 2004). In another study of pediatric steroid-responsive nephrotic syndrome from Poland, no associations of polymorphic genotypes and alleles of the glucocorticoid receptor gene (BclI, rs33389, and rs33388) with nephrotic syndrome were observed. The distribution of individual polymorphisms and three marker haplotypes was similar in healthy children and SRNS patients, but haplotype BclI G, rs33389 T, rs33388 A was associated with a higher glucocorticoid sensitivity (Zalewski *et al.,* 2008). Recently, Teeninga *et al.* (Teeninga *et al.,* 2014) have evaluated GR- 9β, TthIIII and BclI polymorphisms of the NR3C1 gene in a well-defined cohort of 113 children with INS, showing that carriers of GR-9β+TthIIII mutated haplotype had a significantly higher incidence of steroid dependence compared with non-carriers (52% vs. 25%, OR = 3.04, 95% CI = 1.37 to 6.74, log-rank test p = 0.003). While, in another study, De ludicibus *et al.* (De Iudicibus *et al.*, 2007) reported that other NR3C1 SNPs such as N363S (rs6195) and BclI (rs41423247) have been related to an increased sensitivity to endogenous and exogenous GCs. Whereas, a study conducted by Koper *et al.* (Koper *et al.,* 1997) in the Netherlands upon adult population observed no association between five polymorphisms in the NR3C1 gene and GC resistance. Similarly, in our study, we also found no significant association between rs6877893 polymorphism and prednisolone resistance in Bangladeshi children with NS.

In our present study, we studied two polymorphisms of rs10482634 and rs6877893 of the NR3C1 gene, these data suggested that the rs10482634 SNP, not rs6877893 SNP of the NR3C1 gene significantly affected the prednisolone resistance in Bangladeshi NS children.

In addition, we also investigated the correlation between the **CYP3A5*3 (rs776746)** polymorphism of the CYP3A5 gene and prednisolone resistance in children with NS. To the best of our knowledge, no pharmacogenetic study of CYP3A5 genes had been reported on the Bangladeshi population relating those with prednisolone resistance in nephrotic syndrome. We reported this study for the first time in Bangladesh.

Our results revealed that compared with the AA (Pro/Pro) normal homozygous genotype of CYP3A5*3 SNP of the CYP3A5 gene, AG (Pro/Thr) heterozygous and GG (Thr/Thr) mutant homozygous genotypes had 0.57 and 0.87 times lower chance of developing

prednisolone resistance. Whereas, AG + GG combined genotype had 0.72 times lower chance of developing prednisolone resistance in childhood nephrotic syndrome compared with AA (Pro/Pro) normal homozygous genotype. But these results were not statistically significant ($p > 0.05$). The percentage of G allele was slightly higher in cases (65%) compared with controls (64.33%) that also increased the prednisolone resistance risk non-significantly ($p = 0.922$) (Table 3.26).

The human CYP3A subfamily catalyzes the oxidative, peroxidative and reductive metabolism of structurally diverse endobiotic and drugs including prednisolone (Lamba *et al.,* 2002). Substantial interindividual differences in CYP3A expression (Watkins, 1995) contribute significantly to the variation in oral bioavailability and systemic clearance of CYP3A substrates. Within, our study we also investigated the correlation between the CYP3A5*3 polymorphism of the CYP3A5 gene and steroid-resistance in children with NS. The human CYP3A5 gene encodes a member of the cytochrome P450 3A subfamily of enzymes, which plays an important role in drug metabolism, including that of prednisolone (Thummel and Wilkinson, 1998; Lamba *et al.,* 2002; Lin *et al.,* 2002; Pichard *et al.,* 1992). The G allele of CYP3A5*3 SNP causes alternative splicing and produces a truncated protein with a loss of enzyme activity (Kuehl *et al.,* 2001). Polymorphic expression of CYP3A5 may account for some of the interindividual variations in the clearance of CYP3A substrates. Our results showed the association between CYP3A5*3 and steroid-resistance, but this association did not reach statistical significance (OR = 1.03, 95% CI = 0.58 to 1.84; $p = 0.922$). This lack of statistical significance might be due to the relatively small population studied. Thus, a larger number of subjects might be needed to confirm the association. In this study, the frequency of the G allele was slightly higher in PRNS cases (65%) than in PSNS controls (64.33%) that also increased the prednisolone resistance risk non-significantly ($p = 0.922$).

Previous studies have shown that renal transplant recipients with the G allele of CYP3A5*3 SNP displayed higher blood tacrolimus concentrations and required a lower dosage (i.e., the patients were more sensitive) than those with the A allele of CYP3A5*1 SNP (Tada *et al.,* 2005; Ferraris *et al.,* 2011). Thus, it may be expected that the frequency of the G allele would be lower in SR patients than in SS patients. However, a

comparative pharmacokinetic study showed that the pharmacokinetics of prednisolone, unlike that of tacrolimus, was not significantly different between CYP3A5 expressers and non-expressers (Miura *et al.,* 2008). Chiou *et al.* (Chiou *et al.,* 2012) investigated that polymorphic expression of CYP3A5 in 74 children with INS: the frequency of the G allele of A6986G SNP was relatively higher in steroid-resistant subjects (84%) than in steroid-sensitive ones (67%) showing a trend of association that however did not reach statistical significance (OR = 2.63, 95% CI = 0.94 to 7.37; p = 0.059). Our study revealed that the frequency of G allele in PRNS and PSNS were 65% and 64.33%, respectively and we found G allele frequency was slightly higher in PRNS cases than PSNS controls and our results showed trend of association to prednisolone resistance but it did not reach statistically significant (OR = 1.03, 95% CI = 0..58 to 1.84; p = 0.922). So our findings are consistent with this Chiou *et al.* findings. In another study on Tunisian children, Moussa *et al.* (Moussa *et al.,* 2017) reported that the A6986G (CYP3A5) polymorphisms showed a trend of association to GC resistance in NS but these associations did not reach statistical significance (OR = 2.11, 95% CI = 0.53 to 8.38, p = 0.28). These findings were also consistent with our study. The frequency distributions of A and G allele of CYP3A5*3 SNP of our study are consistent with the findings of Moussa *et al.,* Islam *et al.,* and Li *et al.* (Moussa *et al.,* 2017; Islam *et al.,* 2014; Li *et al.,* 2018).

There were several studies of CYP3A5*3 polymorphism conducted on kidney patients in different ethnic groups. Like, barbiturates, carbamazepine, phenytoin, rifampicin (Czock *et al.,* 2005; Frey and Frey, 1990) and HIV protease inhibitors (Penzak *et al.,* 2005) cause CYP3A induction and therefore increase the clearance of prednisolone, while ciclosporin inhibits enzyme activity. Moreover, ciclosporin may increase the absorption of prednisolone, causing both increased availability and decreased clearance (Czock *et al.,* 2005). The latter findings were of clinical relevance in children with NS, as they might explain the beneficial effect from prednisolone combined with ciclosporin for frequent relapse nephrotic syndrome, which had been shown in clinical studies (Hodson *et al.,* 2008). However, a recent study done by Li *et al.* (Li *et al.,* 2018) on the Chinese adult population found no significant correlation between CYP3A4, CYP3A5 (CYP3A5*3) genotype and clinical efficacy of tacrolimus in the treatment of NS patients. A study on Brazilian kidney transplant patients, Cusinato *et al.* (Cusinato *et al.,*

2014) reported that the CYP3A5*3 allele was highly associated with higher tacrolimus bioavailability in renal transplant patients, confirming previous findings and supporting the idea that CYP3A5 was the major enzyme responsible for the marked interindividual variability in tacrolimus pharmacokinetics. On the other hand, Thervet *et al*. (Thervet *et al*., 2010) conducted a prospective clinical trial involving 280 renal transplant recipients, demonstrated that pretransplant tacrolimus dose adaptation according to CYP3A5 genotype resulted in the more rapid achievement of target Co and fewer dose modifications. However, in another study, Kurzawski *et al*. (Kurzawski *et al.,* 2014) suggested that the CYP3A5 gene was an important factor in determining the dose requirement for tacrolimus and individuals with CYP3A5*1 allele had higher tacrolimus metabolism and lower blood tacrolimus concentration. In other tissues, CYP3A5 had also been shown to play an important endogenous function and CYP3A5*3 had been shown to influence the systolic blood and pulse pressure, presumably by altering CYP3A5-mediated glucocorticoid metabolism (Kreutz *et al*., 2005). In a study on Bangladeshi lung cancer patients, Islam *et al.* (Islam *et al.,* 2014) found a non-significant increased risk of lung cancer with CYP3A5*3 polymorphism and a similar result was also found in Caucasians (Dally *et al.,* 2003) and these findings are consistent with our study population. Another study done by Bellah *et al.* (Bellah *et al.,* 2015) revealed a significant association of CYP3A5*3 gene polymorphism with prostate cancer risk in Bangladeshi subjects.

This study highlights the genetic factors that are associated with steroid-resistance and may be useful for the treatment of children with PRNS. For example- our findings suggest that patients with GT heterozygous genotype of 2677G>T polymorphism, CC mutant homozygous genotypes of 3435T>C polymorphism of the ABCB1 gene, TC heterozygous and combined heterozygous and mutant homozygous TC+CC genotype of rs10482634 polymorphism of the NR3C1 gene are significantly associated with increased risk for the development of prednisolone resistance in childhood NS. There were found no significant association of 1236T>C, rs6877893 and CYP3A5*3 SNPs with prednisolone resistance NS. Our limitations of the present study are SNPs of ABCB1, NR3C1 and CYP3A5 genes were investigated upon a relatively small number of patients. So, more extensive multicentric studies and studies across other ethnic groups are needed to elucidate the contradictory implications of SNPs of the ABCB1,

NR3C1 and CYP3A5 genes with PRNS in children. For a better understanding of the correlation between these gene polymorphisms, allele frequency and disease conditions, large cohort studies in different areas need to be conducted. A better understanding of the molecular mechanisms of the disease may also yield new information about etiology and will be helpful in developing targeted therapies against the disease. Despite these limitations, this is the first time study on the Bangladeshi NS children and our results are also promising. We are also successful to develop PCR-RFLP method for detection the SNPs of NR3C1 gene.

CHAPTER FOUR

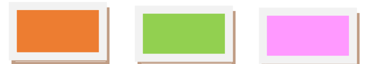
CONCLUSION

4. CONCLUSION

Nephrotic syndrome is a common clinical condition in Asian children. There is a racial variation in susceptibility with a reported incidence in Asian children of 9-16/100,000 in comparison to 2 to 7 children in the USA, 2-4 new cases /100,000 children in the UK. Mortality was observed 50/1000 before starting antibiotic and steroid, which is now 5/1000. Children with nephrotic syndrome aged 1-18 years are unable to achieve a complete remission with corticosteroid therapy within 8 weeks are termed as steroid-resistance nephrotic syndrome. Approximately 10-20% will be classified as steroid-resistant. Children diagnosed with SRNS (initial or late) should undergo renal biopsy before starting specific treatment. The optimal treatment of SRNS remains controversial. SRNS can be managed well with various immunosuppressant drugs such as cyclosporine, cyclophosphamide, mycophenolate mofetil and rituximab. About 10-20% of patients with familial and sporadic SRNS and these patients are usually unresponsive to immunosuppressive medications, progress rapidly to end-stage renal disease. If facilities exist, a mutational analysis should be offered to patients with SRNS. In our present study, we examined a total of six polymorphisms of the ABCB1, NR3C1 and CYP3A5 genes. Those are, three polymorphisms of the ABCB1 gene 1236T>C (rs1128503), 2677G>T (rs2032582), 3435T>C (rs1045642), two polymorphisms of the NR3C1 gene rs10482634, rs6877893 and one CYP3A5*3 (rs774646) polymorphism of the CYP3A5 gene. Therefore, ABCB1, NR3C1 and CYP3A5 genes are having a key role in the drug transporter pathway, drug-receptor binding and drug metabolic pathway and in our current study we had tried to evaluate their effects on the association with prednisolone resistant in childhood nephrotic syndrome patients. From our study, we observed that patient carrying GT heterozygous genotype of 2677G>T allele, CC mutant homozygous genotype of 3435T>C allele of the ABCB1 gene and TC heterozygous genotype and combined heterozygous and mutant homozygous TC+CC genotype of rs10482634 polymorphism of the NR3C1 gene are significantly associated with prednisolone resistance in childhood nephrotic syndrome. However, other SNPs of 1236T>C of the ABCB1 gene, rs6877893 of the NR3C1 gene and CYP3A5*3 of the CYP3A5 gene were not significantly associated with prednisolone resistance in childhood nephrotic syndrome in the Bangladeshi population. So, our

findings open some windows in a further wide range of researches in the field of personalized medicine. This was the first genetic study to demonstrate the association of ABCB1, NR3C1 and CYP3A5 genes polymorphism with NS and resistance to prednisolone treatment in Bangladeshi children with a limited number of cases and controls. Overall, our results demonstrated that different genotypes of ABCB1, NR3C1 and CYP3A5 genes restriction sites were associated with steroid-resistance in childhood NS, suggesting that these genotypes might be associated with some factors which participate in the causation of steroid-resistance. This would both improve drug efficacy and prevent adverse effects, particularly drugs like steroids with multiple toxicities. Further studies in larger cohorts of cases and controls are needed to test the hypothesis that ABCB1, NR3C1 and CYP3A5 variants are associated with steroid-resistance in NS. Though this study had some limitation as the number of patients were small and only six SNPs of three genes are considered, but it opens scope for further research in personalized medicine. From the study, our suggestion is- before starting the treatment of NS, it is essential to find out the types of disease by genetic study then after investigation we can give the patient a safer and effective individualized treatment plan to save many precious lives. Patients' medical costs will be minimized which will contribute economically to developing countries like Bangladesh. The national health budget can cover more people by saving money through a pharmacogenetic application like this.

REFERENCES



REFERENCES

Alam MK, Hossain D, Khan AH, Yasmeen BN, Alam MU, Afroz S, Quader MU, Hasan AS, Talukder SA. Renal Histopathology in Childhood Nephrotic Syndrome at National Institute of Kidney Diseases and Urology, Dhaka, Bangladesh an experience of one decade. Northern International Medical College Journal. 2016; 8(1): 165-9.

Antignac C. Genetic models: clues for understanding the pathogenesis of idiopathic nephrotic syndrome. The Journal of clinical investigation. 2002 Feb 15; 109(4): 447-9.

Arneil GC. The nephrotic syndrome. Pediatr Clin NorthAm. 1971; 18: 547-559.

Bagga A. Management of steroid resistant nephrotic syndrome. Indian pediatrics. 2009 Jan 1; 46(1).

Bagga A, Srivastava RN. Nephrotic syndrome. In: Srivastava RN. Bagga A (eds). Pediatric Nephrology. 5th edition. New Delhi India: Joypee Brothers Medical Publishers Ltd. 2011; 195-234.

Bagga A, Sinha A, Gulati A, Steroid Resistant Nephrotic Syndrome: Protocols in Pediatric Nephrology. 1st edition.CBS Publishers & Distributors Pvt. Ltd. New Delhi. 2012; 110002: 97.

Baxter JD, Rousseau GG. Glucocorticoid hormone action: an overview. Monographs on endocrinology. 1979; 12: 1-24.

Beck J, Handgretinger R, Dopfer R, Klingebiel T, Niethammer D, Gekeler V. Expression of mdrl, mrp, topoisomerase IIα/β and cyclin A in primary or relapsed states of acute lymphoblastic leukaemias. British journal of haematology. 1995 Feb; 89(2): 356-63.

Bellah SF, Ahmed MU, Rabbi SN, Apu MN, Islam MS, Uddin MM, Islam MS, Hasnat A. Prostate Cancer Risk in Relation to CYP3A4 and CYP3A5 Genotypes in the Bangladeshi Population. Dhaka University Journal of Pharmaceutical Sciences. 2015; 14(2): 179-85.

Bierzynska A, McCarthy HJ, Soderquest K, Sen ES, Colby E, Ding WY, Nabhan MM, Kerecuk L, Hegde S, Hughes D, Marks S, Feather S, Jones C, Webb NJ, Ognjanovic M, Christian M, Gilbert RD, Sinha MD, Lord GM, Simpson M, Koziell AB, Welsh GI, Saleem MA. Genomic and clinical profiling of a national nephrotic syndrome cohort advocates a precision medicine approach to disease management. Kidney Int. 2017; 91: 937–947.

Bierzynska A, Saleem M. Recent advances in understanding and treating nephrotic syndrome. F1000 Research. 2017; 6.

Blackstock WP, Weir MP. Proteomics: quantitative and physical mapping of cellular proteins. Trends in biotechnology. 1999 Mar 1; 17(3): 121-7.

Bonilla-Felix M, Parra C, Dajani T, Ferris M, Swinford RD, Portman RJ, Verani R. Changing patterns in the histopathology of idiopathic nephrotic syndrome in children. Kidney international. 1999 May 1; 55(5): 1885-90.

Bourgeois S, Gruol DJ, Newby RF, Rajah FM. Expression of an mdr gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Molecular Endocrinology. 1993 Jul 1; 7(7): 840-51.

Bray PJ, Cotton RG. Variations of the human glucocorticoid receptor gene (NR3C1): pathological and *in vitro* mutations and polymorphisms. Human mutation. 2003 Jun; 21(6): 557-68.

Brodehl J, Krohn HP, Ehrich JH. The treatment of minimal change nephrotic syndrome (lipoidnephrosis): cooperative studies of the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN). Klinische Pädiatrie. 1982 May; 194(03): 162-5.

Brophy NA, *et al*. MDR1 gene expression in childhood acute lymphoblastic leukemias and lymphomas: a critical evaluation by four techniques. Leukemia. 1994; 8: 327-335.

Brumfield RT, Beerli P, Nickerson DA, Edwards SV. The utility of single nucleotide polymorphisms in inferences of population history. Trends in Ecology & Evolution. 2003 May 1; 18(5): 249-56.

Buttgereit F, Saag KG, Cutolo M, da Silva JA, Bijlsma JW. The molecular basis for the effectiveness, toxicity and resistance to glucocorticoids: focus on the treatment of rheumatoid arthritis. Scandinavian journal of rheumatology. 2005 Feb 1; 34(1): 14-21.

Cameron JS. Nephrotic syndrome. British medical journal. 1970 Nov 7; 4(5731): 350-353.

Campbell DA, Valdes A, Spurr N. Making drug discovery a SN (i) P. Drug Discovery Today. 2000 Sep 1; 5(9): 388-96.

Carson PE, Flanagan CL, Ickes CE, Alving AS. Enzymatic deficiency in primaquine-sensitive erythrocytes. Science. 1956 Sep 14; 124(3220): 484-5.

Cascorbi I, Gerloff T, Johne A, Meisel C, Hoffmeyer S, Schwab M, Schaeffeler E, Eichelbaum M, Brinkmann U, Roots I. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clinical Pharmacology & Therapeutics. 2001 Mar; 69(3): 169-74.

Chiou YH, Wang LY, Wang TH, Huang SP. Genetic polymorphisms influence the steroid treatment of children with idiopathic nephrotic syndrome. Pediatric Nephrology. 2012 Sep 1; 27(9): 1511-7.

Choi HJ, Cho HY, Ro H, Lee SH, Han KH, Lee H, Kang HG, Ha IS, Choi Y, Cheong HI. Polymorphisms of the MDR1 and MIF genes in children with nephrotic syndrome. Pediatric Nephrology. 2011 Nov 1; 26(11): 1981.

Cho HY, Choi HJ, Lee SH, Lee HK, Kang HK, Ha IS, Choi Y, Cheong HI. Polymorphisms of the NR3C1 gene in Korean children with nephrotic syndrome. Korean Journal of Pediatrics. 2009 Nov 15; 52(11): 1260-6.

Chrousos GP, Detera-Wadleigh SD, Karl M. Syndromes of glucocorticoid resistance. Annals of Internal Medicine. 1993 Dec 1; 119(11): 1113-24.

Cizmarikova M, Podracka L, Klimcakova L, Habalova V, Boor A, Mojzis J, Mirossay L. MDR1 polymorphisms and idiopathic nephrotic syndrome in Slovak children: preliminary results. Medical science monitor: international medical journal of experimental and clinical research. 2015; 21: 59.

Conyers R, Devaraja S, Elliott D. Systematic review of pharmacogenomics and adverse drug reactions in paediatric oncology patients. Pediatric blood & cancer. 2018 Apr; 65(4): e26937.

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, Biedler JL, Melamed MR, Bertino JR. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proceedings of the National Academy of Sciences. 1989 Jan 1; 86(2): 695-8.

Cusinato DA, Lacchini R, Romao EA, Moysés-Neto M, Coelho EB. Relationship of CYP3A5 genotype and ABCB1 diplotype to tacrolimus disposition in Brazilian kidney transplant patients. British journal of clinical pharmacology. 2014 Aug 1; 78(2): 364-72.

Curran ME. Potassium ion channels and human disease: phenotypes to drug targets? Current opinion in biotechnology. 1998 Dec 1; 9(6): 565-72.

Czock D, Keller F, Rasche FM, Häussler U. Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids. Clinical pharmacokinetics. 2005 Jan 1; 44(1): 61-98.

Daly AK, Monkman SC, Smart J, Steward A, Choletron S. Analysis of Cytochrome P450 polymorphisms. Meth Mol Biol 1998; 107: 405-22.

Dally H, Edler L, Jäger B, Schmezer P, Spiegelhalder B, Dienemann H, Drings P, Schulz V, Kayser K, Bartsch H, Risch A. The CYP3A4* 1B allele increases risk for small cell lung cancer: effect of gender and smoking dose. Pharmacogenetics and Genomics. 2003 Oct 1; 13(10): 607-18.

De Carvalho AP, Carlotti P, Franco PB, Elias LL, Facincani I, Lourenço E, Costa C, Foss N, Moreira AC, De Castro M. Glucocorticoid receptors, *in vitro* steroid sensitivity, and cytokine secretion in idiopathic nephrotic syndrome. Kidney international. 2004 Feb 1; 65(2): 403-8.

De Iudicibus S, Stocco G, Martelossi S, Drigo I, Norbedo S, Lionetti P, Pozzi E, Barabino A, Decorti G, Bartoli F, Ventura A. Association of BclI polymorphism of the glucocorticoid receptor gene locus with response to glucocorticoids in inflammatory bowel disease. Gut. 2007 Sep 1; 56(9): 1319-20.

De Iudicibus S, Stocco G, Martelossi S, Londero M, Ebner E, Pontillo A, Lionetti P, Barabino A, Bartoli F, Ventura A, Decorti G. Genetic predictors of glucocorticoid response in pediatric patients with inflammatory bowel diseases. Journal of clinical gastroenterology. 2011 Jan 1; 45(1): e1-7.

Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. Journal of lipid research. 2001 Jul 1; 42(7): 1007-17.

Den Boer ML, Pieters R, Kazemier KM, Rottier MM, Zwaan CM, Kaspers GJ, Janka-Schaub G, Henze G, Creutzig U, Scheper RJ, Veerman AJ. Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. Blood. 1998 Mar 15; 91(6): 2092-8.

Dhandapani MC, Venkatesan V, Rengaswamy NB, Gowrishankar K, Nageswaran P, Perumal V. Association of ACE and MDR1 gene polymorphisms with steroid resistance in children with idiopathic nephrotic syndrome. Genetic testing and molecular biomarkers. 2015 Aug 1; 19(8): 454-6.

Dilger K, Schwab M, Fromm MF. Identification of budesonide and prednisone as substrates of the intestinal drug efflux pump P-glycoprotein. Inflammatory bowel diseases. 2004 Sep 1; 10(5): 578-83.

Donn R, Payne D, Ray D. Glucocorticoid receptor gene polymorphisms and susceptibility to rheumatoid arthritis. Clin Endocrinol. 2007; 67: 342-5.

Eddy AA, Symons JM. Nephrotic syndrome in childhood. The lancet. 2003 Aug 23; 362(9384): 629-39.

Enabah D, El Baz H, Moselhy H. Higher frequency of C. 3435 of the ABCB1 gene in patients with tramadol dependence disorder. The American journal of drug and alcohol abuse. 2014 Jul 1; 40(4): 317-20.

Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. Science. 1999 Oct 15; 286(5439): 487-91.

Evans DA, Manley KA, McKusick VA. Genetic control of isoniazid metabolism in man. British medical journal. 1960 Aug 13; 2(5197): 485.

Fan Q, Xing Y, Ding J, Guan N, Zhang J. The relationship among nephrin, podocin, CD2AP and α-actinin might not be a true ‘interaction’in podocyte. Kidney international. 2006 Apr 1; 69(7): 1207-15.

Farrell RJ, Murphy A, Long A, Donnelly S, Cherikuri A, O'Toole D, Mahmud N, Keeling PW, Weir DG, Kelleher D. High multidrug resistance (P-glycoprotein 170) expression in inflammatory bowel disease patients who fail medical therapy. Gastroenterology. 2000 Feb 1; 118(2): 279-88.

Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P. Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. Trends in cell biology. 2007 Sep 1; 17(9): 428-37.

Feehally J, Kendell NP, Swift PG, Walls J. High incidence of minimal change nephrotic syndrome in Asians. Archives of disease in childhood. 1985 Nov 1; 60(11): 1018-20.

Fenech AG. Pharmacogenetics: the path to personalized prescribing. Jurnal of the Malta College of Pharmacy Practise. 2007; 13.

Ferraris JR, Argibay PF, Costa L, Jimenez G, Coccia PA, Ghezzi LF, Ferraris V, Belloso WH, Redal MA, Larriba JM. Influence of CYP3A5 polymorphism on tacrolimus maintenance doses and serum levels after renal transplantation: age dependency and pharmacological interaction with steroids. Pediatric transplantation. 2011 Aug; 15(5): 525-32.

Frey BM, Frey FJ. Clinical pharmacokinetics of prednisone and prednisolone. Clinical pharmacokinetics. 1990 Aug 1; 19(2): 126-46.

Frey FJ, Schnetzer A, Horber FF, Frey BM. Evidence that cyclosporine does not affect the metabolism of prednisolone after renal transplantation. Transplantation. 1987 Apr; 43(4): 494-8.

Fujishima SI, Takeda H, Kawata S, Yamakawa M. The relationship between the expression of the glucocorticoid receptor in biopsied colonic mucosa and the glucocorticoid responsiveness of ulcerative colitis patients. Clinical Immunology. 2009 Nov 1; 133(2): 208-17.

Funaki S, Takahashi S, Wada N, Murakami H, Harada K. Multiple drug-resistant gene 1 in children with steroid-sensitive nephrotic syndrome. Pediatrics International. 2008 Apr; 50(2): 159-61.

Gallon L, Leventhal J, Skaro A, Kanwar Y, Alvarado A. Resolution of recurrent focal segmental glomerulosclerosis after retransplantation. New England Journal of Medicine. 2012 Apr 26; 366(17): 1648-9.

Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, O'SHEA JJ, CHROUSOS GP, BORNSTEIN SR. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. The FASEB journal. 2002 Jan; 16(1): 61-71.

Ganesan A, Mini Jacob S, Arvind Selvin Kumar R, Padmaraj R, Anandan B, Sambantham S, Balakrishnan D, Sundaram A, Krishnakumar S, Valarmathi S, Geethalakshmi S, Venkataraman P. Identification Of Functional Single Nucleotide Polymorphisms Of Multidrug Resistance Gene-1 Among Nephrotic Syndrome Children In South India. Asian J Pharm Clin Res. 2017; 10 (2): 418-422.

Gee HY, Saisawat P, Ashraf S, *et al.* ARHGDIA mutations cause nephrotic syndrome via defective RHO GTPase signaling. J Clin Invest. 2013; 123(8): 3243–3253.

Germann UA, Pastan I, Gottesman MM. P-glycoproteins: mediators of multidrug resistance. InSeminars in cell biology: Academic Press. 1993 Feb 1; 4(1): 63-76).

Gipson DS, Massengill SF, Yao L, Nagaraj S, Smoyer WE, Mahan JD, Wigfall D, Miles P, Powell L, Lin JJ, Trachtman H. Management of childhood onset nephrotic syndrome. Pediatrics. 2009 Aug 1; 124(2): 747-57.

Gordillo R, Spitzer A. The nephrotic syndrome. Pediatrics in review. 2009 Mar 1; 30(3): 94.

Gray IC, Campbell DA, Spurr NK. Single nucleotide polymorphisms as tools in human genetics. Human molecular genetics. 2000 Oct 1; 9(16): 2403-8.

Gross KL, Lu NZ, Cidlowski JA. Molecular mechanisms regulating glucocorticoid sensitivity and resistance. Molecular and cellular endocrinology. 2009 Mar 5; 300(1-2): 7-16.

Guo Y, Jamison DC. The distribution of SNPs in human gene regulatory regions. Bmc Genomics. 2005 Dec; 6(1): 140.

Haack D, Schärer K, Asam-Tauscher A, Vecsei P. Glucocorticoid receptors in idiopathic nephrotic syndrome. Pediatric Nephrology. 1999 Sep 1; 13(8): 653-6.

Hakkola J, Pelkonen O, Pasanen M, Raunio H. Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity. Critical reviews in toxicology. 1998 Jan 1; 28(1): 35-72.

Han SH, Park SY, Li JJ, Kwak SJ, Jung DS, Choi HY, Lee JE, Moon SJ, Kim DK, Han DS, Kang SW. Glomerular glucocorticoid receptor expression is reduced in late responders to steroids in adult-onset minimal change disease. Nephrology Dialysis Transplantation. 2007 Sep 22; 23(1): 169-75.

Han SS, Xu YQ, Lu Y, Gu XC, Wang Y. A PRISMA-compliant meta-analysis of MDR1 polymorphisms and idiopathic nephrotic syndrome: susceptibility and steroid responsiveness. Medicine. 2017 Jun; 96(24).

Harris H. "The Principles of Human Biochemical Genetics, 3rd edn, rev." Elsevier. 1980; 246-256.

Heitzer MD, Wolf IM, Sanchez ER, Witchel SF, DeFranco DB. Glucocorticoid receptor physiology. Reviews in endocrine and metabolic disorders. 2007 Dec 1; 8(4): 321-30.

Hillmann AG, Ramdas J, Multanen K, Norman MR, Harmon JM. Glucocorticoid receptor gene mutations in leukemic cells acquired *in vitro* and *in vivo.* Cancer Research. 2000 Apr 1; 60(7): 2056-62.

Hinkes B, Wiggins RC, Gbadegesin R, Vlangos CN, Seelow D, Nürnberg G, Garg P, Verma R, Chaib H, Hoskins BE, Ashraf S. Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. Nature genetics. 2006 Dec; 38(12): 1397.

Hodson E, Knight JJF, Willis NS, Craig JC. Corticosteroid therapy for nephrotic syndrome in children. Cochrane Database of Systematic Reviews. 2002; 3.

Hodson EM, *et al.* Non-corticosteroid treatment for nephrotic syndrome in children. Cochrane Database Syst Rev. 2008; CD002290-doi: 10.1002/14651858.CD002290.pub3.

Hoedemaekers R, Badcott D, Gordijn B. The Advent of the Personal Pill'. Ethical Perspectives. 2001; 1: 50.

Hoffmeyer SO, Burk O, Von Richter O, Arnold HP, Brockmöller J, Johne A, Cascorbi I, Gerloff T, Roots I, Eichelbaum M, Brinkmann U. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity *in vivo.* Proceedings of the National Academy of Sciences. 2000 Mar 28; 97(7): 3473-8.

Honda M, Orii F, Ayabe T, Imai S, Ashida T, Obara T, Kohgo Y. Expression of glucocorticoid receptor β in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. Gastroenterology. 2000 May 1; 118(5): 859-66.

Hossain MM, Management of minimal change nephrotic syndrome in children with frequent relapse. In: Harun Ur Rashid (editor). South Asian renal disease, proceeding of the 2nd International Conference of nephrology Urology and transplantation society of SAARC countries, Feb, 18-20, 1997; Bangladesh: 9-20.

Hoyer PF, Vester U, Becker JU. Steroid-Resistant Nephrotic Syndrome. 2008; Chapter-16.

Huizenga NA. de LP, Koper JW, Clayton RN, Farrell WE, van der Lely AJ, Brinkmann AO, de Jong FH, Lamberts SW: Human adrenocorticotropin-secreting pituitary adenomas show frequent loss of heterozygosity at the glucocorticoid receptor gene locus. J Clin Endocrinol Metab. 1998; 83: 917-21.

Huizenga NA, de Lange P, Koper JW, de Herder WW, Abs R, Kasteren JH, de Jong FH, Lamberts SW. Five patients with biochemical and/or clinical generalized glucocorticoid resistance without alterations in the glucocorticoid receptor gene. The Journal of Clinical Endocrinology & Metabolism. 2000 May 1; 85(5): 2076-81.

Hustert E, Haberl M, Burk O, Wolbold R, He YQ, Klein K, Nuessler AC, Neuhaus P, Klattig J, Eiselt R, Koch I. The genetic determinants of the CYP3A5 polymorphism. Pharmacogenetics and Genomics. 2001 Dec 1; 11(9): 773-9.

Huusko JM, Karjalainen MK, Mahlman M, Haataja R, Kari MA, Andersson S, Toldi G, Tammela O, Rämet M, Lavoie PM, Hallman M. A study of genes encoding cytokines (IL6, IL10, TNF), cytokine receptors (IL6R, IL6ST), and glucocorticoid receptor (NR3C1) and susceptibility to bronchopulmonary dysplasia. BMC medical genetics. 2014 Dec; 15(1): 120.

Ieiri I, Takane H, Otsubo K. The MDR1 (ABCB1) gene polymorphism and its clinical implications. Clin Pharmacokinet. 2004; 43: 553–557.

International Study of Kidney Disease in Children. The primary nephrotic syndrome in children. Identification of patients with minimal change nephrotic syndrome from initial response to prednisone. J. Pediatr. 1981; 98: 561–564.

Ingelman-Sundberg M. The human genome project and novel aspects of cytochrome P450 research. Toxicology and applied pharmacology. 2005 Sep 1; 207(2): 52-6.

Islam MS, Mostofa AG, Ahmed MU, Sayeed MS, Hassan MR, Hasnat A. Association of CYP3A4, CYP3A5 polymorphisms with lung cancer risk in Bangladeshi population. Tumor Biology. 2014 Feb 1; 35(2): 1671-8.

Jafar T, Prasad N, Agarwal V, Mahdi A, Gupta A, Sharma RK, Negi MP, Agrawal S. MDR-1 gene polymorphisms in steroid-responsive versus steroid-resistant nephrotic syndrome in children. Nephrology Dialysis Transplantation. 2011 Apr 2; 26(12): 3968-74.

Jiang T, Liu S, Tan M, Huang F, Sun Y, Dong X, Guan W, Huang L, Zhou F. The phase-shift mutation in the glucocorticoid receptor gene: potential etiologic significance of neuroendocrine mechanisms in lupus nephritis. Clinica chimica acta. 2001 Nov 1; 313(1-2): 113-7.

Johnson JA. Pharmacogenetics: potential for individualized drug therapy through genetics. TRENDS in Genetics. 2003 Nov 1; 19(11): 660-6.

Kalow WB, Tang BK, Endrenyi L. Hypothesis: comparisons of inter-and intra-individual variations can substitute for twin studies in drug research. Pharmacogenetics. 1998 Aug 1; 8(4): 283-90.

Kalow W. Familial incidence of low pseudocholinesterase level. The Lancet. 1956 Sep 15; 268(6942): 576-7.

Kang HG. Treatment of steroid-resistant pediatric nephrotic syndrome. Korean journal of pediatrics. 2011 Aug; 54(8): 317.

Karl MI, Lamberts SW, Detera-Wadleigh SD, Encio IJ, Stratakis CA, Hurley DM, Accili DO, Chrousos GP. Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. The Journal of Clinical Endocrinology & Metabolism. 1993 Mar 1; 76(3): 683-9.

Karl M, *et al.* Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Am Physicians. 1996; 108: 296-307.

Karssen AM, Meijer OC, Van der Sandt IC, De Boer AG, De Lange EC, De Kloet ER. The role of the efflux transporter P-glycoprotein in brain penetration of prednisolone. Journal of endocrinology. 2002 Oct 1; 175(1): 251-60.

Kdigo GN. Work Group. KDIGO clinical practice guideline for glomerulonephritis. Kidney inter., Suppl. 2012; 2: 139-274.

Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM. A" silent" polymorphism in the MDR1 gene changes substrate specificity. Science. 2007 Jan 26; 315(5811): 525-8.

Klimecki WT, Futscher BW, Grogan TM, Dalton WS. P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood. 1994 May 1; 83(9): 2451-8.

Koper JW, Stolk RP, de Lange P, Huizenga NA, Molijn GJ, Pols HA, Grobbee DE, Karl M, de Jong FH, Brinkmann AO, Lamberts SW. Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Human genetics. 1997 Apr 1; 99(5): 663-8.

Koskimies O, Vilska J, Rapola J, Hallman N. Long-term outcome of primary nephrotic syndrome. Archives of disease in childhood. 1982 Jul 1; 57(7): 544-8.

Kozian DH, Kirschbaum BJ. Comparative gene expression analysis.Trends Biotechnol. 1999; 17: 73-78.

Kreutz R, Zuurman M, Kain S, Bolbrinker J, de Jong PE, Navis G. The role of the cytochrome P450 3A5 enzyme for blood pressure regulation in the general Caucasian population. Pharmacogenetics and Genomics. 2005; 15: 831–837.

Kroetz DL, Pauli-Magnus C, Hodges LM, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T, Carlson EJ. Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene. Pharmacogenetics and Genomics. 2003 Aug 1; 13(8): 481-94.

Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nature genetics. 2001 Apr; 27(4): 383.

Kumar V, Husain G M, Ganesan R. The role of Pharmacogenomics in drug development.Pharmainfo. 2007; 5(3).

Kuningas M, Mooijaart SP, Slagboom PE, Westendorp RG, Van Heemst D. Genetic variants in the glucocorticoid receptor gene (NR3C1) and cardiovascular disease risk. The Leiden 85-plus Study. Biogerontology. 2006 Aug 1; 7(4): 231-8.

Kurzawski M, Dąbrowska J, Dziewanowski K, Domański L, Perużyńska M, Droździk M. CYP3A5 and CYP3A4, but not ABCB1 polymorphisms affect tacrolimus dose-adjusted trough concentrations in kidney transplant recipients. Pharmacogenomics. 2014 Feb; 15(2): 179-88.

Lamba JK, Lin YS, Schuetz EG, Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. Advanced drug delivery reviews. 2002 Nov 18; 54(10): 1271-94.

Lande MB, Gullion C, Hogg RJ *et al.* Long versus standard initial steroid therapy for children with the nephrotic syndrome. A report from the Southwest Pediatric Nephrology Study Group. Pediatr Nephrol. 2003; 18: 342–346.

Lane JC, Kaskel FJ. Pediatric nephrotic syndrome: from the simple to the complex. In Seminars in nephrology. WB Saunders. 2009 Jul 1; 29(4): 389-398).

Levine SA. Nephrotic Syndrome. In: Brenner BM, Floyd C, editors. Brenner & Rector's The Kidney. 7th ed. Philadelphia: WB Saunders. 2003; 297-313.

Lewis-Tuffin LJ, Cidlowski JA. The physiology of human glucocorticoid receptor β (hGRβ) and glucocorticoid resistance. Annals of the New York Academy of Sciences. 2006 Jun; 1069(1): 1-9.

Li M, Xu M, Liu W, Gao X. Effect of CYP3 A4, CYP3 A5 and ABCB1 gene polymorphisms on the clinical efficacy of tacrolimus in the treatment of nephrotic syndrome. BMC Pharmacology and Toxicology. 2018 Dec; 19(1): 14.

Lin RC, Wang XL, Morris BJ. Association of coronary artery disease with glucocorticoid receptor N363S variant. Hypertension. 2003 Mar 1; 41(3): 404-7.

Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG, Thummel KE. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. Molecular pharmacology. 2002 Jul 1; 62(1): 162-72.

Liu Y, Song L, Li B. The expression of glucocorticoid receptor beta messenger RNA in peripheral white blood cells of hormone-resistant nephrotic syndrome patients. Zhonghua Nei Ke Za Zhi. 2001 Nov; 40(11): 725-8.

Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir JM, Weigel NL, Wilson EM. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone and androgen receptors. Pharmacological reviews. 2006 Dec 1; 58(4): 782-97.

Malchoff DM, Brufsky A, Reardon G, McDermott P, Javier EC, Bergh CH, Rowe D, Malchoff CD. A mutation of the glucocorticoid receptor in primary cortisol resistance. The Journal of clinical investigation. 1993 May 1; 91(5): 1918-25.

Marsh S, McLeod HL. Pharmacogenomics: from bedside to clinical practice. Human molecular genetics. 2006 Apr 15; 15(suppl_1): R89-93.

Marton MJ, DeRisi JL, Bennett HA, Iyer VR, Meyer MR, Roberts CJ, Stoughton R, Burchard J, Slade D, Dai H, Bassett Jr DE. Drug target validation and identification of secondary drug target effects using DNA microarrays. Nature medicine. 1998 Nov; 4(11): 1293.

Mathieson PW. Immune dysregulation in minimal change nephropathy. Nephrology Dialysis Transplantation. 2003 Aug 1; 18(suppl_6): vi26-9.

Mayor S. Fitting the drug to the patient. Bmj. 2007 Mar 1; 334(7591): 452-3.

McCarthy HJ, Bierzynska A, Wherlock M, Ognjanovic M, Kerecuk L, Hegde S, Feather S, Gilbert RD, Krischock L, Jones C, Sinha MD. Simultaneous sequencing of 24 genes associated with steroid-resistant nephrotic syndrome. Clinical Journal of the American Society of Nephrology. 2013 Apr 5; 8(4): 637-48.

McIntosh N, Helms P, Smyth R. Forfar and Arneil's Test Book of Paediatrics. 6th edition. Edinburgh: ChurchilLinvingstone. 2003; 633-636.

McKinney PA, Feltbower RG, Brocklebank JT, Fitzpatrick MM. Time trends and ethnic patterns of childhood nephrotic syndrome in Yorkshire, UK. Pediatric Nephrology. 2001 Dec 1; 16(12): 1040-4.

McLeod HL, Evans WE. Pharmacogenomics: unlocking the human genome for better drug therapy. Annual review of pharmacology and toxicology. 2001 Apr; 41(1): 101-21.

Mekahli D, Liutkus A, Ranchin B, Yu A, Bessenay L, Girardin E, Van Damme-Lombaerts R, Palcoux JB, Cachat F, Lavocat MP, Bourdat-Michel G. Long-term outcome of idiopathic steroid-resistant nephrotic syndrome: a multicenter study. Pediatric Nephrology. 2009 Aug 1; 24(8): 1525-32.

Mendonca BB, Leite MV, de Castro M, Kino T, Elias LL, Bachega TA, Arnhold IJ, Chrousos GP, Latronico AC. Female pseudohermaphroditism caused by a novel homozygous missense mutation of the GR gene. The Journal of Clinical Endocrinology & Metabolism. 2002 Apr 1; 87(4): 1805-9.

Meyer zu Schwabedissen HE, Kim RB. Hepatic OATP1B transporters and nuclear receptors PXR and CAR: interplay, regulation of drug disposition genes, and single nucleotide polymorphisms. Molecular pharmaceutics. 2009 Jul 10; 6(6): 1644-61.

Miura M, Satoh S, Inoue K, Kagaya H, Saito M, Inoue T, Habuchi T, Suzuki T. Influence of CYP3A5, ABCB1 and NR1I2 polymorphisms on prednisolone pharmacokinetics in renal transplant recipients. Steroids. 2008 Oct 1; 73(11): 1052-9.

Moussa A, Mabrouk S, Hamdouni H, Ajmi M, Tfifha M, Omezzine A, Abroug S, Bouslama A. MDR-1 and CYP3A5 Polymorphisms in Pediatric Idiopathic Nephrotic Syndrome: Impact on Susceptibility and Response to Steroids (Preliminary Results). Clinical laboratory. 2017 Jul; 63(7): 1233-42.

Nash MA, Edelmann CM, Bernstuin J, Barnet HL. Pediatric kidney disease. 2nd edition. Boston/Taronata/London: Little, Brown and company. 1978; 1247-1280.

Nawaz Z, Mushtaq F, Mousa D, Rehman E, Sulaiman M, Aslam N, Khawaja N. Pattern of glomerular disease in the Saudi population: A single-center, five-year retrospective study. Saudi Journal of Kidney Diseases and Transplantation. 2013 Nov 1; 24(6): 1265.

Nei M, Kumar S. Molecular evolution and phylogenetics. Oxford university press; 2000 Jul 27.

Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics. 1996; 6: 1-42.

Neuhaus TJ, Fay J, Dillon MJ, Trompeter RS, Barratt TM. Alternative treatment to corticosteroids in steroid-sensitive idiopathic nephrotic syndrome. Archives of disease in childhood. 1994 Dec 1; 71(6): 522-6.

Niaudet P, Boyer O. Idiopathic Nephrotic Syndrome in Children. In: Ellis D. Avner, William E. Harmon, Patrick Niaudet and NorishigeYosikawa (eds). Pediatric nephrology, 6th edition, Springer Verlag Berlin Heidelberg. 2009; 667-702.

Niaudet P. Treatment of childhood steroid-resistant idiopathic nephrosis with a combination of cyclosporine and prednisone. The Journal of pediatrics. 1994 Dec 1; 125(6): 981-6.

Osborn AM, Moore ER, Timmis KN. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental microbiology. 2000 Feb; 2(1): 39-50.

Oscarson M. Pharmacogenetics of drug metabolising enzymes: importance for personalised medicine. Clinical chemistry and laboratory medicine. 2003 Apr 25; 41(4): 573-80.

Pagani F, Stuani C, Tzetis M, Kanavakis E, Efthymiadou A, Doudounakis S, Casals T, Baralle FE. New type of disease causing mutations: the example of the composite exonic regulatory elements of splicing in CFTR exon 12. Human molecular genetics. 2003 May 15; 12(10): 1111-20.

Palmieri O, Latiano A, Valvano R, D'inca R, Vecchi M, Sturniolo GC, Saibeni S, Bossa F, Latiano T, Devoto M, Andriulli A. Multidrug resistance 1 gene polymorphisms are not associated with inflammatory bowel disease and response to therapy in Italian patients. Alimentary pharmacology & therapeutics. 2005 Dec; 22(11-12): 1129-38.

Penzak SR, Formentini E, Alfaro RM, Long M, Natarajan V, Kovacs J. Prednisolone pharmacokinetics in the presence and absence of ritonavir after oral prednisone administration to healthy volunteers. JAIDS Journal of Acquired Immune Deficiency Syndromes. 2005 Dec 15; 40(5): 573-80.

Pichard L, Fabre I, Daujat M, Domergue JA, Joyeux HE, Maurel PA. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. Molecular Pharmacology. 1992 Jun 1; 41(6): 1047-55.

Prasad N, Jaiswal AK, Agarwal V, Yadav B, Sharma RK, Rai M, Singh H, Chaturvedi S, Singh A. Differential alteration in peripheral T-regulatory and T-effector cells with change in P-glycoprotein expression in childhood nephrotic syndrome: a longitudinal study. Cytokine. 2015 Apr 1; 72(2): 190-6.

Provenzani A, Santeusanio A, Mathis E, Notarbartolo M, Labbozzetta M, Poma P, Provenzani A, Polidori C, Vizzini G, Polidori P, D’Alessandro N. Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients. World Journal of Gastroenterology: WJG. 2013 Dec 28; 19(48): 9156.

Pujols L, Mullol J, Roca-Ferrer J, Torrego A, Xaubet A, Cidlowski JA, Picado C. Expression of glucocorticoid receptor α- and β-isoforms in human cells and tissues. American Journal of Physiology-Cell Physiology. 2002 Oct 1; 283(4): C1324-31.

Quirk E, McLeod H, Powderly W. The pharmacogenetics of antiretroviral therapy: a review of studies to date. Clinical infectious diseases. 2004 Jul 1; 39(1): 98-106.

Rahman MH, Jesmin T, Muinuddin G. An update of Management of idiopathic nephrotic syndrome: a review article. Bangladesh Journal of Child Health. 2013 Dec 4; 37(2): 102-21.

Rao MR, Gorey S. Pharmacogenomics and modern therapy. Indian Journal of Pharmaceutical Sciences. 2007; 69(2): 167.

Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. New England Journal of Medicine. 2005 Oct 20; 353(16): 1711-23.

Robson-WL, Leung-Ak. Nephrotic syndrome in childhood. Adv-Pediatr. 1993; 40: 287-323.

Roses AD. Pharmacogenetics and the practice of medicine. Nature. 2000 Jun; 405 (6788): 857.

Rosmond R, Chagnon YC, Holm G, Chagnon M, Pérusse L, Lindell K, Carlsson B, Bouchard C, Björntorp P. A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin and dysregulation of the hypothalamic-pituitary-adrenal axis. Obesity research. 2000 May; 8(3): 211-8.

Rostin M, Barthe P, Houin G, Alvinerie M, Bouissou F. Pharmacokinetics of prednisolone in children with the nephrotic syndrome. Pediatric Nephrology. 1990 Sep 1; 4(5): 470-3.

Roussel R, Reis AF, Dubois-Laforgue D, Bellanné-Chantelot C, Timsit J, Velho G. The N363S polymorphism in the glucocorticoid receptor gene is associated with overweight in subjects with type 2 diabetes mellitus. Clinical endocrinology. 2003 Aug; 59(2): 237-41.

Roy RR, Haque SM, Mamun AA, Muinuddin G, Rahman MH. Steroid resistant nephrotic syndrome in children: clinical presentation, renal histology, complications, treatment and outcome at Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh. IOSR J Pharm. 2014; 4(11): 1-7.

Russcher H, Smit P, Akker EL. van den, Rossum EF van, Brinkmann AO, Jong FH de. *et al.* Two polymorphisms in the glucocorticoid receptor gene directly affect glucocorticoid-regulated gene expression. J Clin Endocrinol Metab. 2005; 90: 5804-10.

Sachidanandam R, Weissman D, Schidt SC, Kakol JM, Stein LD, Marth G *et al.* A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature. 2001; 409: 928-933.

Safan MA, Elhelbawy NG, Midan DA, Khader HF. ABCB1 polymorphisms and steroid treatment in children with idiopathic nephrotic syndrome. British journal of biomedical science. 2017 Jan 2; 74(1): 36-41.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988 Jan 29; 239(4839): 487-91.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985 Dec 20; 230(4732): 1350-4.

Saito H, Takahashi S, Nagata M, Tsuchiya T, Mugishima H, Yan K, Kondo Y, Matsuyama T, Sekine T, Igarashi T. Reevaluation of glomerular charge selective protein-sieving function. Pediatric Nephrology. 2009 Mar 1; 24(3): 609-12.

Sakaeda T, Nakamura T, Okumura K. Pharmacogenetics of MDR1 and its impact on the pharmacokinetics and pharmacodynamics of drugs. Pharmacogenomics. 2003 Jul 1; 4(4): 397-410.

Sakaeda T, Nakamura T, Okumura K. Pharmacogenetics of drug transporters and its impact on the pharmacotherapy. Current topics in medicinal chemistry. 2004 Sep 1; 4(13): 1383-96.

Sakaeda T. MDR1 genotype-related pharmacokinetics: fact or fiction? Drug metabolism and pharmacokinetics. 2005; 20(6): 391-414.

Salama NN, Yang Z, Bui T, Ho RJ. MDR1 haplotypes significantly minimize intracellular uptake and transcellular P-gp substrate transport in recombinant LLC-PK1 cells. Journal of pharmaceutical sciences. 2006 Oct 1; 95(10): 2293-308.

Salusky IB, Brandis M, Greifer I, *et al.* Pediatric Nephrology Around The World. In: Avner, ED. Harmon, WE. Niaudet, P.Yoshikawa, N. (ed). Pediatric Nephrology. 6th edition. Springer, New York. 2009; 1643-90.

Sambuelli AM, Negreira SM, Gil AH, Huernos SP, Goncalves S, Toro MA, Kogan Z, Cabanne A, Camarero S, Bai JC, Lazarowski AJ. Multidrug resistance gene (MDR1) expression in the colonic mucosa of patients with refractory ulcerative colitis. Acta Gastroenterologica Latinoamericana. 2006; 36(1): 23-32.

Sarker MN, Islam MM, Saad T, Shoma FN, Sharmin LS, Khan HA, Afrooz F, Fatmi LE, Alam A, Salimullah AS, Uddin MR. Risk factor for relapse in childhood nephrotic syndrome-a hospital based retrospective study. Faridpur Medical College Journal. 2012 Apr 8; 7(1): 18-22.

Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, Gottesman MM. Silent polymorphisms speak: how they affect pharmacogenomics and the treatment of cancer. Cancer research. 2007 Oct 15; 67(20): 9609-12.

Schijvens AM, ter Heine R, De Wildt SN, Schreuder MF. Pharmacology and pharmacogenetics of prednisone and prednisolone in patients with nephrotic syndrome. Pediatric Nephrology. 2019 Mar 1; 34(3): 389-403.

Schwartz AG, Yang P, Swanson GM. Familial risk of lung cancer among nonsmokers and their relatives. American journal of epidemiology. 1996 Sep 15; 144(6): 554-62.

Shankland SJ. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. Kidney international. 2006 Jun 2; 69(12): 2131-47.

Sharples PM, Poulton J, White RH. Steroid responsive nephrotic syndrome is more common in Asians. Archives of disease in childhood. 1985 Nov 1; 60(11): 1014-7.

Sher ER, Leung DY, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Steroid-resistant asthma. Cellular mechanisms contributing to inadequate response to glucocorticoid therapy. The Journal of clinical investigation. 1994 Jan 1; 93(1): 33-9.

Shimada T, Terada AI, Yokogawa K, Kaneko H, Nomura M, Kaji K, Kaneko S, Kobayashi KI, Miyamoto KI. Lowered blood concentration of tacrolimus and its recovery with changes in expression of CYP3A and P-glycoprotein after high-dose steroid therapy. Transplantation. 2002 Nov 27; 74(10): 1419-24.

Siddique AB, Hanif M, Ahmed F, Hossain N, Uddin M. Correlation between Serum Prednisolone and Serum Albumin level in Childhood with Nephrotic Syndrome: A study in tertiary care hospital in Bangladesh. IOSR Journal of Dental and Medical Sciences. 2018; 17(9): 62-66.

Siegsmund M, Brinkmann U, Scháffeler E, Weirich G, Schwab M, Eichelbaum M, Fritz P, Burk O, Decker J, Alken P, Rothenpieler U. Association of the P-glycoprotein transporter MDR1 C3435T polymorphism with the susceptibility to renal epithelial tumors. Journal of the American Society of Nephrology. 2002 Jul 1; 13(7): 1847-54.

Soranzo N, Cavalleri GL, Weale ME, Wood NW, Depondt C, Marguerie R, Sisodiya SM, Goldstein DB. Identifying candidate causal variants responsible for altered activity of the ABCB1 multidrug resistance gene. Genome research. 2004 Jul 1; 14(7): 1333-44.

Srivastava T, Simon SD, Alon US. High incidence of focal segmental glomerulosclerosis in nephrotic syndrome of childhood. Pediatric nephrology. 1999 Feb 1; 13(1): 13-8.

Stachowski J, Zanker CB, Runowski D, Zaniew M, Peszko A, Medyńska A, Zwolińska D, Rogowska-Kalisz A, Hyla-Klekot L, Szprygner K, Weglarska J. Resistance to therapy in primary nephrotic syndrome: effect of MDR1 gene activity. Polski merkuriusz lekarski: organ Polskiego Towarzystwa Lekarskiego. 2000 Apr; 8(46): 218-21.

Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE, *et al.* Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. J Clin Endocrinol Metab. 2004; 89: 892-7.

Surendiran A, Pradhan SC, Adithan C. Role of pharmacogenomics in drug discovery and development. Indian journal of pharmacology. 2008 Aug; 40(4): 137.

Suvanto M, Jahnukainen T, Kestilä M, Jalanko H. Single nucleotide polymorphisms in pediatric idiopathic nephrotic syndrome. International journal of nephrology. 2016; 2016: 1-12.

Szilagyi K, Podracka L, Franke NE, Mojzis J, Mirossay L. A new link between steroid resistance, glucocorticoid receptor and nuclear factor kappa B p65 in idiopathic nephrotic syndrome. Neuro endocrinology letters. 2009; 30(5): 629-36.

Tada H, Tsuchiya N, Satoh S, Kagaya H, Li Z, Sato K, Miura M, Suzuki T, Kato T, Habuchi T. Impact of CYP3A5 and MDR1 (ABCB1) C3435T polymorphisms on the pharmacokinetics of tacrolimus in renal transplant recipients. In Transplantation proceedings. 2005 May; 37(4): 1730-1732.

Tanabe M, Ieiri I, Nagata N, Inoue K, Ito S, Kanamori Y, Takahashi M, Kurata Y, Kigawa J, Higuchi S, Terakawa N. Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene. Journal of Pharmacology and Experimental Therapeutics. 2001 Jun 1; 297(3): 1137-43.

Tecile Prince Andolino, Jessica Reid-Adam. Nephrotic Syndrome. Pediatrics in Review. 2015; 36: 117-126.

Teeninga N. Glucocorticoid Treatment in Childhood Nephrotic Syndrome: weighting the cornerstone. 2013.

Teeninga N, Kist-van Holthe JE, Van Den Akker EL, Kersten MC, Boersma E, Krabbe HG, Knoers NV, Van Der Heijden AJ, Koper JW, Nauta J. Genetic and *in vivo* determinants of glucocorticoid sensitivity in relation to clinical outcome of childhood nephrotic syndrome. Kidney international. 2014 Jun 1; 85(6): 1444-53.

Tesar V, Zima T. Recent progress in the pathogenesis of nephrotic proteinuria. Critical reviews in clinical laboratory sciences. 2008 Jan 1; 45(2):139-220.

Thervet E, Anglicheau D, King B, Schlageter MH, Cassinat B, Beaune P, Legendre C, Daly AK. Impact of cytochrome P450 3A5 genetic polymorphism on tacrolimus doses and concentration-to-dose ratio in renal transplant recipients. Transplantation. 2003 Oct 27; 76(8): 1233-5.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan IR, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proceedings of the National Academy of Sciences. 1987 Nov 1; 84(21): 7735-8.

Thervet E, Loriot MA, Barbier S, Buchler M, Ficheux M, Choukroun G, Toupance O, Touchard G, Alberti C, Le Pogamp P, Moulin B, Le Meur Y, Heng AE, Subra JF, Beaune P, Legendre C. Optimization of initial tacrolimus dose using pharmacogenetic testing. Clin Pharmacol Ther. 2010; 87: 721–6.

Thummel KE, Wilkinson GR. *In vitro* and *in vivo* drug interactions involving human CYP3A. Annual review of pharmacology and toxicology. 1998 Apr; 38(1): 389-430.

Tissing WJ, Meijerink JP, den Boer ML, Brinkhof B, van Rossum EF, van Wering ER, Koper JW, Sonneveld P, Pieters R. Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. Clinical Cancer Research. 2005 Aug 15; 11(16): 6050-6.

Ueda K, Clark DP, Chen CJ, Roninson IB, Gottesman MM, Pastan I. The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation. Journal of Biological Chemistry. 1987 Jan 15; 262(2): 505-8.

Ukkola O, Perusse L, Chagnon YC, Despres JP, Bouchard C. Interactions among the glucocorticoid receptor, lipoprotein lipase and adrenergic receptor genes and abdominal fat in the Quebec Family Study. International journal of obesity. 2001 Sep; 25(9): 1332.

Uszycka-Karcz M, Stolarczyk J, Wrzolkowa T. Mesangial proliferative glomerulonephritis in children. Int J Pediatr Nephrol. 1982; 3(4): 251-6.

Van Den Berg JG, Aten J, Chand MA, Claessen N, Dijkink L, Wijdenes J, Lakkis FG, Weening JJ. Interleukin-4 and interleukin-13 act on glomerular visceral epithelial cells. Journal of the American Society of Nephrology. 2000 Mar 1; 11(3): 413-22.

Van Den Berg JG, *et al.* Role of the immune system in the pathogenesis of idiopathic nephrotic syndrome. Clin Sci (Lond). 2004; 107: 125-136.

van Rossum EF, Koper JW, Huizenga NA, Uitterlinden AG, Janssen JA, Brinkmann AO, Grobbee DE, de Jong FH, van Duyn CM, Pols HA, Lamberts SW. A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids *in vivo,* is associated with low insulin and cholesterol levels. Diabetes. 2002 Oct 1; 51(10): 3128-34.

Vesell ES. Pharmacogenetic perspectives gained from twin and family studies. Pharmacology & therapeutics. 1989 Jan 1; 41(3): 535-52.

Viswanath D. Nephrotic syndrome in children. Journal of Indian Academy of Oral Medicine and Radiology. 2013; 25(1): 18.

Wasilewska A, Zalewski G, Chyczewski L, Zoch-Zwierz W. MDR-1 gene polymorphisms and clinical course of steroid-responsive nephrotic syndrome in children. Pediatric Nephrology. 2007 Jan 1; 22(1): 44-51.

Wasilewska A, Zoch-Zwierz W, Tomaszewska B, Wierciński R, Stasiak-Barmuta A. Expression of glucocorticoid receptors in mononuclear cells in nephrotic syndrome. Pediatric Nephrology. 2003 Aug 1; 18(8): 778-82.

Wasilewska AM, Zoch-Zwierz WM, Pietruczuk M. Expression of P-glycoprotein in lymphocytes of children with nephrotic syndrome treated with glucocorticoids. European journal of pediatrics. 2006 Dec 1; 165(12): 839-44.

Watkins PB. Cyclosporine and liver transplantation: will the midazolam test make blood level monitoring obsolete? Hepatology. 1995 Sep; 22(3): 994-6.

Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. Kidney international. 2007 Jun 2; 71(12): 1205-14.

West DV, Eede FVD, Delfavero J, *et al.* Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. Neuropsychopharmacology. 2006; 31(3): 620–7.

Wiggins RC. The spectrum of podocytopathies: a unifying view of disease glomerular. Kidney Int. 2007; 71(12): 1205–1214.

Woodahl EL, Ho RJ. The role of MDR1 genetic polymorphisms in interindividual variability in P-glycoprotein expression and function. Current drug metabolism. 2004 Feb 1; 5(1): 11-20.

Yan K, Kudo A, Hirano H, Watanabe T, Tasaka T, Kataoka S, Nakajima N, Nishibori Y, Shibata T, Kohsaka T, Higashihara E. Subcellular localization of glucocorticoid receptor protein in the human kidney glomerulus. Kidney international. 1999 Jul 1; 56(1): 65-73.

Yang G, Zou LP, He B, Ding YX, Wang J, Shi XY, Sun YH, Jia FY. NR3C1 gene polymorphism for genetic susceptibility to infantile spasms in a Chinese population. Life sciences. 2012 Jul 26; 91(1-2): 37-43.

Yang R, Roden RB. Genetic polymorphism and tumor immunotherapy. Current Pharmacogenomics. 2003 Mar 1; 1(1): 37-57.

Yang Y, Du H, Li Y, Guan W, Tang F, Ga Q, Ge RL. NR3C1 gene polymorphisms are associated with high-altitude pulmonary edema in Han Chinese. Journal of physiological anthropology. 2019 Dec; 38(1): 4.

Ye J, Yu Z, Ding J, Chen Y, Huang J, Yao Y, Xiao H, Yang J, Shen Y, Meng Q. Genetic variations of the NR3C1 gene in children with sporadic nephrotic syndrome. Biochemical and biophysical research communications. 2006 Sep 22; 348(2): 507-13.

Youssef DM, Attia TA, Amal S, Abduelometty FA. Multi-drug resistance-1 gene polymorphisms in nephrotic syndrome: impact on susceptibility and response to steroids. Gene. 2013 Nov 10; 530(2): 201-7.

Yu J, Zhou Z, Tay-Sontheimer J, Levy RH, Ragueneau-Majlessi I. Intestinal drug interactions mediated by OATPs: a systematic review of preclinical and clinical findings. Journal of pharmaceutical sciences. 2017 Sep 1; 106(9): 2312-25.

Zalewski G, Wasilewska A, Zoch-Zwierz W, Chyczewski L. Response to prednisone in relation to NR3C1 intron B polymorphisms in childhood nephrotic syndrome. Pediatric Nephrology. 2008 Jul 1; 23(7): 1073-8.

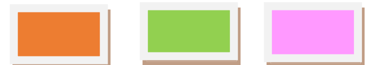
Zhang X, Clark AF, Yorio T. Regulation of glucocorticoid responsiveness in glaucomatous trabecular meshwork cells by glucocorticoid receptor-β. Investigative ophthalmology & visual science. 2005 Dec 1; 46(12): 4607-16.

Zhang X, Ognibene CM, Clark AF, Yorio T. Dexamethasone inhibition of trabecular meshwork cell phagocytosis and its modulation by glucocorticoid receptor β. Experimental eye research. 2007 Feb 1; 84(2): 275-84.

Zheng H, Webber S, Zeevi A, Schuetz E, Zhang J, Bowman P, Boyle G, Law Y, Miller S, Lamba J, Burckart GJ. Tacrolimus dosing in pediatric heart transplant patients is related to CYP3A5 and MDR1 gene polymorphisms. American Journal of Transplantation. 2003 Apr; 3(4): 477-83.

Zheng HX, Webber SA, Zeevi A, Schuetz E, Zhang J, Lamba J, Boyle GJ, Wilson JW, Burckart GJ. The impact of pharmacogenomic factors on steroid dependency in pediatric heart transplant patients using logistic regression analysis. Pediatric transplantation. 2004 Dec; 8(6): 551-7.

APPENDIX



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APPENDIX-1

PATIENT CONSENT FORM

I, the undersigned, authorize the research student to consider my son/daughter as a volunteer/patient for his/her research work. I understand that I can change my mind at any time to withdraw my son/daughter as volunteer during this research work.

Volunteer consent to study treatment

Please tick as appropriate

1.	Do you have complete idea about the type, ultimate goal and methodology of the research?	Yes	No
2.	Are you aware that you child don't have to face any physical, mental and social risk for this?	Yes	No
3.	There will be no chance of injury in any of your child's organs; are you aware of this?	Yes	No
4.	Have you got any idea about the outcome of this experiment?	Yes	No
5.	Have you decided intentionally to participate in this experiment?	Yes	No
6.	Do you think this experiment violate your child's human rights?	Yes	No
7.	Are you sure that all the information regarding your child will be kept confidentially?	Yes	No
8.	No remuneration will be provided for this experiment, are you aware of this?	Yes	No

After reading the above mentioned points, I am expressing my consent regarding my son/daughter to participate in this experiment as a **volunteer**.

Patient's guardian signature and date: ________________________________

Patient' Name: ____
Address:____
Witness:____

Please return the signed copy to the research student and keep an extra copy for yourself

____

Signature of the Researcher

Department of Clinical Pharmacy and Pharmacology

Faculty of Pharmacy, University of Dhaka

Dhaka University Institutional Repository

APPENDIX-2

DATA COLLECTION FORM

Questionnaires

Pharmacogenetic Study of Prednisolone Resistance in Childhood Nephrotic Syndrome Patients of Bangladesh

1. Identification

1.1 I. D. Code:

1.2 Name:

1.3 Father's Name:

1.4 Mother's Name:

1.5 Sex:

Male		Female	

1.6 Date of Birth (dd/mm/yy): 1.7 Age: (yrs)

1.8 Mailing Address:

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1.9 Permanent Address:

1.10 Telephone No.:

1.11 Religion:

1.12 Nationality:

2. Personal History

2.1 Area of Residence:

	Rural	Urban	S-Urban	Others
• Where have you spent your childhood (1-15yrs)?				
• Where have you spent at least ¾ th or more of your life time?				

2.2 Education Level:

- Illiterate
- <5th grade
- <10th grade
- < School
- SSC or equivalent
- HSC or equivalent
- Graduate or higher

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2.3 Occupation:

	Student		Unemployed
	Professional		Housewife
	Business		Skilled worker
	Technical		Other

2.4 Family Expense/month:

2.5 Impression about Social Class:

Rich		Upper		Poor	

2.6 Dietary Habit:

Poor fibrous		Rich fibrous		Moderately fibrous	

2.7 Food Habit (24 hours recall method):

Morning	
Lunch	
Afternoon	
Dinner	

2.8 Family History of Nephrotic Syndrome:

Yes		No	

2.9 Habit of Exercise:

Yes		No	

3. Biophysical Characteristics:

3.1 Height (cm):		3.5 Temperature (^oF):	
3.2 Weight (kg):		3.6 BP (Sys/Dias):	
3.3 Pulse/min:		3.7 BSA:	
3.4 BMI:			

4. Renal Biopsy Result:

Done		**Not done**	

5. Histopathological Findings of the Renal Biopsy:

	Minimal change disease
	Focal segmental glomerulosclerosis
	Mesangioproliferative glomerulonephritis
	Membranoproliferative glomerulonephritis
	Immunoglobulin A nephropathy
	Immunoglobulin M nephropathy
	Inadequate tissue

6. Biochemical Values:

	Features	Values
	Serum albumin (gm/l)	
	Serum cholesterol (mg/dl)	
	Urine protein/creatinine ratio	

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7. Clinical Presentating Complaints:

	Edema at the presentation moment
	Massive proteinuria
	Microhematuria
	Hypertension
	Allergic and atopic constitution
	Oliguria
	Respiratory distress
	Poor renal function

8. Complications in Patients with Nephrotic Syndrome:

	Urinary tract infection
	Respiratory tract infection
	Cellulitis
	Peritonitis
	Obesity
	Malnutrition
	Growth and development retardation
	Thrombosis
	Severe infections

Name of the Investigator:

Signature:

Date: