FUNCTIONAL AND MOLECULAR VARIATIONS OF α_2-MACROGLOBULIN, APOLIPOPROTEIN E AND PROTEIN S IN PATIENTS WITH NEPHROTIC SYNDROME AND CONTROL INDIVIDUALS

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DECLARATION

I, Margaretha Johanna Nel, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Masters of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

I further declare that the work presented was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, and granted the clearance number M950612.

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Twelfth day of February 2001

ABSTRACT

Nephrotic syndrome is a group of kidney diseases in which the glomerular basement membrane is far more permeable than normal, such that large amounts of proteins are lost in the urine. Tubular abnormalities are present, and cause abnormal reabsorption or lack of reabsorption of certain substances. In children, nephrotic syndrome is often an idiopathic disorder, whilst secondary nephrotic syndrome due to diabetes, amyloidosis or systemic lupus erythematosis also occurs but is more prevalent in adults. General complications of nephrotic syndrome include oedema; hyponatraemia; infections; low ionised calcium; diarrhoea; vomiting; thrombosis; hyperlipidaemia and abnormal thyroid function.

Little is known about the genetic factors that contribute to this group of disorders. Nephrotic syndrome is a polygenic disease and the approach of this study was to determine whether molecular variation of certain candidate genes might play a role in the prevalence of nephrotic syndrome in black and white South African children. The present study involved comparing α_2Macroglobulin levels; apolipoproteinE allele frequencies and genotypes as well as protein S allele frequencies in nephrotic syndrome patients and control individuals. These three factors were chosen for this study as α_2Macroglobulin and apolipoproteinE share the same receptor, α_2Macroglobulin levels are high in children, whilst apolipoproteinE is involved in lipid metabolism and hyperlipidaemia is a complication of nephrotic syndrome, and protein S is implicated in thrombosis which is also a complication of nephrotic syndrome.

This study determined α_2Macroglobulin levels, using the elastase binding capacity assay on plasma of paediatric nephrotic syndrome patients and control individuals. The results confirmed that the levels found in paediatric patients were similar to those found in control children, but were significantly increased compared to adults. A significant difference was thus found between the α_2Macroglobulin plasma levels of children compared to adults, which confirms the literature. No significant difference was found between the α_2Macroglobulin plasma levels of paediatric patients with FSGS compared to MLNS/(MCNS), as all children have high α_2Macroglobulin levels.

The polymerase chain reaction was used to screen for the various apolipoproteinE genotypes in FSGS patients compared to MLNS/(MCNS) patients, but no significant difference was found in ›er of the three apoE allele frequency distributions between the two groups of patients. A significant difference was found in the apolipoprotein E genotypes between white nephrotic syndrome patients and white control individuals, as the $apoE_3/E_3$ genotype frequency is decreased and the $apoE_3/E_4$ and the $apoE_2/E_3$ genotype frequency is increased in white nephrotic syndrome patients. The $apoE_3$ allele frequency is decreased in black as well as white nephrotic syndrome patients, indicating less efficient binding and removal of lipids in these patients. The $apoE_2$ allele, which is the least efficient in lipid binding, was significantly increased in the black nephrotic syndrome patients. This could contribute to the fact that focal segmental glomerulosclerosis, which is the more severe form of this disease and associated with renal scarring, is more prevalent in the black population, as was also shown by this study.

It is known that protein S deficiency cause thrombosis, which is common in nephrotic syndrome, but little is known about the genetic contribution of protein S to thrombosis. In the present study, the polymerase chain reaction and Bst XI restriction enzyme analysis were used to determine the prevalence of the Bst XI cut (+) protein S allele, and the Bst XI uncut (-) protein S allele in black and white nephrotic syndrome children patients and control individuals. The + allele, which is the mutated protein S allele, was significantly more prevalent in the black nephrotic syndrome children patients. A significant difference was found in the frequency distribution of the + and the – protein S allele between black and white individuals as a group, as the + protein S allele was far more prevalent in the black population. This finding could further contribute to the fact that black individuals are predisposed to more severe glomerular pathology, and secondarily to interstitial pathology as well i.e. increased renal scarring. Regarding thrombosis, there could be an increased risk with the + protein S allele. This could also possibly increase the risk of scarring in the glomerular microcirculation if thrombosis occurs there.

In conclusion, the present study demonstrated the extent and complexity of nephrotic syndrome and it highlighted the need for further investigations.

In memory of my parents

MJ Nel (Visser)

1929-1997

and

FP Nel

1928-1994

to whom I will always be grateful and who are sorely missed.

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LIST OF ABBREVIATIONS

A	adenine
ACE	angiotensin converting enzyme
AD	Alzheimer's disease
AMP	cyclic adenosine monophosphate
α_1PI	α_1-proteinase inhibitor
α_2M	alpha-2-macroglobulin
α_2MR	α_2M-receptor
α_2MRAP	α_2M-receptor associated protein
α_2MSR	α_2M-signalling receptor
Apo	apolipoprotein
Arg	arginine
ARMS	amplification refractory mutation system
AT III	anti-thrombin III
bFGF	basic fibroblast growth factor
βA	beta-amyloid
bis	N,N'-methylene –bis-acrylamide
bp	base pair
BSA	bovine serum albumin
C	cytosine
C3	third component of complement
Ca^{2+}	calcium ion
CEPT	cholesteryl ester transfer protein
CNF	congenital nephrotic syndrome of the Finnish type
C4b-bp	C4b-binding protein
CG	collapsing glomerulopathy
CNS	congenital nephrotic syndrome
COPD	chronic obstructive pulmonary disease

CyA	cyclosporin A
Cys	cystein
dATP	2' deooxyadenosine 5'-triphosphate
dCTP	2' deoxyadenosine 5'-triphosphate
dGTP	2' deoxyguanosine 5'-triphposphate
DMH	diffuse mesangial hypercellularity
DMS	diffuse mesangial sclerosis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	2' deoxythymidine 5'-triphosphate
DVT	deep vein thrombosis
EBC	elastase binding capacity
EDTA	ethylene diaminetetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ESRD	end stage renal disease
Et Br	ethidium bromide
f-α_2M	fast-α_2M
FSGS	focal segmental glomerular sclerosis
G	guanine
GBM	glomerular basement membrane
Gla	glutamic acid
Gln	glutamine
HDL	high-density lipoprotein
HIV	human immunodeficiency virus

HMG-CoA	β-hydroxy-β-methylglutaryl-Co A
hr	hour
HCl	hydrochloride
IDL	intermediate-dense lipoprotein
IEF	isoelectric focussing
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP_3	inositol triphosphate
IV	intravenous
kb	kilobase
KCl	potassium chloride
kDa	kilodalton
LCAT	lecithin : cholesterol acetyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LPL	lipoprotein lipase
LPLC	C-terminal of LPL
LRP	lipoprotein receptor protein
Lys	lysine
M	molar
MCNS	minimal change nephrotic syndrome
MembGN	membranous glomerulonephritis
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
MLNS	minimal lesion nephrotic syndrome

μg	microgram
μl	microlitre
mM	millimolar
MPGN	membranoproliferative glomerulonephritis
mRNA	messenger ribonucleic acid
NaAc	sodium acetate
ng	nanogram
nm	nanometre
NP40	"nonidet" P40
NS	nephrotic syndrome
NSAID	non-steroidal anti-inflammatory drug
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	protease inhibitor
pM	picomolar
RAP	receptor-associated protein
RIA	radioimmune assay
RNA	ribonucleic acid
RP	retinitis pigmentosis
SAPNA	succinyl-trialanyl-p-nitroanilide
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
s-α_2M	slow-α_2M
T	thymine
Taq	thermus acquaticus

TBE	tris borate EDTA
TEMED	N, N, N', N' – tetramethylene diamine
TFPI	tissue factor pathway inhibitor
TGF	transforming growth factor
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
Tris	tris (hydroxymethyl) aminomethane
uPA	urokinase plasminogen activator
VLDL	very low-density lipoprotein
VLDLR	VLDL-receptor
w/v	weight-to-volume

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Chapter 4

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW ON NEPHROTIC SYNDROME

1.1 The physiologic anatomy of the kidney

1.1.1 Function of the kidney

The kidney has two major functions: (i) Excretion of most of the end products of bodily metabolism such as urea, creatinine, uric acid and urates, (ii) control of concentrations of most of the constituents of the body fluids such as sodium-, potassium-, chloride- and hydrogen ions. The two kidneys together contain approximately $2x10^6$ nephrons and each nephron is capable of independently forming urine (Figure 1.1). The mechanism by which the nephron clears the plasma of unwanted substances is as follows: (i) it filters plasma in the flowing glomerular blood through the glomerular membrane into the tubular system of the nephron, (ii) as this filtered fluid flows through the tubules, the unwanted substances fail to be reabsorbed while wanted substances such as water and electrolytes are reabsorbed back into plasma. The nephron has a second mechanism by which it clears plasma of unwanted substances by secretion directly through the epithelial lining of the tubules into the tubular fluid. Thus, the urine eventually formed is composed of filtered substances and also small amounts of secreted substances (Guyton, 1991).

1.1.2 Anatomy of the nephron

Blood enters the glomerulus through the afferent arteriole and leaves through the efferent arteriole. The glomerulus is a network of branching and anastomosing capillaries covered by epithelial cells and encased by the Bowman's capsule. Blood pressure in the glomerulus causes fluid to filter into the Bowman's capsule and from here it flows into the proximal tubule, situated in the cortex of the kidney along with the glomerulus. Fluid passes from the proximal tubule into the loop of Henle, which dips deep into the kidney mass, sometimes even as deep as the bottom of the renal medulla. Each loop consists of a descending- and an ascending limb. The walls of the descending- and ascending limbs are very thin and are called

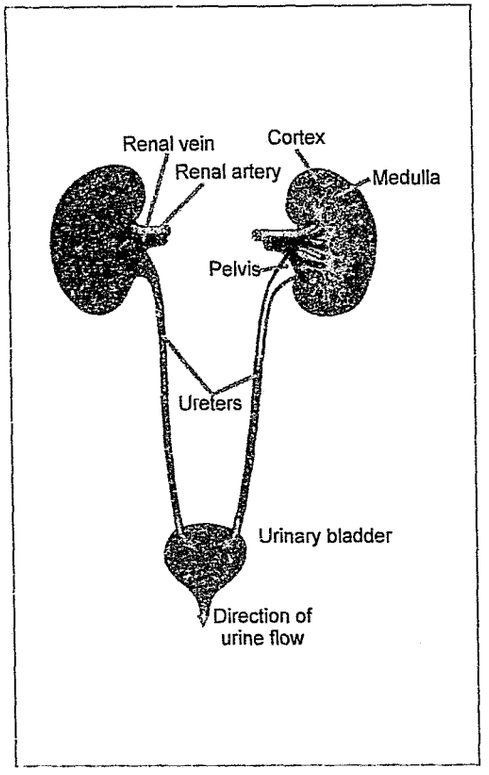


Figure 1.1 The urinary system (Guyton, 1991).

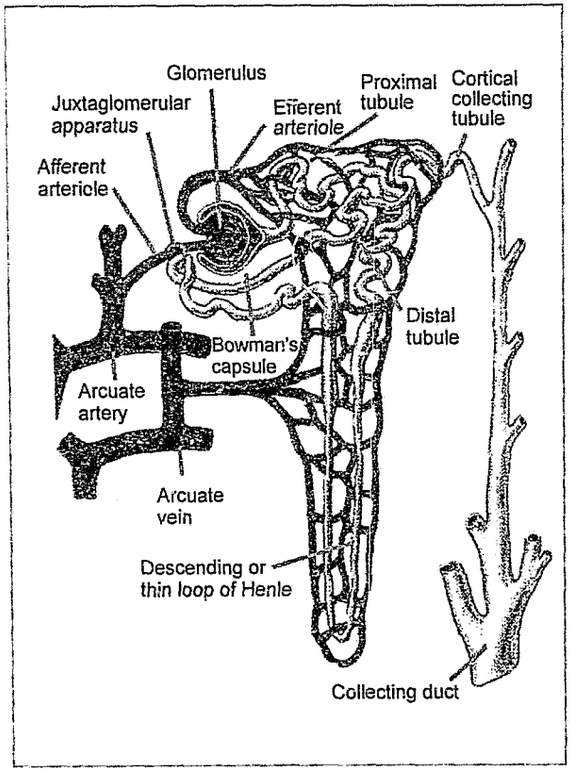


Figure 1.2a Structure of the nephron (Guyton, 1991)

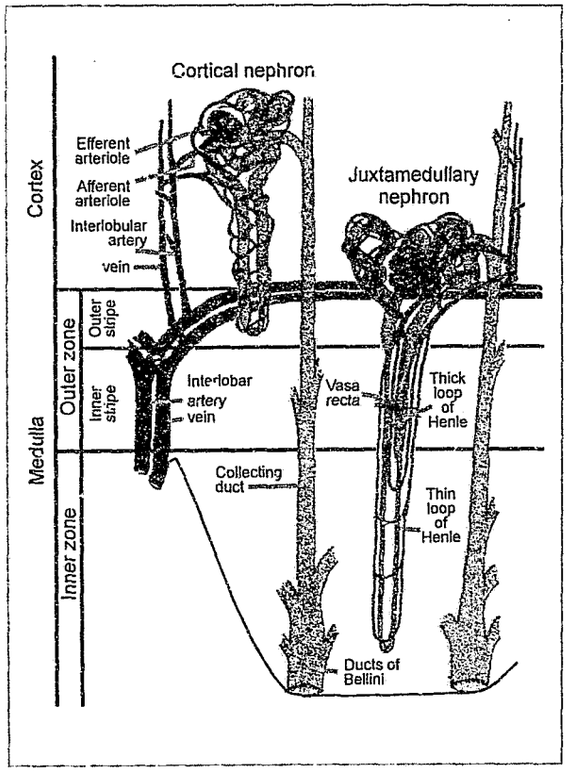


Figure 1.2b Structures of the cortical and the juxtamedullary nephron (Guyton, 1991)

the thin segment of the loop of Henle. The ascending limb of the loop turns back in the cortical direction, where it becomes thick like the other parts of the tubular system (Figure 1.2A). This portion of the loop of Henle is called the thick segment of the ascending limb (Guyton, 1991).

After the fluid has passed through the loop of Henle, it enters the distal tubule lying in the renal cortex (Figure 1.2B). Groups of distal tubules coalesce to form the cortical collecting duct, which turns downward into the medulla of the kidney to become the medullary collecting duct. The collecting ducts form progressively larger ducts, which empty into the renal pelvis through the tips of the renal papillae. Renal papillae are conical projections of the medulla, which protrude into the renal calyces, which are themselves, recesses of the renal pelvis. Each kidney has approximately 250 very large collecting ducts, each of which transmits urine from approximately $4x10^3$ nephrons (Guyton, 1991).

As the glomerular filtrate flows through the tubules, more than 99% of water and varying amounts of solutes are reabsorbed into the vascular system. Small amounts of some substances are secreted into the tubules. The remaining tubular water and dissolved substances become urine (Guyton, 1991).

1.1.3 The glomerular membrane and glomerular permeability

The membrane of the glomerular capillaries is called the glomerular membrane which differs from other capillaries in the body as it consists of three layers: The endothelial layer, a basement membrane and an epithelial layer on the outer surfaces of the capillaries (Figure 1.3). The permeability of the glomerular membrane is 100-500 times as great as that of other capillaries. The endothelial cell lining of the glomerulus is perforated by thousands of small holes called fenestrae (Guyton, 1991). The fenestrated endothelium of the capillary has intracellular and intercellular openings of 40-70 nm in diameter (Tryggvason, 1993). The basement membrane is a 350 nm thick meshwork of collagen and proteoglycan fibrillae with large spaces through which fluid filters. The epithelial cells consist of fingerlike projections that cover the basement membrane. These "fingers" form slits called slit-pores (Guyton,

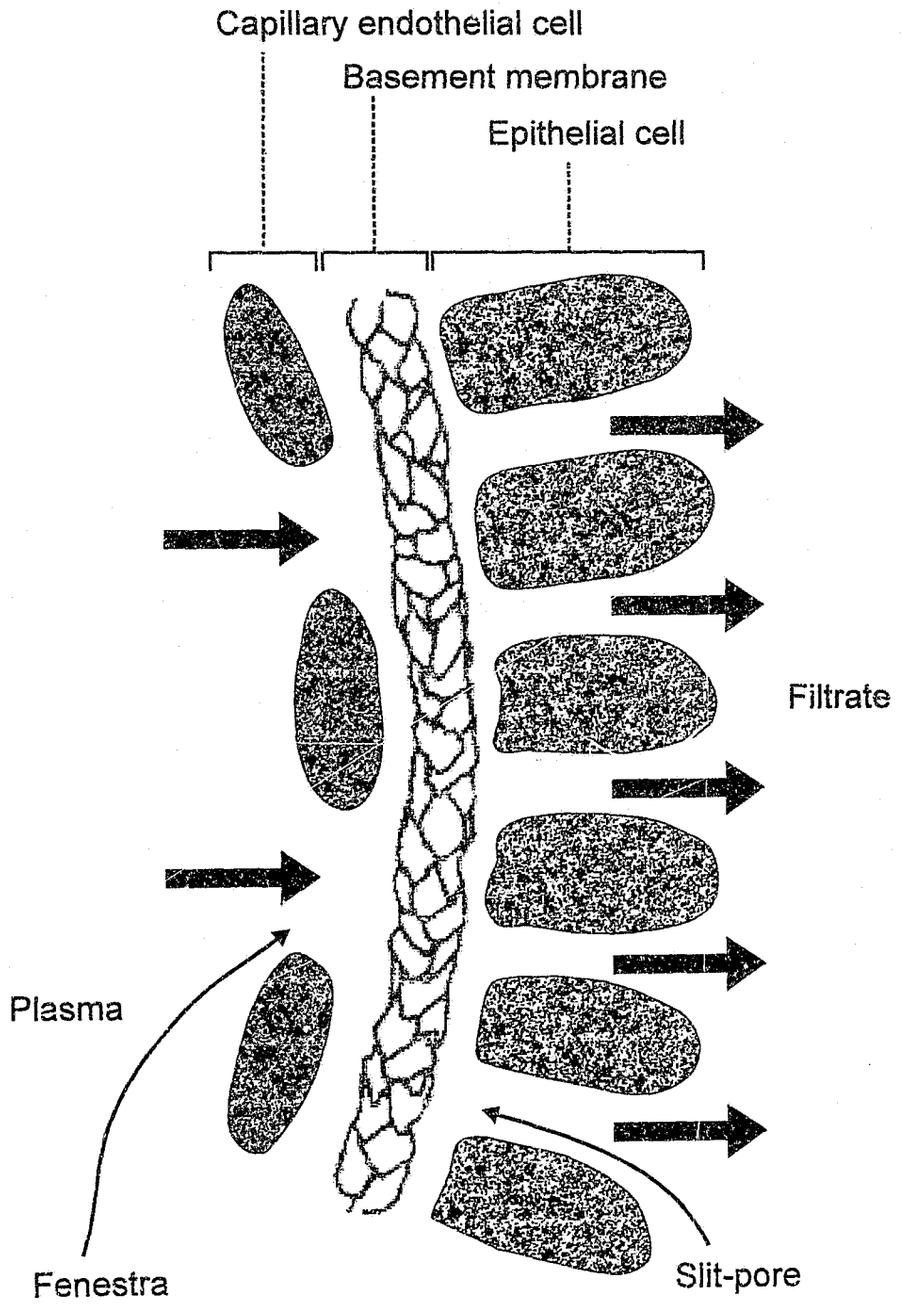


Figure 1.3 Structure of the glomerular membrane (Guyton, 1991).

1991), bridged by thin 25-60 nm wide diaphragms between the epithelial foot processes (Tryggvason, 1993), through which the glomerular filtrate filters (Guyton, 1991).

Despite the tremendous permeability of the glomerular membrane, it has an extremely high degree of selectivity for the size of molecules it allows to pass. The glomerular membrane is almost completely impermeable to all plasma proteins. The smallest plasma protein is albumin with a molecular weight of 69 kDa of which only 0,5% can be filtered. The glomerular membrane is highly permeable to essentially all other dissolved substances in normal plasma. The pores of the membrane are large enough to allow molecules with diameters up to about eight nm/80 angstroms to pass through. The plasma protein, albumin has a molecular diameter of only six nm, but the basement membrane portion of the glomerular pores are lined with a complex of proteoglycans that have a strong negative electrical charge. Plasma proteins also have strong negative electrical charges. Therefore, electrostatic repulsion of the molecules by the pore walls keeps virtually all protein molecules larger than 69 kDa from passing through. For all practical purposes, glomerular filtrate is the same as plasma except that it has no significant amount of proteins (Guyton, 1991).

Structural and functional glomerular membrane changes result in proteinuria, haematuria and renal failure. Alport syndrome (hereditary nephritis), characterised by haematuria and progression to renal failure, and nephrotic syndrome (NS) with characteristic massive proteinuria, are two genetic diseases which affect the glomerular basement membrane (GBM) (Tryggvason, 1993).

1.1.4 Kidney pathology

Renal disease can be classified into five different physiological categories: (i) acute renal failure in which the kidneys stop functioning entirely or almost entirely, (ii) chronic renal failure, in which progressively more nephrons are destroyed until the kidney simply cannot perform the necessary functions, (iii) hypertensive kidney disease in which vascular or glomerular lesions cause hypertension but not renal failure, (iv) NS in which the glomeruli are far more permeable than normal such that large amounts of protein are lost into the urine, and

(v) specific tubular abnormalities that cause abnormal reabsorption or lack of tubular reabsorption of certain substances (Guyton, 1991).

1.2 Definition of nephrotic syndrome

Physiological symptoms that occur with NS are proteinuria, hypoproteinaemia, oedema and hyperlipidaemia (Robson & Leung, 1993). Nephrotic proteinuria is defined as urinary protein excretion of more than 1 $g/m^2/hr$ (Robson & Leung, 1993) which is a result of increased permeability of the glomerular membrane (Guyton, 1991). Clinically speaking, proteinuria refers to the amount of urinary protein lost which leads to hypoproteinaemia and oedema (Robson & Leung, 1993).

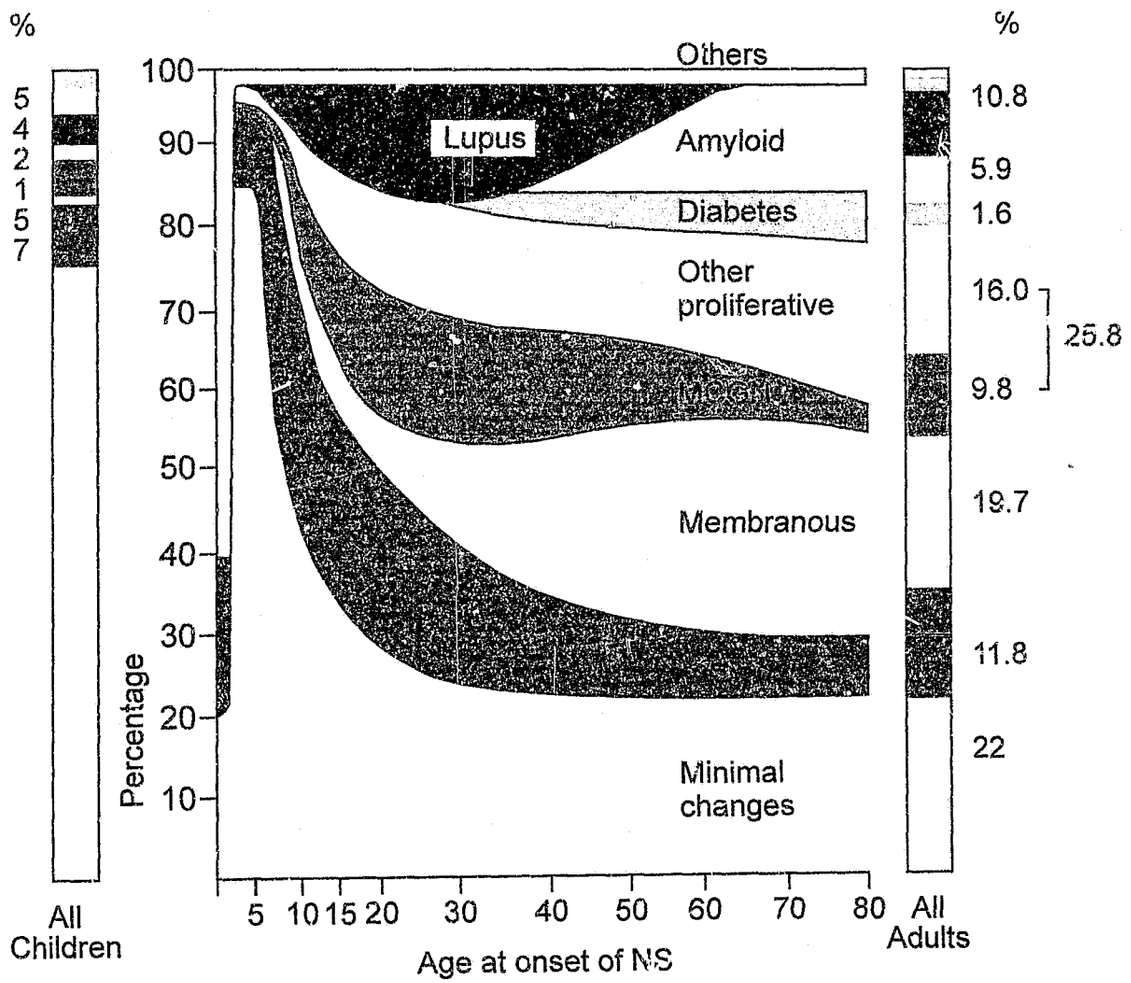
NS in childhood is when proteinuria is sufficiently severe to result in hypoalbuminaemia, oedema and hyperlipidaemia (Melvin & Bennett, 1991).

1.3 History of nephrotic syndrome

Hippocrates was the first person to describe NS as: "Bubbles floating on the surface of the urine denote affections of the kidneys, and that the disease will be long". In 1764 Cotugno described a soldier with massive oedema whose urine coagulated with heat. Bright noted in 1836, that proteinuria is certain in oedematous patients with renal disease. "Nephrosis" was first used by Muller to anatomically differentiate a group of kidney disease. Lipoid droplets were first noticed in the urinary sediment of patients with nephrosis, and Munk then suggested the term "lipoid nephrosis". "Nephrotic syndrome" was first used by Calvin and Goldberg to describe patients with oedema, proteinuria and hyperlipidaemia (Robson & Leung, 1993).

1.4 Nephrotic syndrome in general

The majority of children have an idiopathic disorder, while NS in adults is often due to secondary causes such as systemic lupus erythematosus (SLE), diabetes mellitus or amyloidosis (Figure 1.4). General symptoms reported at the onset of NS are swollen eyelids



MCGN = mesangio-capillary glomerulonephritis
FSGS = focal segmental glomerulosclerosis

Figure 1.4 Prevalence of histological NS types in children and in adults (Adapted from: Melvin and Bennett, 1991).

in 42% of cases, rapid weight gain in 23% of cases and upper respiratory tract infection two to three days before onset of oedema. Forty-one percent of the patient population are male. The onset age is approximately 3,5 years (SD = 2,1 years). The prognosis of steroid dependent minimal lesion nephrotic syndrome (MLNS)/minimal change nephrotic syndrome (MCNS) is good while the outcome of patients with focal segmental glomerular sclerosis (FSGS) is poor (Melvin & Bennett, 1991).

Clinically, the most important decision is whether or not to perform a renal biopsy. In general, if a South African Caucasian child is six or less years of age, has a normal third component of complement (C3), normal creatinine and blood pressure without haematuria, there is a high likelihood of MLNS/(MCNS) and corticosteroid responsiveness. However, if a black South African child presents with these same features and is hepatitis B e antigen negative, a renal biopsy is done even if the child is less than six years of age (Thomson, 1997; Thomson, 2000). If no systemic disease such as SLE, diabetes mellitus or nephrosis is present in a Caucasian child, therapy can proceed without a biopsy. Not all MLNS/(MCNS) patients fit this pattern, some have microscopic haematuria, high blood urea nitrogen, hypertension and non-selective proteinuria (Melvin & Bennett, 1991).

General complications of NS include: oedema, hyponatraemia, infection, low ionised calcium, diarrhoea and vomiting, thrombosis due to anti-thrombin III (AT III) loss in urine, increased fibrinogen and platelet aggregation, abnormalities in anticoagulants such as proteins S and C, low zinc, copper and iron, low ceruloplasmin, low transferrin and abnormal thyroid function tests (Melvin & Bennett, 1991).

Nephrotic syndrome in adults

MLNS/(MCNS) is seen in 30% of adult patients and has the same favourable prognosis as in children. Although MLNS/(MCNS) is highly responsive to steroid treatment, adults respond much slower than children and thus need a longer course of treatment. Forty four to 80% of adults with idiopathic NS have FSGS, which is found in the majority of black NS patients. FSGS patients do not respond well to steroid treatment, but with prolonged steroid courses,

remission rates of up to 60% have been seen. Steroid resistance in adults should only be assumed after failure to respond to a four month course of daily steroid treatment (Korbet, 1995).

Nephrotic syndrome in children

Idiopathic nephrosis include MLNS/(MCNS), diffuse mesangial hypercellularity (DMH)/ diffuse mesangial sclerosis (DMS) and FSGS. These patients are often steroid responsive, and may even recover renal function (Habib, 1993).

DMH/(DMS) occurs in isolated cases or in association with male pseudohermaphroditism and /or Wilm's tumour. DMH/(DMS) has a very characteristic pattern of glomeruli involvement, which distinguishes this disease morphologically from congenital NS (CNS) of the Finnish type (CNF). Clinically DMH/(DMS) presents as NS with two distinct features: it is diagnosed in the first two years of life and i progresses to end stage renal disease (ESRD) before three years of age (Habib, 1993).

Idiopathic nephrosis commonly appears after one year of age and less commonly between three months to one year of age, when some form of congenital NS could still present. Some of these patients in the MLNS/(MCNS), DMH/(DMS) or the FSGS morphological groups respond to steroids and their prognosis is better than that of CNS. The risk of a sibling being affected is higher in a family in which an infant has developed idiopathic nephrosis in the first twelve months of life, than when nephrosis has a later onset (Habib, 1993).

The most common childhood glomerular disorder is associated with NS (Wheeler *et al.*, 1989). This depends on whether the population is from a developing or developed country and their social background. In the South African setting it is post-streptococcal glomerulonephritis (Professor U.K. Kala, personal communication). NS is a group of disorders with the common features of massive proteinuria, hypo-albuminaemia, oedema and hyperlipidaemia. Proteinuria of more than 50 mg/kg body weight per day is the diagnostic factor. Hyperlipidaemia is not universally present, but proteinuria, hypoalbuminaemia and

oedema are seen commonly (Wheeler *et al.*, 1989). Hyperlipidaemia is present in 95% of children with MLNS/(MCNS) where their serum cholesterol is more than 250 mg/dl, while 68% of children with membranoproliferative glomerulonephritis have cholesterol at similar concentrations (Strauss *et al.*, 1987).

1.5 Aetiology of glomerular disease

In developed countries, glomerular disease often has no known etiological agent. Infective causes include malaria, syphilis and hepatitis B. Drugs such as gold, penicillamine, angiotensin converting enzyme (ACE) inhibitors and non-steroidal anti-inflammatory drugs (NSAID's) are implicated in development of glomerular disease (Robertson *et al.*, 1995).

Approximately 40% of diabetics develop glomerular disease after 20 years of illness. Other multisystem disorders such as amyloid, SLE, Goodpasture's syndrome and systemic vasculitis can cause glomerular disease (Robertson *et al.*, 1995).

MLNS/(MCNS) is sometimes associated with lymphoma. Membranous nephropathy is associated with carcinoma. Myeloma produces heavy proteinuria and may present with NS (Robertson *et al.*, 1995).

1.6 Epidemiology of nephrotic syndrome

Robson and Leung (1993) reported NS in two to seven children out of 1000 per year and that this disease is 15 times more common in children than in adults, whilst Robertson *et al.*, (1995), reported that NS occurs in 40 out of 10^6 per year of children under the age of five years, opposed to less than ten out of 10^6 per year of adults (Robertson *et al.*, 1995). Primary NS accounts for approximately 90% of paediatric NS cases. NS occurs in all populations, but is more common in black individuals. Sex and onset age varies with the type of NS. In childhood the male to female ratio is approximately 2:1 but changes to 1:1 in adulthood (Robson & Leung, 1993).

There are definitive differences in the population distribution of glomerular lesions, as FSGS accounts for 50% of nephrotic black adult patients whereas in white adult patients, membranous glomerulonephropathy is the most common (Korbet, 1995). Human immunodeficiency virus related nephropathy leading to FSGS, may especially be important in adults, as the pathogenesis may be different from HIV negative patients with FSGS (Professor U. K. Kala, personal communication). Membranoproliferative glomerulonephritis, immunoglobulin A nephropathy and immunotactoid glomerulopathy are less common causes of idiopathic NS and are rarely seen in black adult individuals (Korbet, 1995) (Figure 1.5).

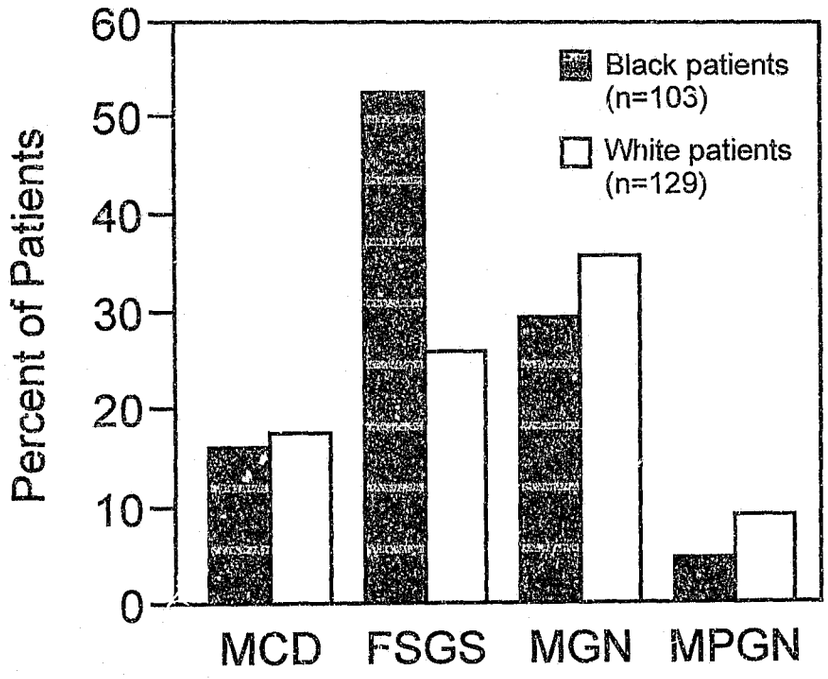
Population groups also have a prognostic significance in FSGS patients as 78% of black children, but only 33% of white children progressed to end stage renal disease (ESDR) over an eight and a half-year study. Black patients present less often with non-nephrotic range proteinuria, (14%), than white patients (52%). The rate of decline of renal function or cumulative renal survival in nephrotic patients does not indicate a significant populatic n difference (Korbet, 1995).

NS is familial in two to eight percent of patients with siblings most commonly affected (White, 1973). Involvement of more than one generation is uncommon. NS is more common in monozygotic twins than in dizygotic twins, which suggests that environmental factors are not as important as genetic factors (Robson & Leung, 1993).

1.7 Classification of nephrotic syndrome

1.7.1 Congenital nephrotic syndrome

There are two main groups of CNS namely, DMH/(DMS) and CNF (Robson & Leung, 1993). Pathological changes in CNS are restricted to the cortex of the kidney, leaving the medulla unchanged (Rapola *et al.*, 1984). CNS presents in the first three months of life and implies an inborn basis of the disorder, which can be idiopathic or secondary to some causative factor (Robson & Leung, 1993) CNS is a heterogeneous group of diseases characterised by extensive persistent proteinuria at or soon after birth. It can be acquired or be part of other



MCD = minimal change disease
FSGS = focal segmental glomerulosclerosis
MGN = membranous glomerulonephropathy
MPGN = membranoproliferative glomerulonephritis

Figure 1.5 Glomerular lesions in idiopathic NS in black and white adult patients (Korbet, 1995).

syndromes which often only affect the GBM (Tryggvason, 1993). Haematuria and leukocyturia are usually present along with generalised aminoaciduria and glucosuria are often present (Robson & Leung, 1993).

NS in the neonatal period and in infancy has phenotypic features such as widely separated skull sutures, high arched palate, wide nasal bridge, distended abdomen, umbilical hernia and flexion deformities of limbs. Characteristic facial features reflecting intra-uterine malnutrition, not described before include: a small mouth, tented upper lip, small nose, fullness of cheeks giving a "jowly" appearance and overhanging outer thirds of upper eyelids. These changes are not seen in children with chronic renal failure and do not reflect oedema and are not inherited. Chronic proteinuria and oedema in uterine and early postnatal life may cause soft tissue contour changes of the face. Consequent foetal malnutrition could result in maldevelopment of the face, a wide, flat nasal bridge and a high arched palate (Barret *et al.*, 1995).

CNF patients are usually born prematurely (Robson & Leung, 1993; Habib, 1993) and some already have proteinuria in utero (Robson & Leung, 1993; Tryggvason, 1993). CNF is characterised by intrauterine onset of massive urinary loss of proteins of which 90% is albumin (Holmberg *et al.*, 1995). The majority of these infants show oedema within the first month of life (Robson & Leung, 1993), with up to 50% showing oedema in the first week of life (Habib, 1993).

The placenta is usually more than 25% of the birth weight and NS is present from birth (Tryggvason, 1993; Habib, 1993), with the latest detection at three months of age in a particular study done by Habib, (1993). The rest of the clinical picture include proteinuria in all patients in the first week of life, distinctive facies without other malformation, poor somatic development, high susceptibility to infections and resistance to steroids and immunosuppressants (Habib, 1993).

CNF patients are susceptible to vascular complications and their growth and development are retarded. The treatment involves renal transplantation after which normal development

without any extrarenal manifestations occurs. This implies that the gene defect involves a highly specific GBM protein or a protein essential for GBM function (Tryggvason, 1993). No major qualitative changes of the GBM components have been found in CNF kidneys. The anionic sites of the GBM are not reduced and urinary heparan sulphate excretion is normal. (In CNS, the incorporation of anionic components into the GBM is altered). After the first three to six months of disease, renal histology becomes typical of CNF. This histology includes expansion of the mesangial area, fusion of the podocyte foot processes and typical tubular cysts (Holmberg *et al.*, 1995).

CNF occurs sporadically in various ethnic groups throughout the world. CNF is common in Finland and often familial. These patients are steroid resistant (Habib, 1993), which means that the patient does not experience remission after eight weeks on a conventional corticosteroid dose (Robson & Leung, 1993). The cause of death is usually not uraemia, but infection or diarrhoea with electrolyte imbalance (Habib, 1993).

The CNF gene is localised on the long arm of chromosome 19 (Holmberg *et al.*, 1995). CNF is an autosomal recessive transmitted disease (Tryggvason, 1993; Habib, 1993), with an incidence of $1{:}10^4$ at birth, which accounts for more than half of all CNS cases world-wide (Tryggvason, 1993).

In the past, CNF patients passed away within the first six months of life. Nowadays a CNF child does better, but is generally not well. This is improvement is achieved by early intravenous albumin supplementation, nutritional support, aggressive treatment of complications and early renal transplantation, after bilateral nephrectomy and peritoneal dialysis (Holmberg *et al.*, 1995).

NS as well as CNF patients have low serum thyroid-binding globulin and low serum thyroid hormone concentrations, but they respond well to thyroxine substitution therapy when the dose is adjusted according to thyroid stimulating hormone (Holmberg *et al.*, 1995).

As is the case in NS patients, CNF patients develop various plasma deficiencies. Due to urinary excretion of plasminogen and AT III, protein synthesis is increased so that levels of macroglobulin, fibrinogen, thromboplastin and Factors II, V, VII, VIII, X and XIII are increased. These increased levels contribute to hypercoagulopathy. Due to urinary losses of gamma globulin and complement factors B and D, nephrotic children are prone to infections caused by pneumococci. Prophylactic penicillin is recommended (Holmberg *et al.*, 1995).

Post-transplant NS is a special problem in CNF patients as these episodes usually follow a cytomegalo- or Epstein-Barr viral infection. Holmberg *et al.*, (1995), reported that 24% of grafts transplanted to CNF patients developed NS, while none developed in patients with other diseases (Holmberg *et al.*, 1995).

1.7.2 Primary nephrotic syndrome

Primary NS is associated with primary glomerular disease (Robson & Leung, 1993). Differentiation of the histological types of primary NS is based on age of onset, corticosteroid response, presence or absence of hypertension, haematuria and hypocomplementemia (Robson & Leung, 1993) (Table 1.1).

Approximately 60-80% of steroid sensitive NS patients experience at least one relapse after complete remission (Takeda *et al.*, 1996). Steroid sensitivity indicates that proteinuria disappears within eight weeks of starting corticosteroid treatment. Remission of NS is when only traces of protein are present in the urine or when the urine is negative for protein for at least three days. Relapse of NS is when proteinuria occurs for three consecutive days in a patient in remission (Robson & Leung, 1993). The risk factors for relapse are young age of onset and a low serum level of total protein. The occurrence risk is not associated with sex, percent body weight gain, blood urea nitrogen level, creatinine serum level, haematocrit or administration of human albumin (Takeda *et al.*, 1996).

In children, the upper limit of normal protein in urine is 4 mg/m^2/hr or 166 mg/1,73m^2/day (Robson & Leung, 1993). FSGS is the most common cause of NS in black children, with

Condition	Age at Presentation <6yr	Hypertension	Microscopic Hematuria	Hypo-complementemia	Response to Corticosteroids
Minimal-lesion nephrotic syndrome	80*	20	25	<5	95
Diffuse mesangial hypercellularity	75	10-45	45-90	0	50-55
Focal glomerulonephritis	50	35-50	50-60	<5	40
Membranous glomerulonephritis	NA	35	50-80	Rare	0
Membranproliferative glomerulonephritis	<5	50	60	75	25

* values are the percentages of patients with specific clinical variables rounded off to the nearest multiple of 5.

NA = not available.

Table 1.1 Clinical differentiation of histological types of primary NS (Robson and Leung, 1993).

MLNS/(MCNS) and hepatitis B-associated membranous nephropathy being the second most common causes. Membranous nephropathy appears to be less prevalent, while mesangial proliferative- and post-infectious nephropathy are more prevalent inland in South Africa. In contrast, on the Natal coast, it seems to be the opposite. This observation could be due to the fact that hepatitis B membranous nephropathy is more common in rural areas. Approximately 40% of black FSGS patients have previously had tuberculosis, opposed to only 5% in other NS types and in the general black population. MLNS/(MCNS) has a much lower prevalence in black NS patients compared to Asians and Caucasians (Thomson, 1997). Autosomal recessive polycystic kidney disease is common in Afrikaners in South Africa but is unusual in black children (Lombard *et al.*, 1989).

FSGS is the most common cause of renal failure and transplantation in black patients in South Africa, with rapidly progressive glomerulonephritis second and congenital NS third (Thomson, 1997). Black children require more repeat transplants due to the use of cadaver kidneys as few relatives are prepared to donate a kidney, and due to poor compliance in the lower socio- economic group, which decrease the graft survival (Thomson, 1997).

1.7.2.1 Minimal lesion nephrotic syndrome / Minimal change nephrotic syndrome

Pathological examination of the renal tissue in patients with MLNS/(MCNS) appears normal by light microscopy and immunofluorescence, but electron microscopy reveals fusion of the foot processes. Light microscopy reveals increased mesangial cells in diffuse mesangial hypercellularity, and electron microscopy shows increased mesangial cells and matrix (Robson & Leung, 1993).

In adults

In adults, MLNS/(MCNS) may be indistinguishable from FSGS, as MLNS/(MCNS) and FSGS are associated with hypertension or microscopic haematuria or both in 20-30% of nephrotic adult patients. Renal insufficiency is also seen in up to 35% of adult patients with MLNS/(MCNS). MLNS/(MCNS) differs from FSGS in that 50% of MLNS/(MCNS) patients

experience remission. Most patients relapse within a year of steroid treatment withdrawal. Remission can be re-established with another course of steroid treatment (Korbet, 1995). Frequently relapsing patients experience at least two relapses in six months or at least four in twelve months. Steroid dependent patients have two relapses during the steroid taper period or a relapse within one month of ending treatment (Korbet, 1995). Steroid dependent patients are not able to discontinue corticosteroid treatment without experiencing a relapse (Robson & Leung, 1993). In these frequently relapsing or steroid dependent patients, cytotoxic therapy such as oral cyclophosphamide can induce more sustained remission. Patients who do not respond to cyclophosphamide may respond to cyclosporin A (CyA). If no response occurs by four to six months on CyA, it is unlikely to occur at all. Normally, these patients become CyA dependent so that steroid dependency is just replaced by CyA dependency which has long term renal complications (Korbet, 1995).

In children

MLNS/(MCNS) is the most common type of NS in children (Robson & Leung, 1993) as it accounts for more than 75% of cases (Korbet, 1995) and typically develops in association with an upper respiratory tract infection. A history of allergy or recent immunisation is often present. Apart from oedema, these children look well, proteinuria is selective and haematuria and hypertension are uncommon. MLNS/(MCNS) is not usually associated with hypocomplementaemia (Robson & Leung, 1993). These patients respond well to corticosteroid treatment (Robson & Leung, 1993), as MLNS/(MCNS) patients are usually steroid responsive (Robertson *et al.*, 1995).

Survival rates, especially in children with MLNS/(MCNS) improved substantially after the introduction of corticosteroids in their treatment (Robson & Leung, 1993) as MLNS/(MCNS) patients are usually steroid responsive (Robertson *et al.*, 1995). Long-term prognosis for children with MLNS/(MCNS) is good, but deaths from infections or complications of treatment continue to occur. Before the use of corticosteroids, death from infections in the first two years after diagnosis was high and antibiotics yielded very little improvement in the overall mortality from NS (Barnett *et al.*, 1984). Patients, who respond poorly to prednisone

initially, will continue to do so and thus be at a high risk of toxicity and death. It is speculated that increased vulnerability to infection and steroid resistance share a common biologic origin (Barnett *et al.*, 1984). Young patients, (less than four years of age), are more likely to relapse than older patients (Korbet, 1995).

1.7.2.2 Diffuse mesangial hypercellularity / Diffuse mesangial sclerosis

In a study done by the International Study of Kidney Disease in Children, DMH/(DMS) is only found in about two percent of children with NS. The main feature of DMH/(DMS) is the insidious onset of heavy proteinuria with or without nephrotic state biochemical features. Approximately half the children with DMH/(DMS) have hypertension and a quarter of children have decreased renal function. Only half the children with DMH/(DMS) are corticosteroid sensitive and frequent relapsing episodes are common (Robson & Leung, 1993).

Due to the early onset of DMH/(DMS), it can be confused with CNF, but DMH/(DMS) differs from CNF by its rapid progression to ESRD and the involved glomeruli has a very characteristic pattern. Under light microscopy the glomerular lesions are characterised by fibrillar increase in mesangial matrix at the early stage of disease. When DMH/(DMS) is fully developed, lesions are characterised by a thickened basement membrane and spongy appearance of expanded mesangial zones and accumulation of mesangial matrix. Under electron microscopy, cells appear hypertrophic and the mesangial cells are surrounded by abundant mesangial matrix containing collagen fibrils (Habib, 1993).

1.7.2.3 Focal segmental glomerulosclerosis

In FSGS, only some glomeruli are affected by sclerosis while others are completely normal. Electron microscopy reveals the collapse of capillaries with wrinkled basement membranes in segmental lesions, sclerosis as well as vacuolation of podocytes, which may even be detached from the basement membrane (Grishman & Churg, 1975).

Focal refers to the fact that only some of the glomeruli are affected by renal disease. Segmental refers to the fact that only a part of each glomerulus is affected by renal disease (Robertson *et al.*, 1995).

Histologically, the location of the segmental scar, mesangial proliferation, presence of IgM, collapsing glomeruli and interstitial fibrosis predict the outcome of FSGS. Only the extent and presence of interstitial fibrosis constantly predicts a poor prognosis (Korbet, 1995).

The use of CyA could accelerate the course of FSGS to ESRD, especially in those patients with pre-existing renal insufficiency and tubulointerstitial disease (Korbet, 1995).

The FSGS lesion is the most commonly found renal lesion in patients with human immuno-deficiency virus (HIV)-associated nephropathy (Korbet, 1995).

FSGS develops and progresses to renal failure in some patients with idiopathic NS resistant to cytotoxic drugs as well as to corticosteroids and 40% of the latter patients have recurrences after kidney transplantation. Some patients with recurrent FSGS respond to plasmaphoresis which indicates that there is a circulating factor that alters the glomerular barrier to protein filtration. Savin *et al.*, (1996) reported that when glomeruli are incubated *in vitro* with protamine, superoxide, activated leukocytes or Heymann antibody and complement, the permeability to albumin increases. Serum from FSGS patients increased the glomerular permeability to albumin, with the highest activity observed in serum from patients with recurrent disease after transplantation. No activity was detected in the serum of healthy individuals or in patients with corticosteroid-sensitive NS whilst low activity levels were detected in the serum of patients with membranous nephropathy after transplantation or in renal failure (Savin *et al.*, 1996).

In patients with recurrent FSGS, plasmaphoresis lowered the level of activity in their serum and decreased the proteinuria. This is consistent with removal of a substance confined to the plasma space and that the serum factor is not rapidly synthesised after its removal. The latter supports the idea that a serum factor is responsible for the glomerular filtration barrier injury

in FSGS patients and may also contribute to the persistent proteinuria in these patients (Savin *et al.*, 1996). Characterisation of this active factor found that it is larger than any known lymphokine, is fairly hydrophobic and can be distinguished from the majority of the serum proteins by its 70% ammonium sulphate solubility. This factor has a weak anionic charge at pH 6.0, and binds to protein A, but does not precipitate with immunoglobulins. Thus it is postulated to be a non-immunoglobulin protein or an immunoglobulin fragment. Savin *et al.*, (1996) postulated that cellular effects could be responsible for the permeability increase and concluded that a serum factor in FSGS patients cause immediate and marked changes in glomerular permeability to albumin. This serum factor is strongly associated with the recurrence of FSGS after renal transplantation and may also cause the proteinuria in patients with this disorder (Savin *et al.*, 1996).

In adults

In the 1970's and early 1980's, FSGS was thought to be responsible for 10-15% of idiopathic NS cases in adults, and a similar percentage for MLNS/(MCNS). At that stage, membranous nephropathy was believed to be the most common primary cause of adult NS at 35-50% of cases (Haas *et al.*, 1995). D'Agati, (1994) reported an increase in FSGS incidence from 2,5% in 1974 to 18,7% in 1993 in native kidney biopsies in New York City. Now, FSGS constitutes the most frequent diagnosis on native kidney biopsies in this medical practice (D'Agati, 1994). FSGS occurs in 44-80% of adults with NS and is the most common cause of idiopathic NS in black adults. Most adults with FSGS present with NS, but often a third of the patients are not nephrotic (Korbet, 1995).

FSGS presents with many different histology patterns, which could occur secondarily in HIV infected patients, intravenous drug abusers, unilaterally nephrectomised patients, sickle cell disease patients, eclampsia/pre-eclampsia, reflux nephropathy or patients with chronic lesions of focal proliferative glomerulonephritis (Haas *et al.*, 1995).

The most aggressive form of FSGS, which is the collapsing variety, in which the glomeruli collapse, occurs with much higher prevalence in African Americans with the prognosis in

children being much poorer (Bakir *et al.*, 1996). In primary FSGS, the type associated with the worst renal survival is the collapsing glomerulopathy (CG) type variant, which occurs primarily in black NS patients and is characterised by a rapid progression to irreversible ESRD (Haas *et al.*, 1995).

A marked progressive increase in the incidence of FSGS has been reported over the period of 1974 - 1993 in all native kidney biopsies and among membranous nephropathy, MLNS/(MCNS) and FSGS cases (D'Agati, 1994; Haas *et al.*, 1995). It was reported that patients with CG have much higher levels of serum creatinine and urinary protein excretion, than patients with FSGS (non-CG). Patients with CG progress more rapidly to ESRD. CG is a rare form of FSGS, which only accounts for five percent of total FSGS cases and showed no significant incidence increase from 1980-1993 (Haas *et al.*, 1995). The increase in FSGS incidence does not include cases secondary to other conditions such as HIV, IV drug abuse, sickle cell disease, obesity, eclampsia/pre-eclampsia, reflux nephropathy or prior unilateral nephrectomy (Haas *et al.*, 1995).

Several research groups have reported that FSGS is more common in black patients than in the general renal biopsy population ((Korbet *et al.*, 1986; Bakir *et al.*, 1989; Pontier & Patel, 1994; Haas *et al.*, 1995). In addition it has been reported that CG has a stronger black population predominance and that CG patients progress more rapidly and more frequently to ESRD than do FSGS patients without CG (D'Agati, 1994; Detwiler *et al.*, 1994; Haas *et al.*, 1995).

Idiopathic FSGS constitute 30% of primary glomerular disease in adult African Americans and it is the most common primary non-proliferative glomerulopathy in this population (Bakir *et al.*, 1989). FSGS occurs in 80% of men and 44% of women African American patients with primary glomerular disease, in contrast to 21% in white men (Pontier & Patel, 1994). In four series of primary NS studies in Caucasian adults, FSGS only accounted for 14% of cases. FSGS is rare in the Chinese population where it only constitutes four percent of primary glomerulopathies in Hong Kong (Bakir *et al.*, 1996).

No correlation was found between the development of ESRD and age, gender, hypertension, oedema, NS, albumin or cholesterol (Bakir *et al.*, 1996). Some studies indicated that hypertension is associated with development of renal failure (Pei *et al.*, 1987). NS was reported to predict renal failure (Velosa *et al.*, 1983), but other studies did not support this relationship. Previously, hypercholesterolaemia was found to correlate with renal failure progression in FSGS children, however Velosa *et al.*, (1983) found no such correlation in children or adults. Elevated serum creatinine at presentation of disease, does predict progression of renal disease (Velosa *et al.*, 1983). Bakir *et al.*, (1996) found elevated serum creatinine at presentation in a higher proportion of patients who developed ESRD than in those who did not. They concluded that increased serum creatinine at entry usually predict progression of renal disease (Bakir *et al.*, 1996). Patients with proteinuria in the nephrotic range usually develop ESRD over six to eight years whilst those with proteinuria of greater than 14 g/24hr develop ESDR within two to three years (Table 1.2). Non-nephrotic patients have an 80% renal survival over ten years. Patients with chronic renal insufficiency with serum creatinine greater than 1,3 mg/dl have a much poorer survival than patients without chronic renal insufficiency (Korbet, 1995).

Pregnancy adversely affects the course of FSGS, causing an exacerbation of hypertension and NS. Rapid deterioration of renal function and adverse foetal outcome is common (Bakir *et al.*, 1996).

In a study conducted by Bakir *et al.*, (1996) almost half the adult African American patients with treated FSGS developed ESRD or chronic renal insufficiency (Bakir *et al.*, 1996), while prognosis at five years was comparable to that in non-African American patients, but the long-term renal survival may not be as good (Bakir *et al.*, 1996).

Remission of NS not only occurred in patients who maintain renal function, but also in those who developed ESRD. The patients who developed ESRD were more likely to present with increased serum creatinine (Bakir *et al.*, 1996).

Condition	Percentage with ESRD
Minimal-lesion nephrotic syndrome	Rare
Diffuse mesangial hypercellularity	4
Focal glomerulonephritis	21
Membranous glomerulonephritis	12
Membranproliferative glomerulonephritis	45-75

Table 1.2 End-stage Renal Disease in primary NS (Robson and Leung, 1993)

In children

Primary FSGS occurs in 7-20% of children and adults with idiopathic NS. FSGS is more prevalent in the black population with 32% of NS children having FSGS. Renal insufficiency is present in approximately 24% of cases at biopsy (Korbet, 1995).

Children with FSGS present in a fashion very similar to MLNS/(MCNS) but the median age of onset is older in the case of FSGS patients. Hypertension and haematuria is present in approximately half of the patients. Patients with FSGS are usually steroid resistant, steroid dependent or frequently relapsing (Robson & Leung, 1993). Steroid resistance may initially be present or may develop after months or years of responsive treatment (Srivastava *et al.*, 1986). Histological diagnosis of NS type can change with time (Robson & Leung, 1993). In some NS patients, FSGS may develop after the original diagnosis of MLNS/(MCNS) or DMH/(DMS) by biopsy so that when a later biopsy is done, FSGS is found (Srivastava *et al.*, 1986).

1.7.2.4 Membranous glomerulonephritis

Characteristic of membranous glomerulonephritis (MembGN) is the presence of diffuse subepithelial deposits and the absence of mesangial proliferation. Immunofluorescence shows vast IgG deposits in glomeruli and electron microscopy reveals a thick glomerular basement membrane, a spike-and–dome pattern as well as subepithelial electron-dense deposits (Habib, 1993).

MembGN occurs mainly in older children and is the most common cause of NS in adults as its incidence increases with age. Almost all patients develop haematuria during the course of the disease with hypertension and renal failure occurring late in the course of this disease. Several extra renal conditions are associated with MembGN which include diabetes mellitis, streptococcal infection, hepatitis B surface antigen, arthralgia, SLE, idiopathic thrombocytopaenia, sickle cell disease, D-penicillamine side effects, syphilis and leukemia (Robson & Leung, 1993). In South Africa, hepatitis B membranous nephropathy also occurs

in younger children and has a strong association with hepatitis B e antigenaemia (Thomson, 1997).

1.7.2.5 Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis (MPGN) can be subdivided into three types with frequencies and characteristics as follows: Type 1 (44%)- glomerular deposits are subendothelial and the GBM is intact. Type 2 (20%)- electron density of the lamina densa is greatly increased and the GBM is thickened. Type 3 (36%)-subepithelial and subendothelial deposits are present. Deposition of immune complexes in the glomeruli appears to be the cause of glomerular sclerosis which occurs in all three these types of MPGN (Robson & Leung, 1993). MPGN differs from MLNS/(MCNS) in presentation of disease at an older age as well as the presence of haematuria, hypertension and hypocomplementaemia. In half the cases, glomerular filtration rate is increased however if decreased glomerular filtration rate is present at onset, the prognosis is usually poor (Robson & Leung, 1993).

1.7.3 Secondary nephrotic syndrome

Secondary nephrotic syndrome is when NS occurs as part of a recognised systemic disease or it could result from some evident cause. In addition to proteinuria and oedema, other clinical manifestations depend on the underlying etiology. For instance a child with NS secondary to SLE could present with symptoms of pallor, unexplained fever, skin rash, joint pain and swelling or chest pains (Robson & Leung, 1993).

1.8 Pathophysiology of nephrotic syndrome

NS is arbitrarily defined by oedema, proteinuria greater than 3 g/24hr and serum albumin less than 30 g/l, but while NS proteinuria is present, it is not uncommon to have no oedema and no drop in serum albumin concentration. NS can occur with or without haematuria, uraemia and hypertension (Robertson *et al.*, 1995).

Although not easy to identify, glomerular red blood cells (RBC's) have bizarre shapes, while non-glomerular RBC's are uniformly round. This is an essential microscopic diagnosis used to confirm presence of blood in urine where chemical blood tests are positive, but it could be a false-positive result (Robertson *et al.*, 1995).

Renal biopsy is indicated for proteinuria greater than 2 g/24hr, macroscopic haematuria after urological cause is excluded, NS and unexplained acute/chronic renal failure in patients with kidneys of a normal size. Renal biopsy gives a histological diagnosis, allows estimation of prognosis, the chance of steroid resistance or responsiveness and the recurrence of a renal transplant (Robertson *et al.*, 1995). Normal protein excretion should not exceed 200 mg/24hr. Proteinuria persisting at more than 1 g/24hr mostly indicates glomerular pathology (Robertson *et al.*, 1995).

1.8.1 Proteinuria

Proteins have a conformational structure; size or negative charge preventing them from crossing the small negatively charged pores in the lamina densa of the basement membrane or the split between foot processes of the glomerulus (Robson & Leung, 1993).

In NS, proteinuria is caused by increased glomerular loss of protein, which may be due to increased glomerular capillary permeability or an increased pore size of the GBM (Robson & Leung, 1993). Reduction in the number of anionic sites in the glomerular capillary wall results in loss of a negative charge and thus reduces the glomerular barrier to negatively charged plasma proteins such as albumin. Thus there is increased glomerular capillary permeability. In some cases of NS, both charge and size abnormalities occur and lead to proteinuria (Robson & Leung, 1993).

1.8.2 Hypoalbuminaemia

NS patients do not show decreased albumin synthesis (Gitlin *et al.*, 1956), but hypoalbuminaemia occurs due to excessive loss of albumin in the urine as well as loss through

the gastrointestinal tract. Increased albumin catabolism also occurs in the proximal renal tubule (Robson & Leung, 1993). Hypoalbuminaemia is a characteristic sign of NS in which albumin synthesis can increase four fold, but the plasma albumin concentration may decrease to less than 25% of normal. The main reason for development of hypoalbuminaemia is the fact that the liver is not able to increase albumin synthesis to such an extent that it can replace the urinary loss (Kaysen, 1994).

In the past, high protein diets were used to increase albumin synthesis, thereby increasing the albumin concentration and oncotic pressure (π). This causes further loss of glomerular permselectivity, which results in greater albumin loss and increased albumin catabolism, and negates the effect of the increased synthesis (Kaysen, 1994). A soy protein diet can reduce urinary protein excretion significantly in NS patients (D'Amico *et al.*, 1992), as the protein composition is as important as the quantity of dietary protein taken in by NS patients (Kaysen, 1994).

NS patients have a decreased ability to catabolise chylomicrons and very low-density lipoprotein (VLDL), as well as to take up released triglycerides into tissue. This is partly mediated by reduced lipoprotein lipase (LPL) on vascular endothelium. Chylomicron remnants are taken up by the liver at a reduced rate and therefore accumulate in plasma. These particles may be taken up by macrophages and are very atherogenic (Kaysen, 1994).

1.8.3 Oedema

Oedema occurs as an abnormal amount of fluid accumulates within interstitial tissue (Robson & Leung, 1993). Capillary hydrostatic pressure forces fluid from the vascular- into the interstitial space (Kaysen, 1994). Hypoalbuminaemia decreases plasma oncotic pressure, which leads to oedema formation in NS (Robson & Leung, 1993). Oedema in NS is due to reduced plasma oncotic pressure with reduced sodium excretion, which leads to plasma volume expansion (Figure 1.6). Even MLNS/(MCNS) patients have increased blood volume (Robson & Leung, 1993).

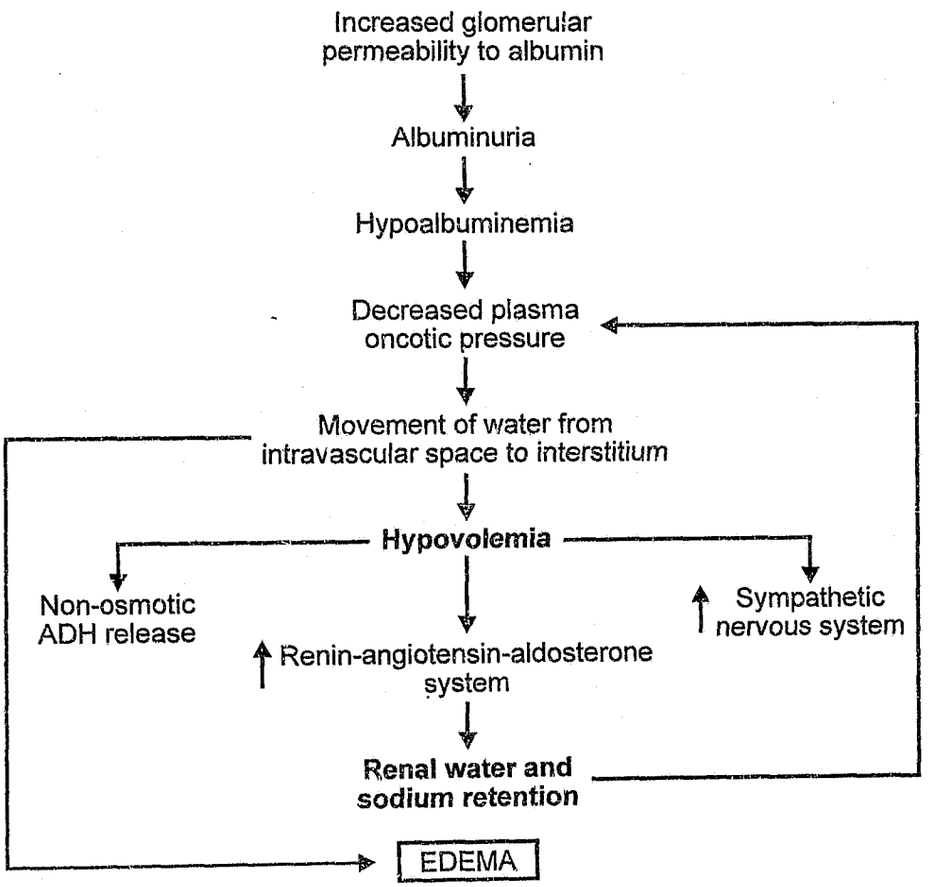


Figure 1.6 The mechanism of oedema formation in NS (Perico and Ramuzzi, 1993)

In the case of children, when serum albumin concentration is less than 20 g/l, oedema develops and when the concentration drops to less than 15 g/l, pleural effusion and ascites develop (Robson & Leung, 1993).

Oedema formation in NS involves two parallel processes namely: reduced plasma oncotic pressure and the reduced ability of the kidney to excrete sodium, either in response to plasma volume expansion or to atrial natriuretic factor (Kaysen, 1994). NS patients retain sodium to maintain the oedematous state by restoring fluid lost from the vascular space (Robson & Leung, 1993). In NS patients the inability to excrete a sodium load during volume expansion is due to enhanced reabsorption of sodium by the collecting duct or abnormal function of deep nephrons (Bernard *et al.*, 1978; Koomans *et al.*, 1984).

Increased levels of vasopressin (the antiduiretic hormone) could be responsible for water retention in some patients (Usberti *et al.*, 1984). Prostaglandin E_2 levels are elevated in NS children, which could add to the urinary reabsorption of sodium (Garin *et al.*, 1983). Noradrenalin levels are elevated in oedematous NS children and may enhance proximal renal tubular sodium reabsorption (Robson & Leung, 1993).

1.8.4 Lipid abnormalities in nephrotic syndrome

In normal lipoprotein metabolism, cholesterol and triglycerides are transported in association with proteins as macromolecular complexes in the circulation. Lipoprotein particles consist of an inner non-polar lipid core surrounded by a polar surface layer. Dietary lipids are delivered to the liver from the intestines and while cholesterol is transported to and from extra-hepatic tissues. The apo components serve to maintain the structural integrity of the particles and influence the enzyme activity involved in their metabolism and act as ligands for cell-surface lipoprotein receptors. Lipoprotein (a) (Lp (a)) is a variant of low-density lipoprotein (LDL), which contains an extra protein, apolipoprotein (a), which is structurally similar to plasminogen (Wheeler & Bernard, 1994).

Plasma cholesterol and triglyceride concentrations are increased in NS patients with heavy proteinuria. Usually, the magnitude of lipid abnormality correlates with disease severity. The nature of the glomerular lesion does not seem to have an effect on the pattern or magnitude of lipid abnormality. The number of LDL particles present in NS is increased and the VLDL concentration is often increased as well. Total high-density lipoprotein (HDL) concentration could be high, normal or low. Two subtypes of HDL exist, namely, HDL_2 and HDL_3. In NS HDL_2 is reduced while HDL_3 is increased (Figure 1.7). This disturbance in HDL together with increased VLDL and LDL cholesterol levels are associated with a risk of atherosclerosis. HDL_2 is involved in the recycling of apo CII to VLDL and chylomicrons. Lp (a) levels are increased in NS with proteinuria and this represents another risk factor for atherosclerotic vascular disease in these patients (Wheeler & Bernard, 1994).

Other abnormalities in nephrotics include higher cholesterol to triglyceride ratio in apo-B-containing particles and an increase in the proportion of cholesterol, cholesterol ester and phospholipid relative to protein. Accumulation of abnormal lipoproteins implies defective catabolism, which is also associated with an increased atherosclerosis risk in non-nephrotic populations (Wheeler & Bernard, 1994).

Plasma levels of apo-B, -C and -E are increased in NS, which is in keeping with an increase in the number of circulating LDL and VLDL particles. Apo-AI and –AII and the major HDL apolipoproteins are no different in nephrotics than in control individuals. Apo-B, which is the major LDL protein, is increased proportionately more than the other apolipoproteins which is consistent with the pattern of lipoprotein abnormality Plasma levels of apo CII and CIII subclasses are elevated and the ratio of CIII to CII is increased. Apo CII activates while apo CIII competitively inhibits LPL. Therefore, ratio changes in these two components may contribute to the defective enzyme activity of LPL and result in a reduced catabolism of lipoprotein in NS (Wheeler & Bernard, 1994).

Elevated total and LDL cholesterol is common in NS patients. In cases of severe proteinuria or hypoalbuminaemia, increased triglycerides with VLDL cholesterol is found. Apo B (the VLDL and the LDL component) is elevated. Apo CII is elevated in plasma even though there is renal loss. Apo CII is necessary for LPL activity (Thabet *et al.*, 1993). Serum apo CII is the

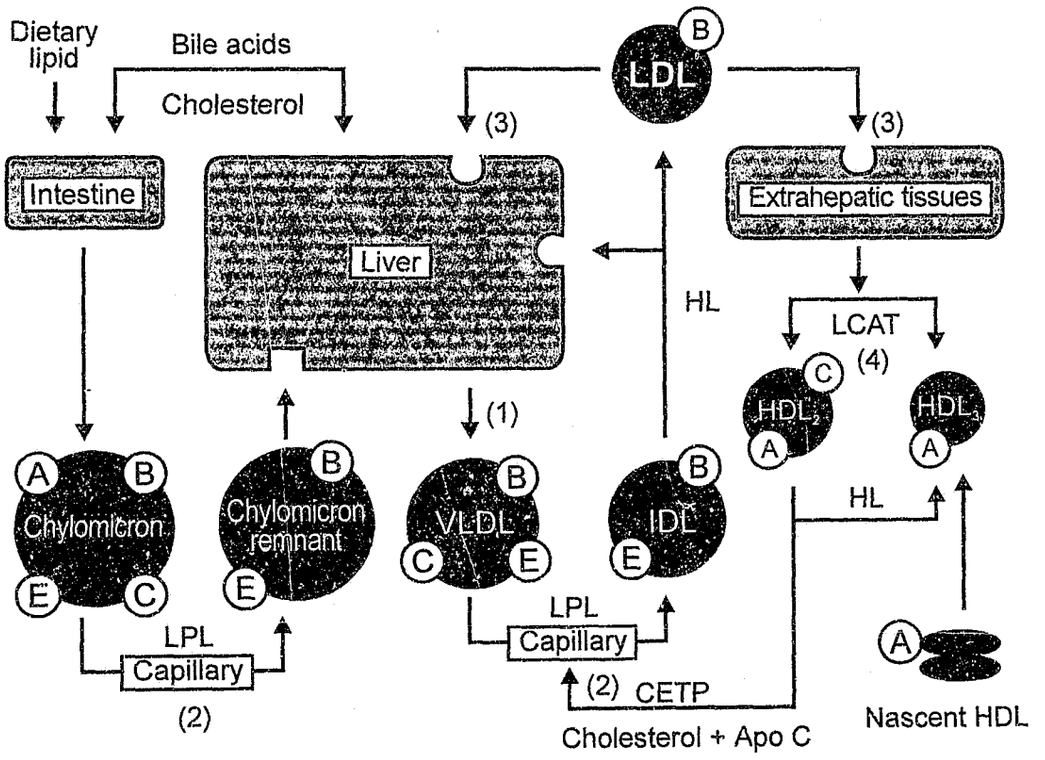


Figure 1.7 Lipoprotein metabolism in NS. In NS, hepatic VLDL production is increased (1), leading to elevated circulating levels of VLDL, IDL and LDL. This is compounded by defective catabolism of these particles in the peripheral circulation as a result of reduced LPL activity (2). In addition, receptor-mediated uptake of LDL particles may be impaired (3). HDL maturation is inhibited as a result of diminished LCAT activity (4). These defects in the HDL pathway are likely to contribute to impaired catabolism of triglyceride-rich lipoproteins (Wheeler and Bernard, 1994).

competitive inhibitor of apo CII and is increased in nephrotic patients. When the apo CIII/apo CII ratio is increased, LPL activity is inhibited (Kaysen, 1991).

The levels of cardioprotective HDL was found to be high, normal or low depending on the drug therapy of the patient. Low HDL cholesterol concentration is observed in untreated nephrotics, normal HDL concentration when the patient is on non-steroidal drugs and high HDL concentration in patients on steroids. HDL subtype distribution is normal in that the HDL_3 subtype is elevated, while the HDL_2 subtype is very low in NS patients. The HDL_2 subtype is known to prevent atherosclerosis (Thabet *et al.*, 1993). The omega-6 fatty acids have an abnormal distribution in nephrotic children. The arachidonic acid in plasma phospholipids and the linoleic acid in triglycerides of subcutaneous adipose tissue are increased, compared to age-matched healthy children (Strauss *et al.*, 1987).

1.8.4.1 Hyperlipidaemia

Hyperlipidaemia does not necessarily persist in NS, but it can be transient and correlate with disease activity and in some cases it can persist indefinitely even during remission (Strauss *et al.*, 1987). Zilleruelo *et al.*, (1984) demonstrated that 24 out of 51 children with MLNS/(MCNS) in remission still had elevated cholesterol, triglycerides, LDL and VLDL (Zilleruelo *et al.*, 1984). High concentrations of cholesterol are also seen in patients with longer and more frequent relapses (Thabet *et al.*, 1993).

The pathology of nephrotic hyperlipidaemia is multifactorial. Increased hepatic synthesis together with decreased clearance of circulating lipoproteins add to the hyperlipidaemia (Kaysen, 1991). The increased hepatic lipogenesis is possibly due to changes in the serum albumin concentration or the plasma oncotic pressure. A viscosity change at the hepatic sinusoid level could send the necessary signal. Loss of urinary proteins, including lipoproteins could also trigger the hepatic synthesis of lipids (Thabet *et al.*, 1993).

Children with NS often have increased cholesterol, triglyceride, LDL and VLDL concentrations. There is an inverse relationship between serum albumin and serum lipoprotein

concentrations (Joven *et al.*, 1990; Keane & Kasiske, 1990). Serum lipid and lipoprotein changes are caused by increased hepatic synthesis, decreased clearance and altered enzyme activities (Appel *et al.*, 1985). Circulating lipoprotein clearance is impaired and lipoprotein lipase activity is decreased by 30–60% in NS patients (Kashyap *et al.*, 1980).

Serum lathosterol to cholesterol ratio is a measure of whole body cholesterol synthesis. Lathosterol is produced along the major route of cholesterol synthesis and is a precursor of cholesterol. Serum lathosterol is bound to lipoproteins and its concentration is positively correlated with total cholesterol and with apo B. The lathosterol to cholesterol ratio is a reliable index of whole body cholesterol synthesis in normocholesterolaemia and in hypercholesterolaemia. Therefore this ratio is a valid measure of cholesterogenesis in NS patients. It is believed that hepatic cholesterol synthesis is unaltered in human NS (Dullaart *et al.*, 1996).

The lathosterol to cholesterol ratio is lower in moderately hyperlipidaemic NS patients compared to healthy controls (Dullaart *et al.*, 1996). These findings cast doubt on the hypothesis that increased cholesterol synthesis provides an important mechanism responsible for maintenance of hypercholesterolaemia associated with NS in humans. The patients investigated in this study had established NS and their cholesterol synthesis was probably elevated in the initial stage of their renal disease. This study showed no relationship between either proteinuria, or serum albumin level and cholesterol to lathosterol ratio at baseline or during treatment. It is unlikely that an improved lipoprotein profile after antiproteinuric treatment is associated with cholesterol synthesis inhibition (Dullaart *et al.*, 1996).

Impaired catabolism of VLDL and LDL in NS patients cause accumulation of these lipoproteins in circulation. This impaired lipoprotein catabolism may be related to a defective metabolism of a lipid regulatory factor, together with urinary protein loss (Dullaart *et al.*, 1996).

Cholesterol synthesis is known to be influenced by dietary factors where low fat, low cholesterol diets increase hepatic cholesterol synthesis. Dullaart *et al.*, (1996) reported that

increased cholesterogenesis is not involved in hypercholesterolaemia associated with human NS. It is unlikely that the decrease of apo B-containing lipoproteins is attributable to cholesterol synthesis inhibition (Dullaart *et al.*, 1996).

1.8.4.2 Pathophysiology and mechanisms of hyperlipidaemia in nephrotic syndrome

There are three postulated causes of hyperlipidaemia in nephrosis: (i) increased hepatic synthesis of cholesterol, triglycerides and lipoproteins, (ii) decreased post-hepatic LPL activity leading to diminished conversion of LDL to HDL, and (iii) decreased LDL receptor activity and increased urinary loss of LDL causing disturbed lipoprotein metabolism (Thabet *et al.*, 1993).

1.8.4.2.1 Increased hepatic lipoprotein production

In response to hypoalbuminaemia in NS, hepatic production of albumin as well as other plasma proteins is increased and the synthesis of cholesterol and triglycerides may be enhanced in parallel with the apolipoproteins. The synthesis of triglycerides and cholesterol is increased in humans with NS. The apo-B moiety of VLDL and LDL synthesis is also increased. There could be strong evidence in favour of enhanced hepatic synthesis of VLDL in NS. However, interpretation of kinetic studies rely on complex mathematical modelling which involves many assumptions which may not hold true in the context of NS (Wheeler & Bernard, 1994).

It is suspected that the stimulus for enhanced hepatic lipoprotein synthesis in NS is directly related to hypoalbuminaemia, as in nephrotic humans and rats, albumin infusions normalise plasma lipid and lipoprotein levels. Dextran which is an oncotically active macromolecule is just as effective, which suggests that a drop in plasma oncotic pressure could be a more important trigger to increased lipoprotein production by the liver (Wheeler & Bernard, 1994). ACE inhibitors lower urinary protein excretion and improve hyperlipidaemia, but it does not change the albumin synthesis rate. These agents may partially correct the increased lipid levels in nephrotic patients. This suggests that urinary loss of a substance, which regulates

lipid metabolism, may play a role in the pathogenesis of nephrotic hyperlipidaemia (Wheeler & Bernard, 1994).

Another explanation for increased hepatic lipogenesis in NS is that the kidney is the major organ responsible for the metabolism of a cholesterol synthesis precursor, mevalonate. Impaired renal mevalonate metabolism could lead to increased plasma levels and increased hepatic production of cholesterol. Increased hepatic cholesterol concentrations may enhance VLDL production and decrease LDL receptor expression which reduces the cholesterol clearance rate from circulation (Wheeler & Bernard, 1994).

Increased hepatic production of proteins other than lipoproteins could contribute to hyperlipidaemia in NS. Plasma levels of cholesteryl ester transfer protein (CEPT) are increased in patients with heavy proteinuria due to over synthesis by the liver. CEPT mediates the transfer of esterified cholesterol from HDL to triglyceride-rich lipoproteins. On the basis of the observation that genetic CEPT deficiency leads to low VLDL and LDL cholesteryl ester concentrations, it is proposed that high levels in nephrotic patient plasma may result in cholesterol enrichment of these lipoproteins (Wheeler & Bernard, 1994).

1.8.4.2.2 Defective lipoprotein catabolism

There is little doubt that lipoprotein catabolism is impaired in NS. LPL enzyme function is impaired which is presumed to be the major cause of the catabolic defect. Factors causing the reduced LPL activity are postulated to be: (i) enzyme is lost in the nephrotic urine, or (ii) hypoalbuminaemia results in free fatty acid accumulation which inhibits enzyme activity (albumin augments LPL by binding free fatty acids generated from lipoprotein hydrolysis), or (iii) urinary loss of a LPL co-factor could contribute to reduced plasma activity of this enzyme (this is most likely as it is consistent with the observation that urinary albumin clearance correlates with plasma lipid abnormalities) (Wheeler & Bernard, 1994).

Other molecules which may influence LPL activity and are small enough to be lost in urine of nephrotic patients are the enzyme co-factor apo-CII, HDL particles (transfer apo CII to

VLDL) and glycosaminoglycans (tether LPL enzyme to vascular endothelium) Absolute plasma levels of apo CII are not decreased in cases of heavy proteinuria, but a relative deficiency exists as the proportion of apo CII per VLDL unit is reduced. A glycosaminoglycan deficiency alone is not responsible for impaired lipoprotein catabolism. Urinary loss of HDL may impair recycling of apo CII to VLDL and thereby reduce LPL activity (Wheeler & Bernard, 1994).

Other factors than reduced LPL activity are also responsible for impaired lipoprotein catabolism in NS. Reduced activity of lecithin: cholesterol acetyltransferase (LCAT), another key enzyme in lipoprotein catabolism, also occurs in rat and in human nephrotic plasma. Hypoalbuminaemia could be responsible for this as albumin binds lysolecithin (a product of the LCAT reaction). Accumulation of lysolecithin impairs enzyme activity. LCAT could also be depleted due to loss in urine. Low LCAT levels impair cholesterol esterification within the HDL particle which inhibits conversion of HDL_3 to HDL_2. The defect in HDL maturation reduces transfer of apo CII to VLDL, which inhibit catabolism of triglyceride-rich lipoproteins (Wheeler & Bernard, 1994).

1.8.4.2.3 Defective receptor clearance of lipoprotein particles

Another abnormality, which contributes to nephrotic hyperlipidaemia, is the defective removal of intermediate-dense lipoprotein (IDL) and LDL from the circulation by lipoprotein receptors. At this stage there is still a lot of controversy about this mechanism (Wheeler & Bernard, 1994).

1.8.4.3 Complications of hyperlipidaemia in nephrotic syndrome

High plasma lipids have two potential risks. Elevated plasma concentration of cholesterol is associated with atherosclerosis, which leads to cardiovascular disease in NS patients. Secondly a strong possibility exists that lipid deposits could form in the glomerulus and ultimately cause renal failure. These hypotheses have not been proven although data supports both possibilities (Bernard, 1988; Ordoñez *et al.*, 1990).

Hyperlipidaemia has been implicated in cardiovascular diseases (Martin *et al.*, 1986) and is thought to play a role in renal disease as well. It is not sure whether it has an indirect or direct e' ct on the kidneys (Berlyne & Mallick, 1969). An increase in the incidence of ischaemic heart disease in nephrotic patients was disputed by Wass *et al.*, (1979). Hyperlipidaemia might be involved in the progressive glomerular damage, which leads to renal failure (Grundy & Vega, 1989).

Vascular disease due to hyperlipidaemia is the most common cause of death in renal disease patients (Robertson *et al.*, 1995). It should be assumed that hyperlipidaemia in NS patients could be a serious risk for progressive atherosclerosis. Therefore the effort should be made in high-risk patients such as patients with persistent NS, and patients with unfavourable lipoprotein profiles (especially in the presence of atherosclerotic cardiovascular disease or other atherosclerotic risk factors) to correct abnormal lipid levels. The most effective treatment is dietary management together with β-hydroxy-β-methylgluteryl (HMG)-CoA reductase inhibitors (Wheeler & Bernard, 1994).

1.8.4.3.1 Nephrotic hyperlipidaemia and cardiovascular disease

In the general population, atherosclerosis is usually associated with increases in total and LDL cholesterol with low HDL concentrations. High HDL concentrations is considered to be cardioprotective. Cardiovascular disease in NS is believed to be the result of hyperlipidaemia, but these patients also have hypertension, hypercoagulability and other risks contributing to cardiovascular disease development (Thabet *et al.*, 1993).

Atherosclerosis develops over 30 to 50 years and few NS patients have been followed that long. Their cardiovascular disease is not evaluated as the renal disease is predominant and renal failure is stated as the cause of death (Thabet *et al.*, 1993).

Many lip' ' bnormalities found in NS patients such as increased total and LDL and VLDL cholesterol, reduced HDL_2 relative to HDL_3 and increased Lp (a) concentrations are associated with cardiovascular disease risk in other population groups. NS patients often have additional

risk factors for atherosclerosis, such as hypertension and hypercoagulability (Wheeler & Bernard, 1994).

Patients with familial hypercholesterolaemia, with cholesterol levels similar to NS patients, have a coronary artery disease rate of 20% at age 40 and 75% at age 60. At this stage it seems reasonable to conclude that hyperlipidaemia in NS patients, represents a serious risk for development of atherosclerosis at least in some of the patients (Wheeler & Bernard, 1994). Hopp *et al.*, (1994) described a case of a seven year old boy with steroid unresponsive MLNS/ (MCNS) for five years who developed acute myocardial infarction. The boy presented with acute myocardial infarction due to a dissected atherosclerotic plaque. This child had a long history of extreme hypercholesterolaemia and hypertriglyceridaemia together with an apolipoprotein E_4/E_3 phenotype. The mother also had the apo E_4/E_3 phenotype and mild hypercholesterolaemia. This finding suggests that children with long-lasting NS and a mild familial hyperlipidaemia, may have an increased risk for cardiovascular disease, as myocardial infarction is very rare in children (Hopp *et al.*, 1994).

NS patients have many risk factors for developing ischaemic heart disease, which includes an increased clotting tendency, platelet hyperfunction, steroid therapy and hyperlipidaemia. Regardless of this, the incidence of ischaemic heart disease among nephrotic patients is debatable. To date there is no statistical evidence for or against the association between NS and ischaemic heart disease (Hopp *et al.*, 1994). Hopp *et al.*, (1994) propose that persistent NS together with a familial risk factor could increase the risk for ischaemic heart disease in children. A low fat diet, lipid-lowering agents and low-d aspirin could be preventative (Hopp *et al.*, 1994).

Some case studies showed an increased risk of coronary heart disease in NS patients and others failed to do so. All these previous studies were criticised for having small sample sizes, ill-defined comparison groups or incomplete documentation of coronary heart disease among the subjects (Wardle, 1979). Ordoñez *et al.*, (1993) used a large number (142) of NS patients and a control age-matched, diabetes-free group of the same size. Analysis was adjusted for hypertension and smoking. The results suggest that NS patients are at an increased risk for

developing coronary artery disease. The estimated risk for myocardial infarction is five to six times higher in NS individuals than in controls. Events and deaths from coronary heart disease are two to three times higher in NS patients than in control individuals of the same age and sex. Thirteen NS subjects, who had coronary heart disease before NS was diagnosed, were excluded from the analysis. If these individuals were included, the results would have been even more striking (Ordoñez *et al.*, 1993).

Hyperlipidaemia and NS are so closely associated that it is not possible to determine their independent effects on coronary heart disease risk (Ordoñez *et al.*, 1993).

1.8.4.3.2 Nephrotic hyperlipidaemia and glomerular injury

Increased cholesterolaemia could lead to FSGS through several pathways: (i) altered prostaglandin metabolism with increased thromboxanes and decreased prostacyclin which can alter blood viscosity, in turn produce changes in glomerular haemodynamics with increased platelet activation leading to glomerular injury, (ii) an increased ratio of saturated fatty acids to unsaturated fatty acids, which decrease membrane fluidity and cause endothelial damage with release of platelet-derived growth factors, leads to mesangial proliferation and matrix expansion, (iii) increased peroxidated lipoproteins cause the release of free radicals, cytokines and growth factors which could result in glomerular injury (Thabet *et al.*, 1993) (Figure 1.8).

The pathology includes increased mesangial matrix and cellularity, lipid-laden "foam cells" and infiltration of the glomerulus by macrophages and monocytes (a condition resembling arteriosclerosis) (Thabet *et al.*, 1993).

The large LDL particles are not filtered while the smaller HDL particles are excreted in urine. The high LDL concentration is toxic to mesangial cells (Moorhead *et al.*, 1989). Hyperlipidaemia may exacerbate renal injury via a variety of different mechanisms. Lipid becomes deposited in the diseased kidney early in the course of renal disease. In experimental nephrotic rats, lipid deposits of cholesterol, triglycerides, phospholipids, apo A, B and E as well as oxidised LDL particles were found in glomeruli (Wheeler & Bernard, 1994).

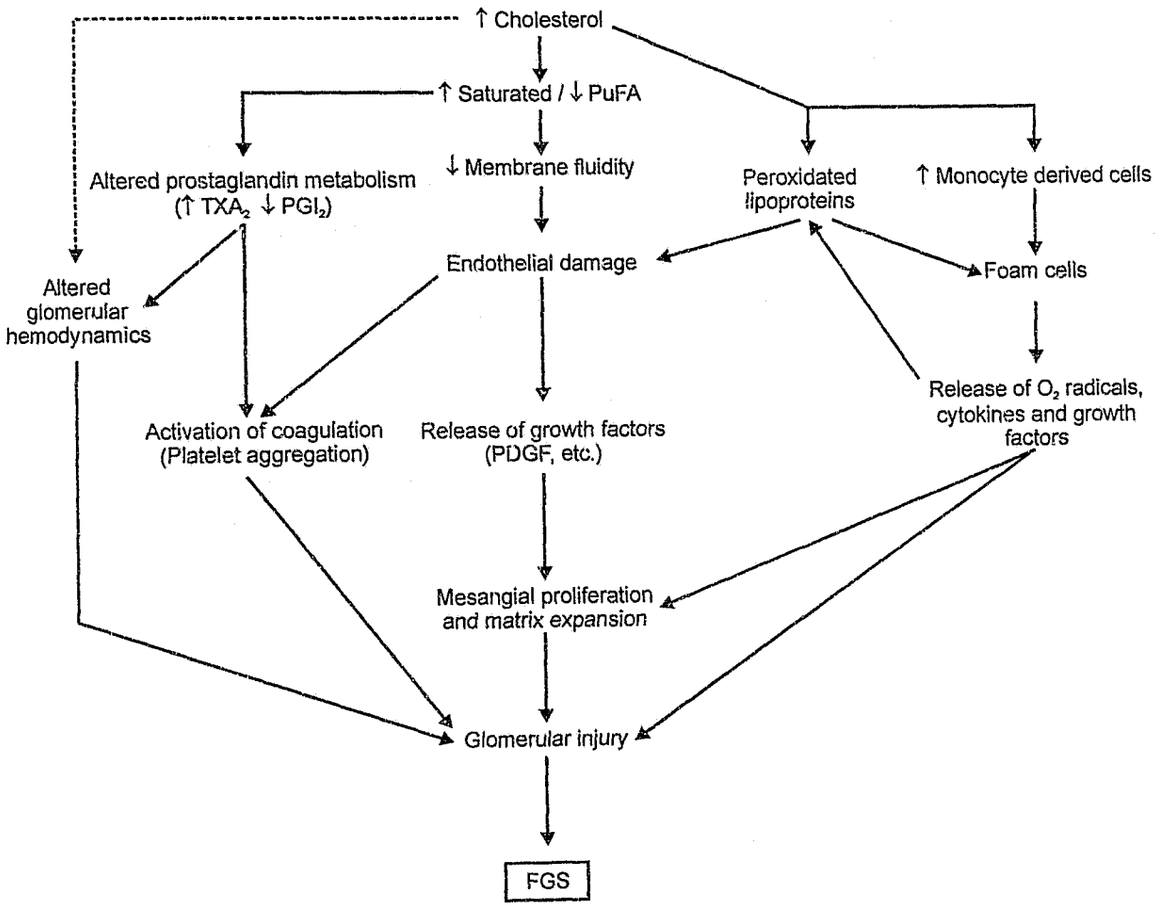


Figure 1.8 Diagram of events possibly involved in cholesterol-induced glomerular injury (Thabet, 1993).

Normal glomeruli capillaries are lined by a fenestrated endothelium, macromolecules the size of lipoproteins may gain unimpeded access to the mesangium, but are usually returned to the capillary via a similar route and are cleared by lymphatic channels. Lipoprotein is deposited in the mesangium when clearance mechanisms are defective or overloaded after loss of functional nephron mass. Phagocytosis of fatty-acid-laden albumin by proximal tubular cells could promote deposition of lipids in the tubulointerstitium (Wheeler & Bernard, 1994).

A feature of early glomerulosclerosis is mononuclear cell infiltration due to lipid deposition in the glomerulus. The monocytes which infiltrate the mesangium, differentiate into tissue macrophages that ingest deposited lipids to become foam cells. These foam cells may release inflammatory mediators, which modify glomerular function. Lipoproteins may act with these inflammatory mediators to promote glomerular injury. LDL stimulates monocyte chemoattractant protein-1 production by mesangial cells and this is quite likely the mechanism by which LDL mediates the inflammatory response (Wheeler & Bernard, 1994). Diets deficient in essential fatty acids inhibit monocyte influx which explains their protective effect in experimental glomerular disease (Wheeler & Bernard, 1994).

LDL enhances proliferative effects of mesangial mitogens and it increases the synthesis of matrix components by cultured cells. LDL stimulates the production of reactive oxygen species when incubated *in vitro* with mesangial cells. Oxidised LDL is cytotoxic to mesangial culture cells and it stimulates the production of thromboxane A_2, which is a potent eicosanoid causing vasoconstriction. Lipoprotein oxidation together with increased thromboxane A_2 production could contribute to lipid-induced renal injury (Wheeler & Bernard, 1994).

In conclusion, accumulation of lipoproteins in the mesangium initiate a chronic inflammatory reaction together with macrophage infiltration, disturbed mesangial cell secretory function, mesangial death and excessive accumulation of matrix components, which lead to irreversible scarring. These events are similar to the process causing fibrous plaques in arterial walls (Wheeler & Bernard, 1994).

When renal function is normal, the kidney is protected from lipid-induced injury. Some dyslipidaemias associated with abnormal circulating lipoprotein particles could cause glomerular lipid accumulation and renal damage (Wheeler & Bernard, 1994).

In adults and children with non-diabetic renal disease, increased plasma lipid levels are associated with a faster rate of renal function deterioration (Maschio *et al.*, 1989). In type I diabetes, hypercholesterolaemia is also an independent risk factor in the development of nephropathy (Mulec *et al.*, 1990). Only one long-term clinical trial showed that lipid-lowering agents reduce proteinuria (Rabelink *et al.*, 1990).

Studies done to investigate whether hyperlipidaemia reduction leads to a decreased risk of renal failure are too short in duration and did not achieve lipid reduction levels associated with regression of atherosclerotic plaques (Keane *et al.*, 1992).

1.9 Complications

NS is the result of the reduced ability of the glomerular barrier to exclude intermediate size (40-200kDa) proteins from urine. Even when barrier function is severely disrupted, the very large proteins remain excluded from the glomerular ultrafiltrate. Urine proteins then include albumin, immunoglobulins, proteins of the clotting cascade, erythropoietin and hormone-binding proteins together with the hormones they carry (Kaysen, 1994).

Complications of NS include: (i) renal complications such as renal failure and ESRD (Robson & Leung, 1993), (ii) increased infection susceptibility due to urinary loss of immunoglobulins, (iii) renal vein thrombosis due to urinary loss of fibrinolytic factors and increased hepatic synthesis of clotting factors, (iv) premature atherosclerosis due to increased protein synthesis to make up for urinary loss of protein (Robertson *et al.*, 1995), (v) cerebral infarctions (Chaturvedi, 1993), (vi) failure to thrive because of anorexia, protein loss in urine, increased metabolism of protein and the frequency of infections, (vii) tetany due to hypocalcaemia (Robson & Leung, 1993), (viii) decreased erythropoietin plasma levels, (ix) increased hepatic synthesis of albumin, transferrin, fibrinogen and apolipoproteins B and E, (x) deficiency

syndrome due to steroid hormones lost in physiologically significant quantities, and the loss of proteins that bind specific nutrients particularly vitamin D and zinc and (xi) muscle wasting due to continuous proteinuria (Kaysen, 1994).

1.10 Prognosis

Congenital NS has the worst prognosis. For secondary NS, the underlying disease and its specific treatment determines the prognosis. Mortality in primary NS used to be as high as 50% but has decreased drastically with a better understanding and treatment of the disease. The most common causes of death in primary NS include infections, thromboemboli and congestive heart failure due to infusion of albumin to promote diuresis (Robson & Leung, 1993).

ESRD occurs commonly in FSGS patients requiring either dialysis or renal transplantation. The risk for developing ESRD in FSGS children is influenced by race as black and Hispanic children with FSGS are more likely to progress to ESRD than white children. Children with MPGN progress very slowly toward ESRD as at age ten approximately 50% of these children have reached ESRD and at age 20, it is approximately 90% (Robson & Leung, 1993).

1.11 Treatment of nephrotic syndrome

The introduction of penicillin treatment in 1944 reduced NS childhood mortality from approximately 40% to 16% and dropped further to three to seven percent in the 1950's when corticosteroid treatment was introduced (Melvin & Bennett, 1991). ACE inhibitors and NSAID's reduce the degree of proteinuria in NS patients, but their use in FSGS has not been positive. The immunosuppressant, FK506 has been helpful in steroid resistant NS. Patients had a 50% proteinuria reduction, but upon discontinuation, relapse of proteinuria to the level prior to treatment results (Korbet, 1995).

Better results and less complications occurred when standard immunosuppressive therapy of CyA, prednisolone and often azathioprine are used after renal transplantation (Thomson,

1997) than when total lymphoid irradiation is done prior to renal transplantation in children (Myburgh *et al.*, 1987). When low-dose aspirin is used in the regimen after renal transplantation, thrombotic complications do not occur with use of CyA (Halkas *et al.*, 1996).

Treatment also involves haemodialysis and transplantation. According to Habib, (1993), disease does not recur after transplantation (Habib, 1993), but Robson and Leung, (1993) reported that NS recurs in the transplanted kidneys in cases of primary NS with FSGS, MPGN, MembGN and in secondary NS due to SLE (Holmberg *et al.*, 1995). FSGS and MPGN are the two most problematic forms of NS regarding recurrence after transplantation, although FSGS has only recurred once in a black South African child with FSGS (Personal communication Prof. PD Thomson, Head Paediatric Nephrology, Johannesburg Hospital S.A.) Renal transplantation is the only curative therapy for CNF (Holmberg *et al.*, 1995).

Therapeutically, NS can be classified into four groups namely: steroid sensitive, steroid resistant and steroid dependent or frequently relapsing (Robson & Leung, 1993).

1.11.1 Diet

Low fat, low cholesterol with high polyunsaturated fat to saturated fat ratio diet is recommended before resorting to pharmacological agents in treatment of hyperlipidaemia in proteinuric patients (Wheeler & Bernard, 1994).

1.11.2 Fish oil supplements

Fish oil enriched diets have good effects on plasma lipids and renal function in nephrotic rats, which have low circulating LDL concentrations. Fish oils reduce blood viscosity, increase RBC deformability and diminish platelet aggregability. Fish oils thereby have the potential to decrease thrombotic event incidence in NS patients (Wheeler & Bernard, 1994).

Fish oils are rich in long chain ω-3 polyunsaturated fatty acids which are incorporated into cell membrane phospholipids and substitute for arachidonic acid (an ω-6 compound) as substrates

for prostaglandin, thromboxane and leukotriene synthesis. The resulting eicosanoids have different properties to the arachidonic acid-derived mediators and have a much wider variety of biological effects. Oxidation of ω-3 fatty acids have adverse renal function effects in rats, therefore anti-oxidants such as vitamin E is given with fish oil (Wheeler & Bernard, 1994).

1.11.3 Lipid-lowering agents

1.11.3.1 Bile acid sequestrants

These agents bind bile acids in the lumen of the intestines and interrupt their enterohepatic cycle, thereby reducing hepatic cholesterol content. Upregulation of hepatic LDL receptors results and this leads to a drop in the LDL concentrations. These agents have limited use in NS patients as it causes gastrointestinal upsets (Wheeler & Bernard, 1994).

1.11.3.2 Fibric acid derivatives

Bezafibrate, fenofibrate and gemfibrozil interfere with hepatic synthesis of triglyceride and cholesterol by enhancing LPL activity. An early fibric acid derivative, clofibrate was associated with acute myositic syndrome in some patients. The newer drugs in this class appear safe, but plasma creatinine phosphokinase levels should be checked regularly. These agents should be avoided or used with caution in cases of severely impaired renal function (Wheeler & Bernard, 1994).

1.11.3.3 Probucol

This drug is thought to enhance LDL catabolism via a non-receptor-mediated mechanism. It is an effective anti-atherogenic agent in animals. The protective effect of probucol is attributed to antioxidant rather than lipid-lowering properties. This drug is relatively free of side effects but its effect on plasma lipids is so moderate that it is not regarded as first-line therapy in NS (Wheeler & Bernard, 1994).

1.11.3.4 β-Hydroxy-β-Methylglutaryl-Co A reductase inhibitors

These drugs inhibit β-Hydroxy-β-Methylglutaryl-Co A (HMG-CoA) reductase, which is the rate-limiting enzyme in the synthesis of cholesterol in the liver. A compensatory increase in LDL receptor expression leads to increased hepatic clearance of LDL particles. A decrease in VLDL triglycerides and an increase in HDL follows. Beta-Hydroxy-β-Methylglutaryl-CoA (HMG-CoA) reductase inhibitors are the most effective lipid-lowering compounds in the treatment of nephrotic hyperlipidaemia. Lovastatin, simvastatin and pravastatin are well tolerated and they reduce total plasma cholesterol, LDL cholesterol and total plasma triglyceride. Combining bile-acid sequestrants or probucol with these agents enhances their efficacy (Wheeler & Bernard, 1994).

1.11.3.5 ACE inhibitors

These agents are not regarded as lipid-lowering agents, but they do lower plasma lipids in nephrotic rats, which results from reduced urinary loss of a factor important in lipoprotein catabolism. Patients treated with these agents who showed no decline in protein excretion, showed reduced cholesterol levels. This indicates the possibility that converting enzyme could play a direct role in the pathogenesis of this lipid disorder (Wheeler & Bernard, 1994).

1.12 Objectives of the present study

A vast amount of clinical knowledge is available on the classification, complications and treatment of the various forms of NS, yet there is comparatively little information about the genetic factors that contribute to this group of disorders. NS is a polygenic disease and therefore our approach was to determine whether molecular variation of certain candidate genes might play a role in the prevalence of NS in black and white South African children.

This syndrome is thought to be caused by the interaction between genetic and environmental factors that include infectious agents. The disease pathophysiology and presentation thus

being determined by the inflammatory response resulting from this interaction. Some of the factors associated with the syndrome include the proteinase inhibitor α_2 macroglobulin (α_2M), apolipoprotein E and protein S.

Alpha$_2$M is a multifunctional and targeting protein (Borth, 1994), which binds proteinases, ligands and cytokines (Gaillard & Kilroe-Smith, 1987). Recent studies of glomerular sclerosis suggest that fibrogenic cytokines, in particular transforming growth factor- β_1 (TGF-β_1), play a central role. TGF-β is known to increase the production of collagens, fibronectin and proteoglycans as well as to upregulate the expression of extracellular matrix receptors (Border *et al.*, 1992; Border & Ruoslahti, 1992). The interaction of TGF-β_1 with the binding proteins such as α_2M, represent potential mechanisms to regulate the multiple and varied growth effect of this cytokine. One of the mechanisms implicated in the cell proliferative response is that α_2M delivers TGF-β_1 to the α_2M receptor/lipoprotein receptor protein (LRP) (Stouffer *et al.*, 1993).

LRP is a receptor for multiple ligands and it also interacts with apolipoprotein E complexes (Hyman *et al.*, 1994). Certain genotypes of apolipoprotein E, particularly apolipoprotein E$_4$, have been shown to be associated with NS (Lerique *et al.*, 1994). Apolipoprotein E$_4$ is not as efficient in interacting with the LRP as the other apolipoprotein E genotypes. This would thus lead to an increased deposition of triglycerides in various tissues and also enhance the α_2M TGF-β_1 complex binding to LRP, thus causing proliferation of various cells in the kidney matrix, resulting in matrix proliferation. These findings prompted studies to determine the levels of activated α_2M in plasma of black and white South African children, with and without NS, as well as to determine the apolipoprotein E genotypes present in these children.

Thrombotic events are observed in many children with NS, due to abnormalities in procoagulant and anticoagulant protein levels (Mehls *et al.*, 1987) such as increased levels of α_2M (Thaler *et al.*, 1978) and protein S deficiency. Protein S is a plasma glycoprotein with an anticoagulant function and it serves as a cofactor for activated protein C. Protein S

directly inhibits factor Va and Xa, independent of protein C (Heeb *et al.*, 1993; Heeb *et al.*, 1994). In plasma, protein S circulates in two distinct forms, free protein S which acts as an anticoagulant and protein S-C4-binding protein complex which is inactive (Dahlback, 1986; Nishioka & Suzuki, 1990). A deficiency of protein S has been associated with thrombosis (Heeb *et al.*, 1993; Kemkes-Matthes, 1992). A polymorphism in the protein S gene was described recently in an Italian family, and was associated with symptomatic protein S deficiency (Marchetti *et al.*, 1993). This finding prompted a study to determine whether this polymorphism can be found in black and white South African children, with and without NS.

1.13 Summary of aims of this study

- To assess the functional levels of α_2M in South African paediatric NS patients and in children and adult control individuals.
- To determine the apolipoprotein E genotypes in South African white, and black children with FSGS or MLNS/(MCNS), and to look for an association of genotype with FSGS or MLNS/(MCNS).
- To determine the prevalence of the two different protein S alleles in South African white, and black children with NS and in black and white control individuals.

CHAPTER 2: α_2 MACROGLOBULIN IN NEPHROTIC SYNDROME

2.1 Introduction to α_2Macroglobulin

2.1.1 Structure of α_2Macroglobulin

Alpha$_2$Macroglobulin (α_2M) is a plasma protein of 725kDa (Kilroe-Smith *et al.*, 1989), which acts as a competitor of α_1 protease inhibitor (PI) for binding to proteinases (Gaillard *et al.*, 1987).

Alpha$_2$M is a tetrameric protein consisting of two non-covalently bound pairs of identical subunits joined by disulphide bonds (Hall & Roberts, 1978). Each of the two subunits contains amino acid sequences, which can be cleaved by proteinases. This cleavage causes a conformational change in the molecule and this in turn results in irreversible protease binding. The enzyme-α_2M complex can still degrade small molecular weight substrates as α_2M traps the enzyme, but does not bind to the active site. This newly formed α_2M-protease complex can bind growth factors and cytokines and modulate their inflammatory effects (Gaillard *et al.*, 1995).

Since 1974, α_2M has been known to be micro-heterogeneous (Frenoy & Bourrillon, 1974) which is due to the differences in carbohydrate composition of its different molecular forms (Sottrup-Jensen *et al.*, 1984). The carbohydrate portion of the molecule, is responsible for various degrees of steric hindrance in the reaction of the substrate, with the elastase-bound α_2M giving different specific activities to the α_2M-elastase complexes (Gaillard *et al.*, 1989) (Figure 2.1).

2.1.2 The trap mechanism of α_2Macroglobulin

The "trap" hypothesis for the broad reactivity of α_2M with proteinases was suggested for the first time in 1973 (Barrett & Starkey, 1973). Initially there were arguments against this hypothesis but subsequently it was shown that the reaction of α_2M with proteinases does involve proteolytic cleavage and that it occurs at the same point in the molecule despite the

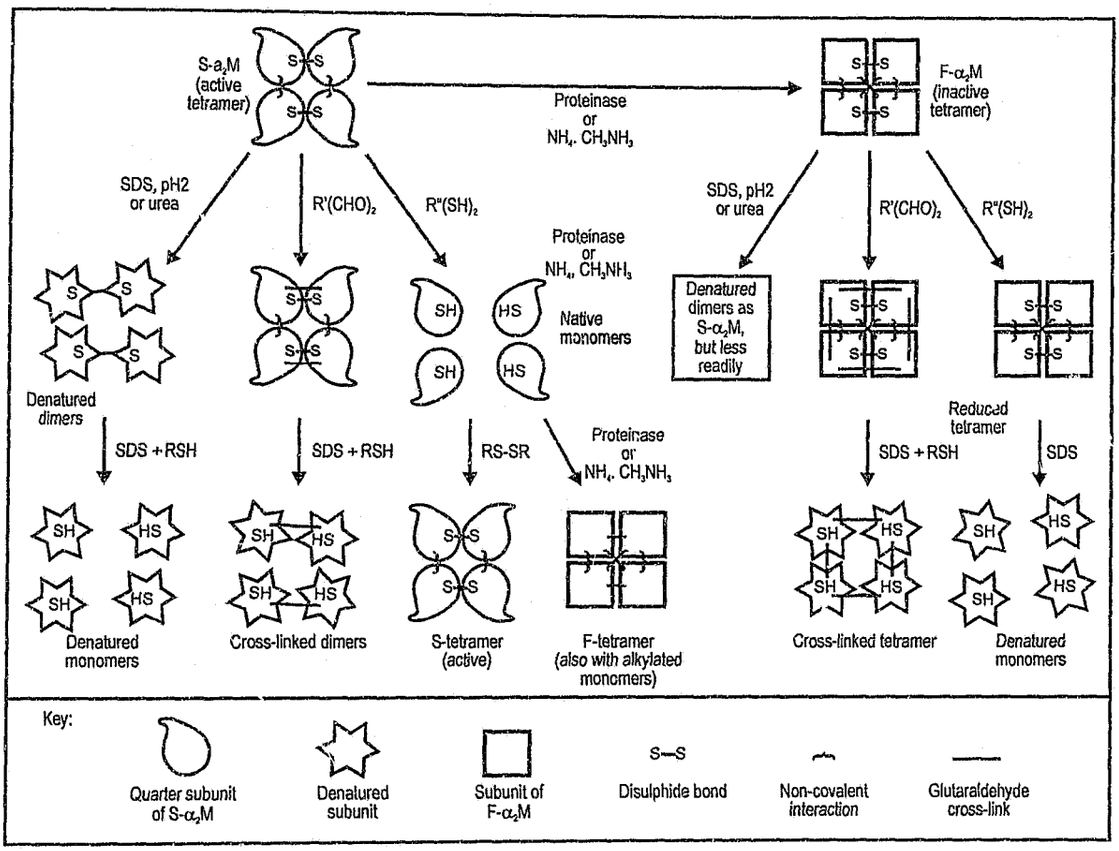


Figure 2.1 Diagrammatic illustration of possible α_2M molecule transitions (Barrett *et al*, 1979).

wide variety of proteinase specificities. It was demonstrated by electron microscopy, that the appearance of the α_2M molecule is consistent with a conformational change associated with the binding of proteinases (Barrett *et al.*, 1979). These two pieces of evidence support the 'trap' hypothesis.

Alpha$_2$M inhibit proteinases by a conformational change of the native α_2M molecule, resulting in a particular trapping mechanism. Native and transformed α_2M tetramers are designated s for slow form (s-form) and f for fast form (f-form) according to their electrophoretic mobility on non-denaturing polyacrylamide gel electrophoresis (PAGE) (Barrett *et al.*, 1979; Legrès *et al.*, 1994). By hydrolysing the internal thiol ester of the s-form with methylamine, the f-form containing free thiol groups, can be obtained (Legrès *et al.*, 1994; McCaffrey *et al.*, 1994) (Figure 2.2).

Alpha$_2$M is a major plasma binding protein for cytokines and growth factors with differences regarding the binding mode and the conformational state of the α_2M molecule. Migration of the α_2M-cytokine complex is regarded as f-form (Legrès *et al.*, 1994).

Alpha$_2$M has different molecular shapes in its native or transformed state and that there are differences in the f-form when the molecule is transformed by proteinase or by methylamine treatment (Legrès *et al.*, 1994).

A "pretrapping" model of α_2M was proposed in which the major structural change of α_2M is separated from the initial holding of a proteinase. Structural change certainly helps to securely retain the proteinase molecule, but the proteinase has already lost its freedom to separate from the α_2M long before any structural change occurs. Fluorescence stopped-flow experiments, indicated that some minor structural changes take place before any major structural change. These minor changes in α_2M conformation could be sufficient to pretrap proteinase molecules (Ikai *et al.*, 1994).

Dimer-dimer interactions are involved in the conformational changes that take place in human α_2M. These interacting domains between two dimers are called contact zones, of which there is one in each sub unit of α_2M. Conformational changes in α_2M brought about by cleavage of the bait region or thiol esters, change the interactions at the contact zones. These contact zones are functionally important for regulation of large conformational

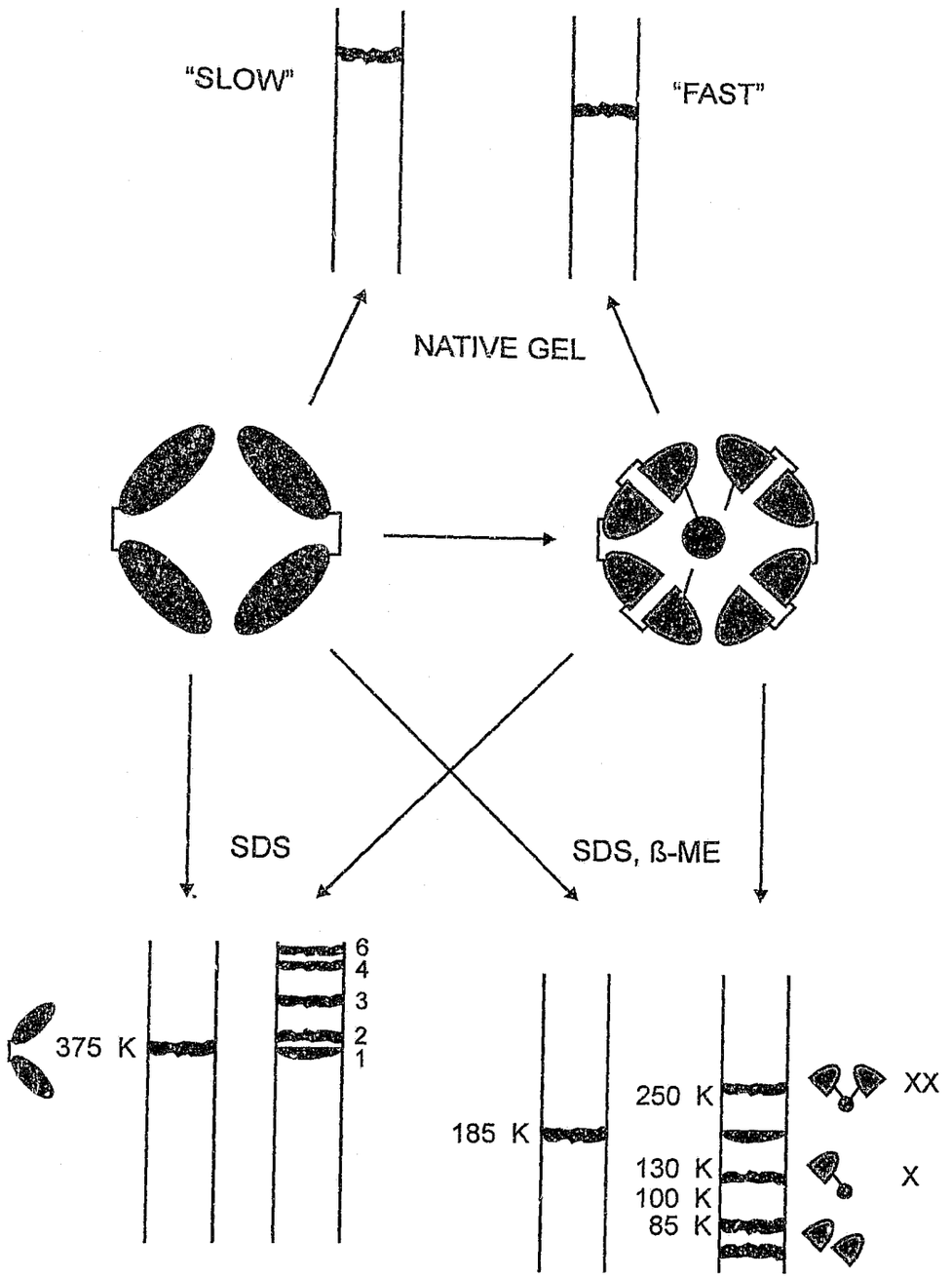


Figure 2.2 Schematic illustration of the subunit structure of the α_2M molecule and the changes occurring upon reaction with poteinases (Feinman, 1994).

changes (Shanbhag *et al.*, 1997). It was previously postulated that large conformational changes, in human α_2M are regulated by the thiol esters (Jensen & Stigbrand, 1992). The location of the contact zone is between the thiol ester and the C terminus. This part of the α_2M subunit involves a large loop generated by a disulphide bond between cysteine (Cys) 898 and Cys 1298. This loop is likely to be the site of the contact zone in α_2M (Shanbhag *et al.*, 1997).

Monomeric, dimeric and tetrameric α_2M all appear to operate by sterically hindering access to the active site of bound proteinases (Chu *et al.*, 1994).

More recently it has been shown that nonproteolytic ligands can be incorporated into the receptor-recognised α_2M form without the proteolytic step, but by a nucleophilic exchange mechanism. The nucleophile cleavage of thiol esters in α_2M is a reversible process. Proteins can be covalently incorporated into the nucleophile-treated-, receptor-recognised α_2M (Grøn & Pizzo, 1998).

During the proteolytic reaction, proteinases can be trapped along with ligands in the internal cavity of α_2M. The size of the ligand and proteinases determine the number of molecules, which can be incorporated. Very small amounts of protein ligand are incorporated into native, slow migrating α_2M. The proteinases compete with lysozymes for reaction with thiol esters (Grøn *et al.*, 1998).

2.1.3 The human α_2Macroglobulin-receptor gene

The human α_2M-receptor gene is located on chromosome 12q13-q14, with the coding sequences of the α_2M-receptor gene covering 90kilobases (kb), which is small in comparison to the large messenger ribonucleic acid (mRNA) and the protein. Eighty-nine exons are flanked by donor and acceptor splice sequences. Exon sizes varied from 65 bases for exon 86 to 925 bases for exon 89, with a mean of 167 bases per exon, which is larger than average. Introns vary in size from 82 bases for intron 53 to more than 8kb for intron 6, with a mean of 0,7kb. The intron sequences revealed at least four complete and many incomplete Alu sequences with intron 44 as a complex intron with repeat sequences (Van Leuven *et al.*, 1994).

The α_2M multifunctional receptor occurs on lymphocytes, monocytes and granulocytes (Gläser *et al.*, 1994). A very large protein of about 600kDa, synthesised as a precursor encoded by a 15kb mRNA, carries the receptor activity (Van Leuven *et al.*, 1994).

2.1.4 Functions of α_2Macroglobulin

2.1.4.1 In general

Proteinase-complexed α_2M is rapidly cleared *in vivo*, by the liver, spleen and bone marrow in dogs and humans (Chu *et al.*, 1994).

Although the biological role of α_2M is unknown, it is considered to be a proteinase inhibitor. However, it possesses several properties that suggest that its function extend beyond that. Various proteinases are capable of cleaving the α_2M-bait region and become entrapped, even though many possess more efficient and specific *in vivo* inhibitors. But since there are no known complete deficiencies in α_2M, it suggests that α_2M has a more central role than merely functioning as a reserve proteinase inhibitor during emergencies. Alpha$_2$M may function in a reserve capacity, when other inhibitors are deficient or overwhelmed. Alpha$_2$M may be important in controlling metalloproteinases associated with ovulation, matrix remodelling, snakebites or microbial infections. (Chu *et al.*, 1994).

Many non-proteolytic proteins besides growth-regulatory proteins have been reported to bind α_2M. Binding of cytokines reflects a generalised ability of α_2M to bind, capture or trap proteins. The high binding level leukaemia inhibitory factor, TGF-β and epidermal growth factor (EGF) is through nucleophilic attack by ϵ-lysyl, α-amino and possibly hydroxyl groups on the metastable thiol ester. This is the case whether the non-proteolytic ligand is an enzyme, growth factor or hormone, be it recombinant or glycosylated, acidic or basic, and regardless of the level of non-covalent binding to native or fast-form of α_2M. Some growth factor binding may result from adherence of basic proteins to acidic α-macroglobulin (Chu *et al.*, 1994).

2.1.4.2 Alpha$_2$Macroglobulin function in relation to cytokines

Alpha$_2$M regulates the function of growth factors and cytokines *in vivo* in the following four ways, referred to as the α_2M-α_2MR/LRP growth regulatory axis: (i) Alpha$_2$M binds cytokines (this is the favoured interaction compared with other plasma protein cytokine interactions). (ii) Activated α_2M which has undergone conformational change due to reaction with primary amines or proteinases, targets cytokines to surfaces of cells expressing the α_2M receptor (α_2M receptor/low density lipoprotein receptor-related protein or α_2MR/LRP). (iii) Alpha$_2$M regulate cytokine activity *in vitro*. The nature of regulation is cell type-specific and may depend on whether the cell expresses α_2MR/LRP. (iv) Cytokines modulate cellular expression of α_2MR/LRP, thereby altering the ability of α_2M to affect the activity of other cytokines in the same cell type (Gonias *et al.*, 1994).

Numerous cytokines bind to α_2M which include TGF-β_1 and -β_2, platelet-derived growth factor (PDGF), tumour necrosis factor (TNF), basic fibroblast growth factor (bFGF), nerve growth factor, interleukin (IL)-1β, IL-6 and insulin (Gonias *et al.*, 1994; Gettins & Crews, 1994). Intracellular mediators such as IL-1; IL-6 and EGF which promote mesangial cell proliferation, also stimulate kidney matrix synthesis (Schnaper, 1995).

2.1.4.3 Alpha$_2$Macroglobulin as a sensor for proteolysis

The α_2M family has the ability to inhibit proteinases displaying different specificities and catalytic mechanisms. Cleavage of a peptide bond triggers a conformational change that encages the proteinase and sterically hinders its access to larger substrates, active-site-direct inhibitors and antibodies. The receptor-recognised f-α_2M conformation, which no longer has the potential to inhibit proteinases, is recognised by cell surface receptors, leading to rapid clearance of α_2M-proteinase complexes from circulation. Alpha$_2$M-bound proteinases retain activity against some substrates and this clearance mechanism is critical for effective proteolytic activity control (Chu *et al.*, 1994).

Alpha$_2$M may be a reservoir of molecules capable of sensing increased proteolysis and causing appropriate cellular responses to injury or inflammation, rather than functioning

solely as a proteinase inhibitor. Expression of two types of α_2M receptors, one endocytic and the other coupled to G-proteins, represent a potential mechanism of regulating specific tissue or cellular responses (Chu *et al.*, 1994).

2.1.4.4 The role of α_2Macroglobulin growth factor complexes on fibroblast proliferation

Alpha$_2$M is a potential modulator of cell growth induced by PDGF (Bonner, 1994).

The concentration of α_2M in vertebrate plasma, range from two to four mg/ml. After vascular injury or during atherosclerosis, PDGF is released from degranulating platelets, and binds to both native and activated forms of α_2M. In serum free *in vitro* studies, it has been shown that α_2M modulates PDGF-stimulated mitogenesis of human skin fibroblasts, Swiss mouse 3T3 fibroblasts and primary passage rat lung fibroblasts (Bonner, 1994) (Figure 2.3).

The modulatory effect of α_2M on the fibroblasts responding to PDGF depends on the binding protein conformation. Conformation of α_2M either as the receptor-recognised fast-form or the slow inactive form which is not recognised by the receptor. Alpha$_2$M-methylamine enhances PDGF-induced cell growth by a yet unknown mechanism. It has also been reported that TGF-β_1-stimulated smooth muscle cell proliferation is synergistically enhanced by α_2M-methylamine. TGF-β_1-stimulated cell growth enhanced by α_2M-methylamine depends on α_2M-methylamine binding to the α_2M receptor/LRP (Bonner, 1994).

2.1.4.5 Modulation of fibroblast chemotaxis by α_2Macroglobulin

Native slow-form α_2M alone has no chemotactic activity, although the receptor-recognised fast-form α_2M inhibits PDGF-stimulated chemotaxis (Bonner, 1994).

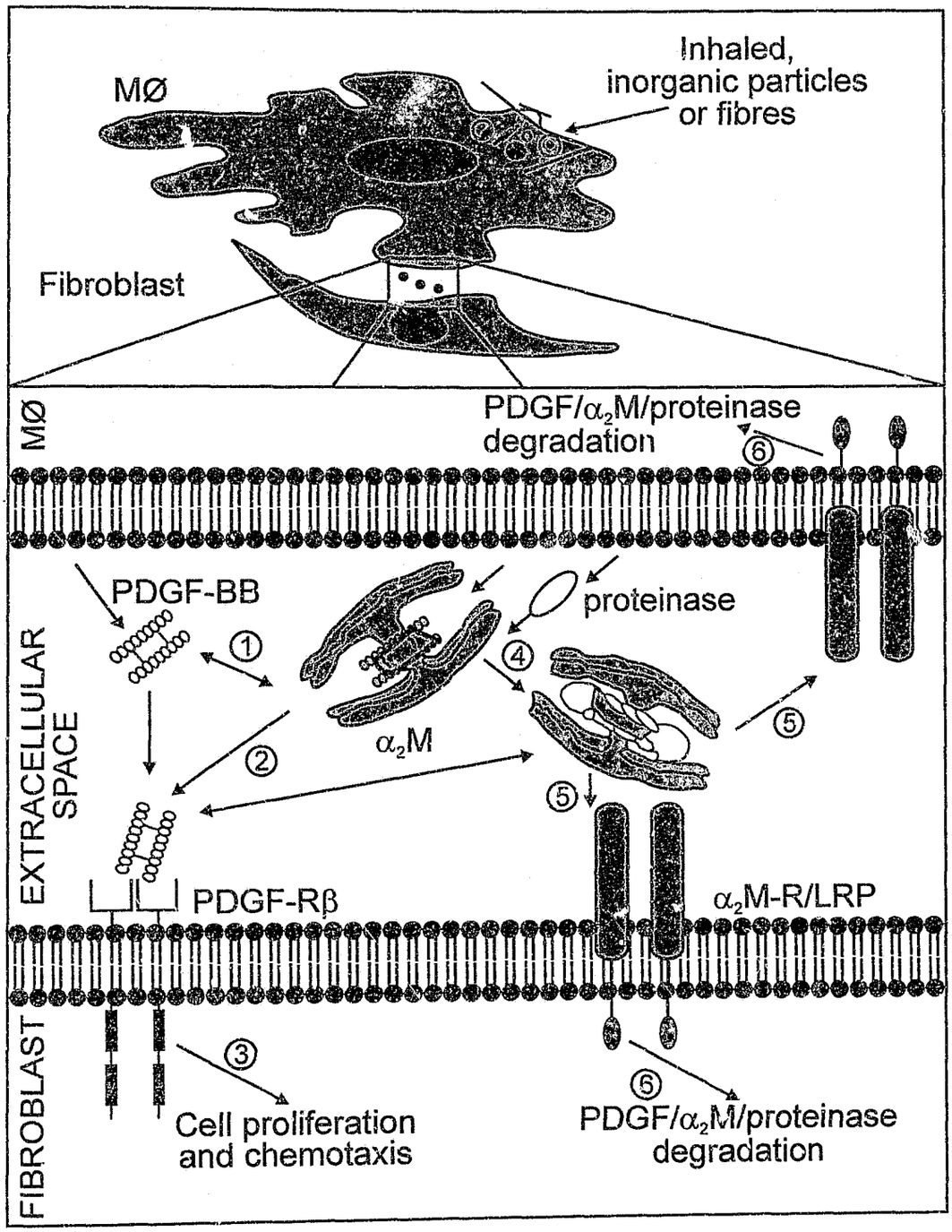


Figure 2.3 Pathway of cellular and protein interactions leading to α_2M-proteinase-complex degradation (Bonner, 1994).

2.1.4.6 Clearance of PDGF/α_2M-proteinase complex by macrophages via the α_2M-receptor/LRP

Alpha$_2$M might be converted to the receptor-recognised form when extracellular proteinase concentrations increase, as is the case at inflammation sites. This is a well-established mechanism whereby proteinases are inactivated and cleared by cells possessing the α_2M receptor/LRP. Little is known about clearance of PDGF or other growth factors via α_2M receptor/LRP-mediated endocytosis. Plasmin-activated α_2M serves as a scavenger of extracellular PDGF. Alpha$_2$M might provide a way of PDGF clearance *in vivo*, where excess growth factors are removed from inflammation sites following tissue repair (Bonner, 1994).

Alpha$_2$M was found to be an important regulator of PDGF-stimulated fibroblast proliferation and chemotaxis *in vivo*. Native α_2M binds to PDGF and prevents it from interacting with its receptor. Alpha$_2$M serves as an extracellular reservoir for PDGF, which can be released over time in a controlled way to interact with PDGF-α or -β receptors. Decreased pH favours the release of bio active PDGF *in vitro* (Bonner, 1994).

Methylamine-activated α_2M synergistically enhances PDGF-induced cell growth, while plasmin-activated α_2M inhibits PDGF-stimulated fibroblast proliferation. The reason for the difference of these two receptor-recognised α_2M effects is not known (Bonner, 1994).

2.1.4.7 Alpha$_2$Macroglobulin / TGF-β_1 interactions

Most cells have the capacity to synthesise and respond to TGF-β_1, which may be activated by limited proteolysis involving enzymes such as plasmin. Active TGF-β_1 can modulate plasmin-dependent activation of TGF-β_1 in an autocrine/paracrine fashion by stimulating plasminogen activator inhibitors or plasminogen activators in macrophages (McCaffrey *et al.*, 1994). The growth factor, TGF-β_1 is known to increase plasminogen activator inhibitor expression whilst increased expression of plasminogen activator inhibitor occurs simultaneously with increased TGF-β_1 expression (Schnaper, 1995).

The predominant serum and plasma form of TGF-β_1 is found as an inactive complex with α_2M. TGF-β_1 may be involved in the antiproliferative effect of heparin. Heparin binds directly to TGF-β_1 and prevents TGF-β_1 from associating with α_2M. TGF-β_1 activity is enhanced in the presence of heparin (McCaffrey *et al.*, 1994).

Various ligands that interact with TGF-β_1 could alter the diffusibility, the stability, cell targeting and biological actions of TGF-β_1. TGF-β_1 is found *in vivo* as an inactive complex with α_2M, but is not cleared by α_2M (McCaffey *et al.*, 1994).

It may be that heparin-like agents modulate TGF-β_1 activity in ways other than by modulating the binding to f-α_2M. It is possible that heparin and its related compounds could directly stabilise TGF-β_1 and protect it from proteolytic degradation. Maybe these two mechanisms combine to allow heparin-like molecules to increase TGF-β_1 activity in the pericellular environment, and increase the TGF-β_1 duration of action in its diverse physiological and pathological roles (McCaffrey *et al.*, 1994).

The feedback mechanisms between α_2M and TGF-β_1 reduce extracellular matrix synthesis of liver fat storing cells (Bachem *et al.*, 1994). Alpha$_2$M bind and inactivate several growth factors including TGF-β_1. Alpha$_2$M is suggested to be a modulator of the TGF-β_1 biological activity. TGF-β_1 increases α_2M synthesis and bind to α_2M. It is not clear whether the α_2M-complex is irreversibly cleared from circulation or whether this complex represents a latent form of TGF-β_1 (Bachem *et al.*, 1994).

2.1.4.8 Modulation of immune cell activities by α_2Macroglobulin

Evidence exists that proteinase trapping, is a separate and much faster process than the major structural change. Cytochrome c becomes highly immunogenic when conjugated with α_2M. At an inflammatory site, not only proteinases and antigenic materials, but also α_2M and macrophages encounter each other within a short sequence of time. Alpha$_2$M can function as an effective carrier of antigenic materials *in vivo*. This has potential for developing artificial "piggyback" vaccines in future (Ikai *et al.*, 1994).

2.1.4.9 The lipoprotein receptor protein (LRP) mediates clearance of coagulation Factor Xa *in vivo*

A few plasma serine proteinase inhibitors such as α_2M, α_1PI, anti-thrombin (AT)-III and tissue factor pathway inhibitor (TFPI) seem to inhibit the activity of Factor Xa. Alpha$_2$M only accounts for 10-15% of Factor Xa inactivation *in vitro*, but up to 90% *in vivo*. It appears that LRP mediates the plasma clearance of Factor Xa after formation of a complex with protease inhibitors such as α_2M (Narita *et al.*, 1998).

2.1.5 The α_2Macroglobulin receptor

2.1.5.1 General information

The α_2M receptor is identical to LRP. As this receptor was thought to play a double role, it was named α_2M/LRP (Gliemann *et al.*, 1994). LRP is widely expressed as a glycoprotein of 4525 amino acids with the extracellular domain structurally resembling four combined LDL receptor molecules (Obermoeller *et al.*, 1998).

The primary structure of LRP has a very high cystein residue content, which is mostly found within clusters of tandemly arranged complement type of EGF-type repeats within the extracellular domain. Each repeat consists of ~40 amino acid residues which includes 6 cysteine residues forming 3 disulfide bonds (Fass *et al.*, 1997). Therefore a single LRP molecule may contain 159 disulfide bonds (Obermoeller *et al.*, 1998). The C-terminal domain of LPL (residues 313-448) (LPLC), mediates interaction with LRP in both cell-suface and solid-phase assays. Proteoglycans mediate binding and uptake of LPL, LPLC and their complexes with lipoproteins independently of LRP (Chappell *et al.*, 1994).

Co-expression of a receptor-associated protein (RAP) is necessary and sufficient for correct folding of the four putative ligand binding domains of LRP (Bu *et al.*, 1996). When RAP co-expression is absent, the four ligand binding domains of LRP misfolds due to formation of intermolecular disulfide bonds and are retained within the endoplasmic reticulum with little secretion (Herz *et al.*, 1991). Gene knockout studies showed that cells without RAP exhibit endoplasmic reticulum-retention of aggregated LRP and a 75% reduction in functional LRP (Willnow *et al.*, 1996 (b)).

RAP consists of three internal repeats (Bu *et al.*, 1995). The carboxyl-terminal repeat of RAP functions the same as the full-length RAP in terms of helping receptor folding. (Obermoeller *et al.*, 1997). This RAP repeat however does not inhibit α_2M binding to LRP. The amino-terminal and central repeats of RAP, which are unable to assist receptor folding, do inhibit α_2M binding to LRP though (Obermoeller *et al.*, 1997). This finding suggests that the effects of RAP in receptor folding and in inhibition of ligand interaction are independent functions (Obermoeller *et al.*, 1998).

Binding of ligands to LDL receptor gene family members is calcium-dependent. Recently a study of the crystal structure of a ligand binding repeat from the LDL receptor revealed that each repeat contains a single calcium (Ca^{2+}) ion trapped in an octahedral structure formed by 4 conserved acidic residues (Fass *et al.*, 1997). The interaction of these acidic residues with the calcium stabilises the receptor and maintains it in its native conformation. These authors found that calcium and RAP are independently required for LRP folding as well as formation of disulfide bonds (Obermoeller *et al.*, 1998).

Depletion of calcium will eventually result in total misfolding of LRP. Participation of RAP in LRP folding is important but not essential as deletion of the RAP gene results in a 75% reduction but not total elimination of functional LRP molecules (Willnow *et al.*, 1996 (b)). RAP has a special chaperone function during biogenesis of LRP (Bu & Schwartz, 1998).

2.1.5.1.1 The multiligand α_2Macroglobulin receptor / low density lipoprotein receptor-related protein (α_2MR/LRP).

A large protein, structurally closely related to LDL receptor (LDLR), named LDLR-related protein or LRP was first cloned in 1988 (Herz *et al.*, 1988). LRP functions as a receptor for chylomicron remnants/ β-migrating very low-density lipoproteins (β-VLDL) rich in apolipoprotein (apo)E. The α_2M receptor (α_2MR) and LRP are identical. The receptor was thought to play the role of a double agent, therefore it has a double name: α_2MR/LRP. The binding, endocytosis and lysosomal degradation of α_2M receptor associated protein (α_2MRAP) mediated by gp330 of the renal tubules, provided the first evidence for its function as a receptor for the endocytosis of proteins. Alpha$_2$MRAP has been renamed as

RAP because it binds to at least two receptors. RAP binds to at least two sites in each α_2MR/LRP molecule. RAP blocks the binding of apoE-enriched β-VLDL (Gliemann *et al.*, 1994).

Alpha$_2$M originally purified from human plasma to be cloned as a candidate for the lipoprotein remnant receptor revealed upon sequencing that it is a LDL receptor-related protein (α_2M/LRP). This purified receptor binds several ligands in addition to the ligands bound by α_2M (Table 2.1). This implicates it as a scavenger receptor. Ligands include lactoferrin, *Pseudomonas exotoxin A*, LPL, apoprotein E-enriched lipoproteins, urokinase and tissue-type plasminogen activator/plasminogen activator inhibitor-1 complexes. Some of these ligands cross-compete for binding to the α_2MR/LRP, with a ligand called the 39kDa RAP, which is capable of competing with all these ligands including α_2M. Maybe RAP modulates ligand binding to α_2MR/LRP, although its location is more consistent with a role in intracellular trafficking (Chu *et al.*, 1994).

Gonias *et al.*, (1994) proposed three models describing how receptor-recognised α_2M may enhance cytokine activity. Model A is focussed on the delivery of cytokines to signalling receptors. Many cytokines that bind to α_2M also interact with binding proteins/proteoglycans in extracellular matrix and in association with the cell surface. Actual cytokine activity is a function of cytokine concentration and the fractions of cytokine that reaches true signal-transducing receptors (binding proteins/proteoglycans are not true receptors, as cytokine binding does not result in cell signalling or cytokine activity). The affinity of cytokines for activated α_2M may change once α_2M is bound to α_2MR/LRP (Gonias *et al.*, 1994).

Fibroblasts, macrophages, hepatocytes, adipocytes and dermal dendritic cells express a f-α_2M receptor (Howard *et al.*, 1996 (b)), demonstrating rapid internalisation and intracellular degradation with recycling of the receptor (Chu *et al.*, 1994). Activated α_2M target cytokines to signalling receptors. Transfer to signalling receptors may occur on the cell surface due to the high affinity of cytokine-signalling receptor interaction (Gonias *et al.*, 1994).

Proteinases and Proteinase-Inhibitor Complexes

α_2M-proteinase complexes

PZP-proteinase complexes

tPA

tPA:PAI-I complexes

uPA

uPA:PAI-I complexes

Lipoproteins

Apolipoprotein E

apolipoprotein E-enriched β-VLDL

lipoprotein lipase

lipoprotein lipase-enriched β–VLDL and VLDL

Toxins and Viruses

Pseudomonas exotoxin A

minor-group human rhinovirus

Other Molecules

Lactoferrin

RAP

Table 2.1 Ligands known to be internalized and degraded upon binding to LRP (Williams *et al*, 1994).

Model B is an extension of model A where the potential role of endocytosis in promoting synergy is considered. This model assumes that activated α_2M-cytokine complexes are transferred as an intact unit once the α_2M has bound to α_2MR/LRP. The α_2M-cytokine complex undergoes endocytosis in vesicles, which are slowly acidified. Alpha$_2$M and α_2MR/LRP dissociate and α_2MR/LRP is recycled to the cell surface. The acidic pH promotes the dissociation of cytokines from α_2M (Gonias *et al.*, 1994).

Model C involves an entirely different mechanism whereby α_2M possibly synergise cytokine activity. This model proposes that the binding of α_2M to its receptor independently regulates cell function (Gonias *et al.*, 1994).

Alpha$_2$M-cytokine interactions vary a lot in affinity and in preference for specific α_2M conformations. TGF-β_2 is unique as it binds with high affinity to both native slow α_2M and to fast transformed α_2M. Alpha$_2$M regulates cytokine activity in cell culture. Whether regulation involves promoting cytokine activity or inhibiting cytokine activity depends on α_2M conformation and or cellular expression of α_2MR/LRP. Many cytokines, particularly interferon (IFN)-γ, regulate α_2MR/LRP-expression *in vitro* (Gonias *et al.*, 1994).

When macrophages are exposed to α_2M, a rapid generation of inositol triphosphate (IP_3) takes place, which is followed by a rise in calcium. Cyclic adenosine monophosphate (AMP) levels also rise and activities of protein kinase C as well as a variety of phospholipases increase too. This does not happen when macrophages are exposed to native α_2M, therefore, the signalling cascade is only activated *in vivo* in cases when proteolysis is involved (Howard *et al.*, 1996 (b)).

Alpha$_2$M-LRP/α_2MR complexes collect in clusters in the clathrin-coated pits on the cell surface and is then endocytosed. These complexes, once inside the cell, are taken up by endosomes where the ligand dissociates from the receptor. The ligand is degraded in the lysosomes and the receptor is recycled to the surface where it can again bind a ligand and go through the same process again (Howard *et al.*, 1996 (b)).

2.1.5.2 The α_2Macroglobulin mechanism of binding

Alpha$_2$M inhibits all four classes of proteinase. The mechanism is as follows: α_2M is cleaved by the proteinase in the "bait region" after which conformational changes activate internal thiol ester bonds, providing attachment sites for the covalent binding of proteinases and other molecules. The conformational change expose receptor-binding domains on the α_2M-proteinase complex. These exposed receptor-binding domains mediate the interaction with specific cell surface receptors and are responsible for removing these complexes from the circulation. The α_2M receptor is identical to LDL receptor–related protein (LRP). The receptor contains a 515kDa heavy chain and an 85kDa light chain, which are non-covalently associated. A polypeptide of 39kDa co-purifies with the receptor and is called RAP (Williams *et al.*, 1994).

2.1.5.3 Functions of the LDL receptor gene family

The LDL receptor gene family in mammals consists of the LDL receptor, the VLDL receptor, LRP and the Heymann nephritis antigen gp330 (Figure 2.4). The last two are large multifunctional proteins. All four are known as endocytic cell surface receptors (Herz & Willnow, 1994).

The physiological role of the LDL receptor is the endocytic uptake of ligands from extracellular space into the cell where ligands are degraded in lysosomes. A variety of structurally as well as functionally different ligands bind to these closely related receptors. Some receptors have overlapping ligand-binding specificity (Herz & Willnow, 1994).

The LDL receptor gene family consists of a group of endocytic receptors that take up ligands from extracellular spaces and from circulation. *In vitro* studies do not give firm conclusions about the physiological importance of receptors for biological processes in which they participate (Herz & Willnow, 1994).

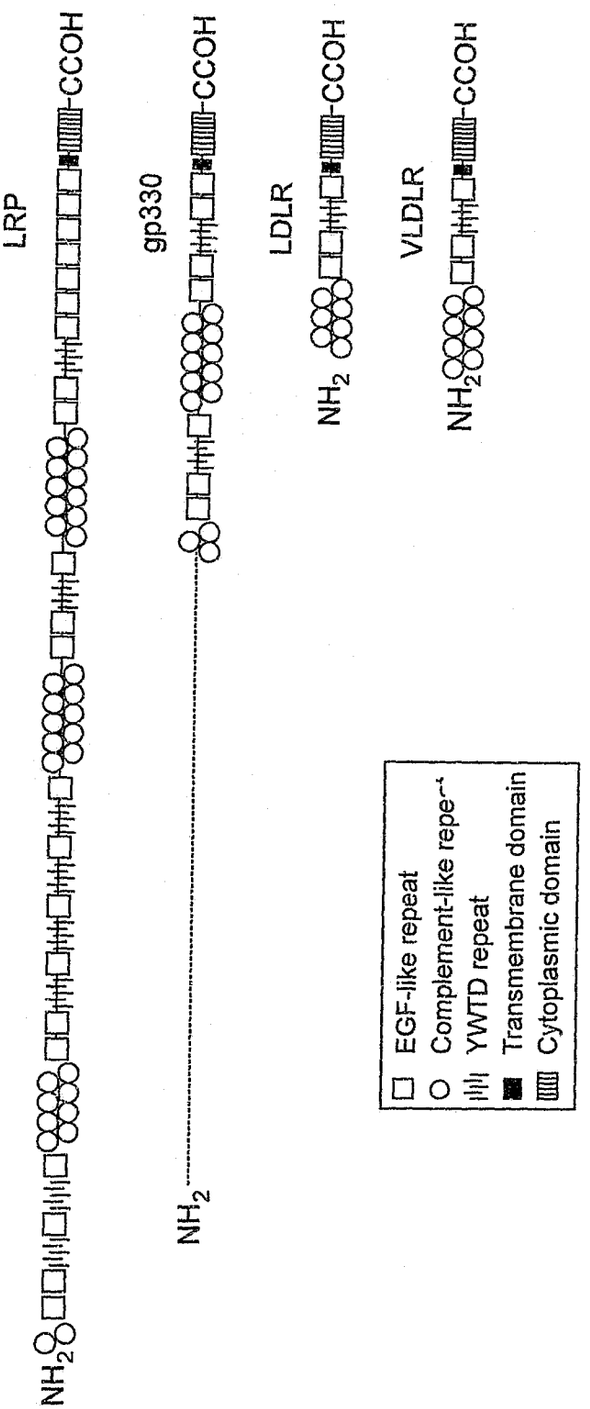


Figure 2.4 Schematic illustration of the LDL receptor family (Williams *et al*, 1994).

2.1.5.3.1 LRP- a multifunctional receptor

LRP belongs to the LDL receptor family, which includes: LRP, epithelial glycoprotein called glycoprotein 330 (gp330) and the VLDL receptor (Williams *et al.*, 1994).

LRP has close structural homology to the LDL receptor. This overall homology of a single membrane-spanning segment and two endocytosis signals lead to the prediction that LRP may physiologically function as a receptor for apoE-containing lipoproteins. LRP is predominantly found in the liver and brain, although it is expressed ubiquitously. LRP is identical to the α_2M receptor and has therefore a multifunctional role. LRP play a central role in plasminogen activation regulation. Cellular activity of LRP is proposed to be regulated by RAP *in vitro*, which is capable of blocking interaction of all known ligands with this receptor (Herz & Willnow, 1994).

LRP mediates internalisation of several proteinase-inhibitor complexes. LRP directly regulates the levels of the proteinases: tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Hepatic clearance of proteinases, proteinase-inhibitor complexes and apoE or lipoprotein lipase-enriched lipoprotein particles from plasma is an important function of LRP, but its widespread cellular distribution suggests additional roles (Williams *et al.*, 1994).

2.1.5.3.2 LDL receptor

Brown, Goldstein and their colleagues have first described the LDL receptor. LDL receptors bind plasma lipoproteins that contain apoB-100 or apoE on their surfaces (Herz & Willnow, 1994).

2.1.5.3.3 Heymann nephritis antigen gp330

The Heymann nephritis antigen gp330 is an abundant kidney membrane protein. It is a constitutively expressed recycling receptor of similar size as LRP (approximately 600kDa). The gp330 has overlapping ligand binding specificity with LRP and ligand interaction is RAP modulated (Herz & Willnow, 1994).

2.1.5.4 Physiological role of α_2MR/LRP

The α_2MR/LRP receptor is essential, as disruption of the α_2MR/LRP gene in mice, blocks embryo development. A wide variety of molecules are endocytosed by α_2MR/LRP. Alpha$_2$M is secreted by activated macrophages which scavenge α_2M proteinase produced locally. Active α_2M binds many cytokines and growth factors especially TGF-β and these are scavenged together with the complex. It has been shown that active α_2M enhances TGF-β-induced growth response in rat aortic smooth muscle cells and this effect depends on the complex binding to α_2MR/LRP. Therefore it might be possible that active α_2M bound to α_2MR/LRP can present the growth factor to a signalling receptor before endocytosis of the complex. Alpha$_2$MR/LRP has many functions via binding of active α_2M, which is just one of its ligands. Lipoprotein uptake by α_2MR/LRP is mediated by LPL and by apoE. This mechanism partly accounts for the systemic clearance of chylomicron remnants and VLDL by the liver. This clearance can continue even when α_2MR/LRP is loaded with active α_2M. Expression of α_2MR/LRP is particularly important for the uptake of lipoprotein in the human arterial smooth muscle cells, which do not express other known lipoprotein receptors (Gliemann *et al.*, 1994).

2.1.5.5 Alpha$_2$Macroglobulin receptor physiology

The LDL-receptor family includes α_2MR/LRP, glycoprotein 330, VLDL-receptor, apo E-receptor and LDL-receptor itself. Structurally these receptors are similar. They all have cystein-rich repeats of the EGF type and complement type. Complement types are responsible for binding of a variety of ligands (Ellgaard *et al.*, 1997).

Proteolysis of the bait region located at residues 667-705 of each unit, initiate complex formation. This cleavage cause interruption of an internal thiol ester which cause conformational changes which result in entrapment of proteinases together with exposure of previously hidden receptor recognition sites on each of the four identical subunits of the inhibitor. Each of the four α_2M subunits have an internal β-cysteinyl-γ-glutamyl thiol ester which can be attacked directly by nucleophilic methylamine or ammonia which will also cause a conformational change to expose receptor recognition sites (Sottrup-Jensen, 1987;

Howard *et al.*, 1996 (a)). The transformed α_2M binds to the α_2M receptor (α_2MR/LRP) via the receptor binding domain located at the tip of the H-like structure of the inhibitor molecule (Sottrup-Jensen, 1987). The receptor-binding domain is the 20kDa C-terminal portion of the 180kDa monomer (Van Leuven *et al.*, 1986).

LRP/α_2MR is a multivalent receptor, which binds many ligands apart from α_2M-proteinase or –methylamine, including RAP, apoE, lactoferrin, lipoprotein lipase, plasminogen activators alone or in complex with inhibitors and other serpin-proteinase complexes. Most ligands do not compete for binding with each other, although RAP is able to block binding of all known ligands to LRP/α_2MR (Howard *et al.*, 1996 (a)).

RAP is a 39kDa glycoprotein, which consists of 323 amino acids. RAP binds to α_2MR/LRP and gp330 with high affinity and with much lower affinity to the VLDL receptor (VLDLR) and to the LDLR. RAP can inhibit binding of all known ligands of the LDLR family members (Ellgaard et al., 1997).

The Heymann nephritis antigen, which is a 319 amino acid RAP homoloque in rats, shows a 73% homology with human RAP. Sequence alignment shows that RAP consists of three similar domains and an N-terminal extension of 17 amino acid residues. All three domains are related in primary, secondary and tertiary structure (Ellgaard *et al.*, 1997).

The lysine-1370 and lysine-1374 residues spaced by three amino acids are essential for receptor binding. In the absence of one of these two lysine residues in α_2M, there is no affinity to the receptor. When a lysine is mutationally inserted in the protein, receptor binding is not reconstituted. This indicates that apart from the two lysine residues, other sites might contribute to receptor binding too (Birkenmeier *et al.*, 1997).

2.1.6 Selective mutations in cloned and expressed αMacroglobulin receptor binding fragments

A second α_2M receptor called the α_2M signalling receptor (α_2MSR) which activates a typical signalling cascade has been identified recently. This receptor is linked to a pertussis toxin-insensitive G-protein. Activation of this cascade causes a rapid increase in

IP_3 synthesis together with an increase in intracellular calcium. Ligation of α_2MSR promotes protein kinase C activation as well as many phospholipases including the phosphorylation and activation of phospholipase Cγ1. Alpha$_2$MSR ligation promotes cell cytoplasm alkalisation. It is suggested that the role of α_2MSR is to detect proteolysis in the environment and to initiate macrophage responses, which may be growth factor-like (Howard *et al.*, 1996 (a)).

2.1.7 Pathophysiology of α_2Macroglobulin

Early-onset Alzheimer's disease (AD) has been linked to mutations in the amyloid precursor gene on chromosome 21 and to an unknown site on chromosome 14. Late-onset AD has been linked to chromosome 19 (Hyman *et al.*, 1994).

Evidence was found of genetic disequilibrium between AD and one allele of the apoE gene. ApoE$_4$ is over-represented three fold in AD. The homozygote E$_4$/E$_4$ genotype is five fold over-represented in AD than the expected frequency (Hyman *et al.*, 1994). The apoE$_4$ isotype binds strongly to the amino acid peptide, β-amyloid (βA), in the plaques. AD patients have an increased prevalence of the apoE$_4$ isotype. Aggregates of the 39-42 amino acid peptide, βA are scavenged by α_2MR/LRP reactive cells (Gliemann *et al.*, 1994).

Reactive astrocytes, microglia and senile plaques are α_2MR/LRP-immunoreactive in AD (Gliemann *et al.*, 1994). Senile plaques contain α_2M/LRP ligands (Gliemann *et al.*, 1994), proteinase inhibitors, anti-chymotrypsin, α_2M as well as the apoE protein (Hyman *et al.*, 1994).

By using quantitative image analysis techniques, it was discovered that individuals homozygous for apoE$_3$/E$_3$ had fewer senile plaques present in the inferior temporal gyrus than those whom inherited one apoE$_4$ allele. Individuals who inherited two apoE$_4$ alleles have an even greater density of senile plaques (Hyman *et al.*, 1994).

It is hypothesised that apoE interacts with βA in such a way as to influence a kinetic equation with the E$_4$ isoform, leading to increased deposition of amyloid. βA is

extracellular accumulations of precipitated peptide. ApoE interacts with lipid-containing molecules and chaperon them to specific receptors (Hyman *et al.*, 1994).

Alpha $_2$MR/LRP is implicated in AD (Gliemann *et al.*, 1994). The receptor is internalised after binding of apoE-containing complexes, so that endosomes and lysosomes can degrade and recycle the internalised components (Hyman *et al.*, 1994).

LDL receptors and α_2M/LRP both occur in the neurons of the normal brain and could interact with apoE complexes. LDL receptors are important in lipid metabolism in the nervous system and LRP is a multifunctional receptor that not only binds apoE but also α_2M, other proteinases and proteinase inhibitors. ApoE complexes are taken up by LRP. If apoE_4 interacts less well with βA or if the apoE_4/βA complex is cleared less well from the neuropil than the apoE_3 complexes, βA could accumulate. (The neuropil is a fibrous network of delicate unmyelinated nerve fibres interrupted by numerous synapses, and found in concentrations of nervous tissue in highly developed parts of the brain). This would lead to increased deposition of senile plaques as observed in individuals with apoE_4 alleles (Hyman *et al.*, 1994).

Other potential ligands for LRP such as α_2M, other proteinases and proteinase inhibitors are also associated with senile plaques. LRP is a receptor for α_2M complexes and other proteinase/proteinase inhibitor complexes (Hyman *et al.*, 1994).

ApoE_4 allele inheritance is associated with an increased risk of developing AD (Hyman *et al.*, 1994).

Elastase binding capacity (EBC) of α_2M is higher in patients with chronic obstructive pulmonary disease (COPD), emphysema and in asthma, than in normal individuals and the functional activity levels of α_2M is also high in these patients (Gaillard & Kirroe-Smith, 1987; Kilroe-Smith *et al.*, 1989). This is important as α_2M-proteinases complexes are still active on small molecular weight substrates (Gaillard *et al.*, 1995).

It has been shown that α_2M levels vary with age. Children have very high levels, more than twice the adult values (Kilroe-Smith *et al.*, 1991).

Alpha$_2$MR/LRP is a scavenger-type receptor for endocytosis of many proteins, complexes and particles. Therefore this receptor may play a role in physiological systems and pathophysiology of many diseases (Gliemann *et al.*, 1994).

2.1.8 Role of apoE and the LDL Receptor-Related Protein in remnant lipoprotein metabolism

ApoE plays an important role in remnant lipoprotein clearance. For example: In a genetic disorder of lipoprotein metabolism, called type III hyperlipoproteinaemia, defective apoE or its complete absence, causes hypercholesterolaemia and hypertriglyceridaemia, characterised by β-VLDL accumulation. Defective apoE is unable to mediate binding of remnants to LDL receptors or to LRP. A unique apoE remnant receptor, possibly LRP, is responsible for mediating uptake of lipoproteins (Mahley *et al.*, 1994).

2.1.9 Immunology

Apart from proteinase inhibition, α_2M has a variety of biological effects. In the case of macrophages, α_2M regulates: the ability to kill tumour cells, respiratory burst, proteinase secretion and prostaglandin production (Chu & Pizzo, 1994). Alpha$_2$M enhances antigen presentation by macrophages to T-cells and stimulates proliferation of smooth muscle cells synergistically with TGF-β (Stouffer *et al.*, 1993).

2.1.10 Determination of total and transformed α_2M by a monoclonal antibody immunosorption assay in patients with different diseases

It has been shown that native and transformed inhibitors behave differently in binding cytokines and growth factors due to functional differences. Determination of the different forms of α_2M has pathological- and clinical diagnostic value in certain conditions. Birkenmeier *et al.*, (1994) developed an enzyme linked immunosorbent assay (ELISA)-α-1

for detecting the transformed α_2M and an ELISA-α-11 for total α_2M (Birkenmeier *et al.*, 1994).

A small group of patients with NS had very high levels of total α_2M in sera. Six of the eight nephrotics investigated in this study had values of 500 mg/100 ml or more, which put them above the upper limit of normal even when compensating for age. There is no explanation for the raised plasma α_2M in NS as absolute catabolic rates are normal. It is suggested that high plasma levels could be due to diminution of plasma volume and selective retention of high molecular weight proteins by the kidney. Other more likely factors are that the half-life could be prolonged or the rate of synthesis could be increased. No significant variation was found in patients with miscellaneous diseases (Birkenmeier *et al.*, 1994). The fact that women have higher levels than men, and even higher levels during pregnancy, may indicate that α_2M play a role in hormone transport (Housley, 1968).

2.1.11 Alpha$_2$Macroglobulin in nephrotic syndrome

Macrophages secrete α_2M which entraps proteinases as well as α_1-proteinase inhibitor (α_1PI), which inhibits cathepsin G and human neutrophil elastase. By clearing active proteinases via α_2MR/LRP, tissue destruction is limited and f-α_2M generated in this way may directly affect macrophage function through signalling α_2MR. Non-proteolytic proteins that forms complexes with f-α_2M at the inflammation site, can lead to increased antigen expression for T-cell detection following endocytosis via α_2MR/LRP (Chu *et al.*, 1994).

Alpha$_2$M might be converted to the receptor-recognised f-form when extracellular proteinase concentrations increase, as is the case at inflammation sites. The exogenous f-α_2M might be used to counteract fibroproliferative diseases where PDGF overexpression may play a role. During inflammation, or the progression of fibrogenesis, the regulation of PDGF might be lost. Oxidative bursts from neutrophils and eosinophils can activate the function of α_2M in inflammation (Bonner, 1994).

The 26kDa activated TGF-β_1 molecule released in its latent form by platelets; Kupffer cells and myofibroblast cells, can interact with multiple cell-surface binding sites; α_2M and with extracellular matrix components such as fibronectin, thrombospondin and type IV collagen (McCaffrey *et al.*, 1994; Bachem *et al.*, 1994). TGF-β_1 is found in high concentrations in platelet α-granules, extracellular matrix and bone. TGF-β_1 can signal changes in cell migration, proliferation and extracellular matrix synthesis and degradation, as well as in the immune response (McCaffrey *et al.*, 1994).

As TGF-β_1 binds to α_2M, the *de novo* matrix synthesis in fat storing cells in the liver was found to decrease. Maybe α_2M plays an *in vivo* role as a scavenger for active TGF-β_1 at sites of liver injury as it is produced in significant amounts by hepatocytes located close to fat storing cells (Bachem *et al.*, 1994). TGF-β_1 may mediate mesangial expansion in glomerulonephritis associated with matrix accumulation (Schnaper, 1995).

A negative feedback regulation exists between α_2M and TGF-β_1. TGF-β_1 is the most important fibrogenic mediator in the body. Therefore, this mechanism may play a role in the self-limitation of fibrogenesis in NS, in a way similar to that associated with fibrogenesis in the liver (Bachem *et al.*, 1994).

In processes such as inflammation, arthritis, pulmonary fibrosis, hepatic fibrosis and cardiovascular disease, the modulation of endogenous TGF-β activity may play a critical role (McCaffrey *et al.*, 1994).

2.1.12 Aim

The aim of this part of the study was to assess the functional levels of α_2M in South African paediatric NS patients and in children and adult control individuals.

2.2 Materials and Methods

2.2.1 Materials

Human α_1PI, porcine pancreatic elastase and the amide substrate used for pancreatic elastase, succinyl-trialanyl-p-nitroanilide (SAPNA) were obtained from Sigma, Atlasville, South Africa. All other reagents used were of analytical grade (Merck, Darmstadt, Germany).

2.2.2 Subjects

Thirteen black (Bantu-speaking) NS children and four white (Caucasoid) NS children were screened for α_2M EBC. Six black control children, and five white control children without any renal disease, were screened for α_2M EBC. Five black control adults and fifteen white control adults without any renal disease were also screened for α_2M EBC.

The black and white NS children patients were recruited from the Paediatric Nephrology Clinic of the Johannesburg hospital. The black and white control children attended the Asthma and Respiratory Clinic of the Johannesburg hospital and were free of any renal disease. The black and white control adults were individuals who presented themselves as blood donors at the South-African Blood Transfusion Services in Johannesburg. None of these control adults had a history of any renal disease.

The children who participated in the study were 5-16 years of age and the parents of each subject gave informed consent to participate in the study, which was passed by the Committee for Research on Human Subjects of the University of the Witwatersrand. The adults who participated in the study were 25-45 years of age and gave informed consent to participate in the study approved by the above mentioned Research Committee.

Each NS patient had a history of renal disease, either of FSGS or MLNS/(MCD) as diagnosed upon clinical investigation by the paediatric nephrologists at the Johannesburg hospital.

2.2.3 Methodology

Determination of elastase binding capacity of α_2M in plasma

The EBC was determined by a previously described method (Gaillard & Kilroe-Smith, 1987).

A volume of 100µl of a 1:50 dilution of plasma in 0,1 M Tris-HCl buffer was incubated with 100µl porcine pancreatic elastase (50µg/ml Tris-HCl buffer pH 8,8) for two minutes at 37°C to allow binding. This was followed by the addition of 100µl of SAPNA (10mg of the substrate was dissolved in a minimum volume of dimethyl sulphoxide and then diluted to 8ml with Tris-HCl buffer, pH 8,8).

An excess of exogenous α_1PI was added. This inhibitor was a semi-purified preparation of α_1PI which was prepared from human plasma, using precipitation with $(NH_4)_2SO_4$ at a final concentration of 2,0 M, thus ensuring that all of the α_2M had been removed.

The reaction between the α_2M-elastase complex and SAPNA was allowed to take place for 15 minutes, after which point the reaction was stopped by adding 1ml of 0,5 M citric acid. The absorbance was read at 405nm (D_2) on a spectrophotometer. The unbound elastase activity (D_0), was determined by substituting buffer for the plasma and absorbance was read at 405nm.

The following formula was used to determine α_2M in KU/l: $\alpha_2M = 19,15 \times (D_2 - D_0)$ KU/l where D_0 = OD405 for elastase only, and D_2 = OD405 for elastase plus patient plasma dilution (Gallard & Kilroe-Smith, 1987).

2.2.4 Statistical analysis

The software used to do the statistical analysis was: STATA version 6.0 from STATA Corporation, College Station, Texas.

The Students t-test was confirmed by the Mann-Whitney-U test in each comparison.

The α_2M EBC of control children of all races without renal disease was compared to the α_2M EBC of children of all races with NS, using both the above mentioned statistical methods.

The α_2M EBC of control children of all races without renal disease was compared to the α_2M EBC of adults of all races without renal disease, using both the above mentioned statistical methods.

The α_2M EBC of paediatric FSGS patients of all races was compared to the α_2M EBC of paediatric MLNS/(MCNS) patients of all races, using both the above mentioned statistical methods.

2.3 Results

Table 2.2 indicates the α_2M EBC comparisons of control individuals and patients.

There was not a significant difference in the mean α_2M EBC between children controls of all races compared to the mean α_2M EBC of children patients of all races (Students t-test: p = 0,146 and Mann-Whitney-U-test: p = 0,126), as α_2M levels are high in all children.

There was a highly significant difference in the mean α_2M EBC between children controls of all races and the mean α_2M EBC of adult controls of all races, (Student t-test: $p < 0,001$ and Mann-Whitney–U-test: $p < 0,001$), as the mean α_2M EBC of children is higher (14,55 KU/l) than the mean α_2M EBC of adults (9,19 KU/l).

Table 2.2 Comparisons of α_2M EBC (mean ± SD) in black and white children patients and in black and white children controls, as well as in adult controls.
(Number of subjects in brackets).

All children controls (11) (14,55 KU/l ± SD 4,14 KU/l)	All children patients (17) (18,47 KU/l ± SD 2,99 KU/l)	STT: p = 0,146 MWUT: p = 0,126	Total 28
All children controls (11) (14,55 KU/l ± SD 4,14 KU/l)	All adult controls (20) (9,19 KU/l ± SD 2,22 KU/l)	STT: p < 0,001 MWUT: p < 0,001	Total 31

Table 2.3 indicates the α_2M EBC comparison of FSGS and MLNS/(MCNS) patients.

There is no significant difference in the mean α_2M EBC between FSGS patients of all races compared to MLNS/(MCNS) patients of all races (STT: p = 0,146 and Mann Whitney U-test p = 0,144).

Table 2.3 Comparison of α_2M EBC (mean ± SD) in FSGS patients of all races to α_2M EBC (mean ± SD) in MLNS/(MCNS) patients of all races.
(Number of subjects in brackets).

FSGS patients (12) (18,02 KU/l ± SD 4,65 KU/l)	MLNS/(MCNS) patients (5) (18,91 KU/l ± SD 1,33 KU/l)	STT: p = 0,146 MWUT: p = 0,144	Total 17

2.4 Discussion

Alpha$_2$M is a multifunctional binding- and targeting protein with possible roles in immunity (Borth, 1994). This protein binds proteinases such as elastase and cytokines in particular, and also competes with α_1PI for proteinases (Gaillard & Kilroe-Smith, 1987). In this study the α_2M EBC assay was used to determine plasma concentrations of α_2M as immunological methods such as the nephelometric assay were found to be less accurate (Gaillard *et al.*, 1989). Studies of glomerulosclerosis suggest that fibrogenic cytokines, particularly TGF-β_1 play a central role. TGF-β_1 is known to increase production of collagens, fibronectin and proteoglycans. TGF-β_1 upregulates cellular expression of extracellular matrix receptors (Border *et al.*, 1992; Border & Ruoslati, 1992). The interaction of TGF-β_1 with binding proteins such as α_2M, represents potential mechanisms to regulate the multiple and varied growth effect of this cytokine. One of the mechanisms implicated in cell proliferative response is that α_2M delivers TGF-β_1 to the α_2M receptor, LRP. Binding of α_2M to LRP is essential for α_2M regulation of TGF-β_1 actions (Stouffer *et al.*, 1993). LRP is a receptor for multiple ligands which is expressed on a variety of cells including fibroblasts and macrophages (Howard *et al.*, 1996 (b)). The α_2M/TGF-β_1 complex binds to LRP, causing proliferation of these cells in the kidney matrix, resulting in matrix proliferation. Furthermore, α_2M might be converted to the receptor-recognised form when extracellular proteinase concentrations increase, as is the case at inflammation sites. Proteinases are inactivated and cleared via endocytosis, by cells that possess α_2MR/LRP. Thus, α_2M provide a way to remove excess growth factors from inflammation sites (Bonner, 1994).

In this study, all children had significantly raised α_2M EBC in comparison to adults. This confirms a study by Kilroe-Smith *et al.*, (1991), who found that children have high levels of α_2M which decrease in the third decade of life (Housley, 1968), to approximately half (Kilroe-Smith *et al.*, 1991). These levels in childhood may relate to the activity of a factor, which varies with age and may be linked to steroid hormone function (Kilroe-Smith *et al.*, 1991). Thus, the concentration of α_2M in plasma is normally about 2 g/l and declines gradually until adulthood (De Sain-Van Der Velden *et al.*, 1998 (a)). Although inherited α_2M deficiency does exist, a total absence may not be compatible with [illegible] (De Sain-Van

Der Velden *et al.*, 1998 (a)). Increased serum levels have been reported in adult patients with burns, diabetes, active SLE and in renal diseases such as glomerulonephritis; pyelonephritis and renal amyloidosis (Hedfors *et al.*, 1971). These authors also found α_2M levels in serum to be 30% higher in adult females, however, no difference was found in the α_2M serum levels of adult patients with or without NS. Alpha$_2$M is an acute phase reactant, and may be increased in inflammatory states such as asthma, and hepatocellular carcinoma (Gaillard *et al.*, 1995). However, in a study done by Kilroe-Smith *et al.*, (1991), it was shown that α_2M levels are the same for asthmatic children (13,1 KU/l) and for normal control children (13,2KU/l). The present study showed no significant difference in α_2M EBC between children control individuals and paediatric NS patients. Mansfield *et al.*, (1980), found high levels of α_2M in paediatric NS patients, by using patient sera in a radial immunodiffusion test. This might be due to a prolonged half-life or due to increased α_2M synthesis, as a result of the inflammatory response (Birkenmeier *et al.*, 1994). Increased α_2M levels are described in adult NS patients, regardless of the primary disease (Vaziri *et al.*, 1994) and may contribute to complications of NS by increasing plasma viscosity (McGinley *et al.*, 1983) and decreasing the availability of zinc (Tumer *et al.*, 1989). However, the present study showed no significant difference in the α_2M EBC of paediatric FSGS patients compared to paediatric MLNS/(MCNS) patients.

A change in plasma volume was reported to be responsible for the increased α_2M plasma levels in NS, but other studies reported normal plasma volumes in adults with NS (De Sain-Van Der Velden *et al.*, 1998 (b); Geers *et al.*, 1984). Therefore, accumulation of α_2M in plasma of adults with NS must be a consequence of metabolic changes, rather than increased plasma concentration (De Sain-Van Der Velden *et al.*, 1998 (a)). As the LRP/α_2M receptor binds LRP as well as apoE, a reduced uptake of α_2M could result if apoE containing lipoproteins such as VLDL (Chappell *et al.*, 1993) are increased as a consequence of decreased clearance in NS patients (De Sain-Van Der Velden *et al.*, 1998 (c)). Thus, a reduced uptake of α_2M could result if the decreased clearance of VLDL is due to alterations in the LRP (De Sain-Van Der Velden *et al.*, 1998 (a)). By using the nephelometric assay, De Sain-Van Der Velden *et al.*, (1998 (a)) found that α_2M plasma levels are increased approximately two-fold in adult NS patients due to increased hepatic synthesis alone (De Sain-Van Der Velden *et al.*, 1998 (a)). Alpha$_2$M interferes with the

removal of lipids as it binds to the same LRP receptor used by apoE to clear lipoproteins from the circulation. The high α_2M levels found in all children, which is independent of the clinical condition contribute to the less efficient lipoprotein clearance present in NS.

The results of the present study, using the EBC assay, showed no statistically significant difference between α_2M levels in children control individuals (mean α_2M level = 14,55 KU/l ± SD 4,14 KU/l) and paediatric NS patients (mean α_2M level = 18,47 KU/l ± SD 2,99KU/l) ($p = 0,146$ (STT) and $p = 0,126$ (MWUT)). This study confirms the finding of Kilroe-Smith *et al.*, (1991) in which α_2M EBC was found to be the same in asthmatic as well as in control children.

The results of the present study, using the EBC assay, showed that α_2M levels in children control individuals (mean α_2M level = 14,55 KU/l ± SD 4,14 KU/l) were significantly raised compared to the adults control individuals (mean α_2M level = 9,19 KU/l ± SD 2,22 KU/l) ($p < 0,001$ (STT) and $p < 0,001$ (MWUT)). The study confirms the finding of Kilroe- Smith *et al.*, (1991) that α_2M EBC start decreasing in the third decade of life to approximately half in adulthood.

The results of the present study, using the EBC assay, showed no statistically significant difference in α_2M plasma levels of children with FSGS (mean α_2M level = 18,02 KU/l ± SD 4,65) compared to children with MLNS/(MCNS) (mean α_2M level = 18,91 KU/l ± SD 1,33 KU/l) ($p = 0,146$ (STT) and $p = 0,144$ (MWUT)).

Variable α_2M concentrations in plasma modulate the inhibitory role of α_1PI in human plasma. *In vivo*, a balance is needed in the synthesis and breakdown of elastin. This homeostasis may be disturbed by the increased concentration of α_2M, causing less inhibitory activity by α_1PI (Gaillard & Kilroe-Smith, 1987).

Proteolytic enzymes are involved in activation of inflammatory mediator systems participating in complement activation, kinin regeneration, coagulation and fibrinolysis (Vaziri *et al.*, 1994; Asami *et al.*, 1992). After release from leukocytes, proteinases can be direct effectors of tissue injury. Of the seven major proteinase inhibitors in plasma, α_2M is

the most abundant. It acts as a defence system against tissue injury because of its broad specificity for various proteinases. Although α_2M binds proteinases it does not inactivate them, and therefore they are still able to degrade small molecular weight substrates. Proteolytic events are dominant over proteolytic control mechanisms of endogenous proteinase inhibitors at sites of inflammation. Proteinase inhibitors participate in proteolytic tissue injury and may have a protective effect. Asami *et al.*, (1992) reported on a four year old male patient with α_2M deposition in the glomeruli. This is the first report on deposition of α_2M in renal tissue, and therefore difficult to evaluate in relation to the pathogenesis of NS. The patient responded to a synthesised proteinase inhibitor, camostat mesylate. (Asami *et al.*, 1992).

A recent study by Halkas *et al.*, (1998), has shown that patients with NS has less effective α_1PI variance than control individuals. Thus, the increased α_2M EBC could aggravate the imbalance of the two main protease binders. The α_2M deposition might indicate plasma leakage through capillary walls due to increased vascular permeability (Asami *et al.*, 1992). Furthermore, this increased concentration could interfere with the binding of apoE to the LRP receptor and cause accumulation of triglycerides in NS patients.

In conclusion, this study has shown that α_2M EBC is raised in patient- and in control children, as well as in paediatric FSGS and MLNS/(MCNS) patients. This is due to the fact that α_2M is inherently raised in children compared to adults.