CHAPTER 3: APOLIPOPROTEIN E IN NEPHROTIC SYNDROME

3.1 Introduction to ApolipoproteinE

3.1.1 ApolipoproteinE structure

Plasma lipoproteins form globular micelle-like particles consisting of a non-polar core of triacylglycerols and cholesterol esters surrounded by an amphiphilic coating of protein, phospholipid and cholesterol. They have been classified into five categories on the basis of physical and functional properties:

1) Chylomicrons which transport exogenous triacylglycerols and cholesterol from intestines to tissues.

2-4) VLDL, IDL and LDL related particles, which transport endogenous triacylglycerols and cholesterol from liver to tissue.

5) HDL which transport endogenous cholesterol from tissue to liver (Voet & Voet, 1990).

Lipoproteins are particles consisting of non-covalently associated lipids and proteins. They function in blood plasma as transport vehicles for triacylglycerols and cholesterol (Voet & Voet, 1990). ApoE is a protein constituent of the triglyceride rich lipoproteins, VLDL, HDL and chylomicrons (Mahley, 1988; Poirier *et al.*, 1995). Mature apoE in human plasma and cerebrospinal fluid is a single glycosylated 34 kDa polypeptide, which contains 299 amino acids (Rall *et al.*, 1982; Mahley, 1988).

3.1.2 ApolipoproteinE gene structure

Human apoE is encoded by a 3,6kb long, four-exon gene on the long arm of chromosome 19 (Paik *et al.*, 1985; Olaisen *et al.*, 1982; Das *et al.*, 1985) at position 19q13.2 (Davignon *et al.*, 1988). The nucleotide sequence and structure of the apoE gene was determined in 1985 (Paik *et al.*, 1985). After the sequencing of the apoE gene, the genotype was determined directly by using the polymerase chain reaction (PCR) and hybridization with radiolabelled oligonucleotide probes (Weisgraber *et al.*, 1988). The Amplification Refractory Mutation System (ARMS) which extends the PCR to allow rapid known mutation analysis in genomic deoxyribonucleic acid (DNA) was employed. The genotype is determined by agarose gel electrophoresis (Newton *et al.*, 1989). In early days,

isoelectric focussing of VLDL or serum was used to detect the apoE genotype based on phenotype assignment (Wenham *et al.*, 1991).

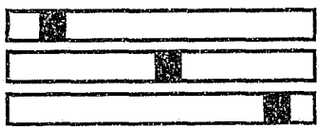
An oligonucleotide specific for the mutation site was used as one of the PCR primers. The technique is based on the fact that when a primer is mismatched at the 3'-nucleotide, no amplification will take place. The allele-specific primer is synthesized in two forms: one with the mutant and the other with a non-mutant 3'-nucleotide. Amplification occurs with the mutant primer if the mutation is present in the template DNA. Amplification also occurs with the non-mutant primer if the mutation is absent. Therefore hybridization with labelled allele-specific oligonucleotides is not necessary. The ARMS technique is applied to determine apoE genotypes (Wenham *et al.*, 1991).

The apoE gene is polymorphic so that three common alleles: E_2; E_3 and E_4 are inherited co-dominantly and then code for three apoE isoforms namely E_2: E_3 and E_4 (Rall *et al.*, 1982). Each of these isoproteins differs by a single amino-acid substitution at one of two positions of the 299-residue protein. $ApoE_3$ is the most common isoform in all human populations. $ApoE_3$ has a Cys at position 112 and an arginine (Arg) at position 158. $ApoE_2$ has a Cys at position 112 and at position 158. $ApoE_4$ has an Arg at position 112 and at position 158 (Figure 3.1). These changes cause major structural differences and also change the physical properties of the protein. These changes are responsible for effects of allelic variation at the apoE gene locus on cholesterol metabolism and the risk for atherosclerotic vascular disease. The fact that the Arg at position 158 of $apoE_3$ is replaced by a Cys in $apoE_2$ cause $apoE_2$ to have defective binding to the LDLR (Davignon *et al.*, 1988; Rall *et al.*, 1982).

3.1.3 ApolipoproteinE function

ApoE is synthesized and secreted in the kidney, adrenal gland, brain and retina. ApoE plays a role in lipid uptake in photoreceptor cells and is important in nerve regeneration. ApoE may be involved in maintenance and repair of photoreceptor membranes and when defective, it may lead to their degeneration. A defect in lipid metabolism can diminish lipid supply to the retina, which will eventually lead to retinal dystrophy (Huq *et al.*, 1993).

	ε4	ε3	ε2
Allele frequency on chromosome 19 in eastern Canada	0.152	0.770	0.078

Phenotypes		Relative charge	%	Isoelectric profile ⊖ ⊕
Homozygotes	ε4/4	+2	3.9	
	ε3/3	+1	61.8	
	ε2/2	0	2.0	
Heterozygotes	ε4/3		20.6	
	ε4/2		9.8	
	ε3/2		2.0	

Protein coded by each allele (Apo E is 299 amino acids long)	ε4	ε3	ε2
Site 112	ARG	CYS	CYS
Site 158	ARG	ARG	CYS

Figure 3.1 Apolipoprotein E polymorphisms, population phenotype distribution in eastern Canada and the biochemical properties (Poirier, 1994).

ApoE is important in metabolism of plasma lipoprotein (Davignon *et al.*, 1988; Wenham *et al.*, 1991), and is one of several apolipoproteins that directs lipid metabolism. It facilitates cholesterol transport into and out of cells and the removal of cholesterol from plasma (Poirier *et al.*, 1995). ApoE mediates the cellular uptake of lipid complexes through interaction with apoB/apoE LDLR and distinct hepatic apoE receptors (Poirier *et al.*, 1995).

3.1.4 ApolipoproteinE receptors

Two ApoE receptors are expressed in the human brain, namely LRP and LDLR (Rebeck *et al.*, 1995). The LRP belongs to the LDLR family and is a multifunctional receptor (Herz, 1993), which binds apoE-containing lipoproteins (Kowal *et al.*, 1989) along with 10 other ligands (Krieger & Herz, 1994). Some of these ligands are apoE; α_2M; tissue factor pathway inhibitor (TFP1); LPL; RAP; tissue plasminogen activator (tPA); lactoferrin and βA precursor protein (Herz, 1993; Du *et al.*, 1997). LPL promotes the binding of apoE-containing lipoproteins to LRP (Nykjaer *et al.*, 1993), and induces uptake of normal triglyceride rich lipoproteins by LRP (Chappell *et al.*, 1993). The LDLR is specific for apoE and apoB and a reduction on the degree of their binding occurs in the presence of activated α_2M (Stewart *et al.*, 1998).

LRP is a 600kDa transmembrane molecule translated as a single polypeptide (Herz *et al.*, 1988; Rebeck *et al.*, 1995). It is cleaved in the trans-golgi network into two sub-units to generate an 85kDa transmembrane fragment and a 515kDa extracellular fragment (Willnow *et al.*, 1996 (a); Rebeck *et al.*, 1995), which remain linked non-covalently. The extracellular domain contains many copies of two types of Cys-rich repeats. One repeat is found in growth factors, another in complement proteins, where these repeats serve as multiple independent binding sites for several ligands (Rebeck *et al.*, 1995).

Alpha$_2$M complexes with βA and mediates the endocytosis of βA via cell surface LRP (Du *et al.*, 1997). LRP binds apoE and the principal function of apoE is to mediate lipoprotein binding to cell surface receptors. Lipoprotein particles are taken up by cells and the lipid components are stored and/or re-utilized for other cellular functions (Bu *et al.*, 1998).

ApoE-containing lipoproteins and various ligands are cleared from the extracellular space by binding to LRP. Binding of each of these ligands to their various domains on LRP is blocked *in vitro* by the 39kDa RAP which could act as a physiological regulator of LRP activity (Rebeck *et al.*, 1995). *In vitro* LRP/RAP complexes are maintained in the endoplasmic reticulum of cells and only when LRP is brought to the cell surface, the two components dissociate (Rebeck *et al.*, 1995).

LRP can be responsible in part for the appearance of the very diverse nature of βA-associated proteins, which are associated with senile plaques. All known LRP ligands, which are all very diverse, are associated with senile plaques (Rebeck *et al.*, 1995). α_2M complexes with βA (Du *et al.*, 1997), and apoE also complexes with βA (Rebeck *et al.*, 1995). LRP becomes associated with large βA aggregates and thus can not function to clear apoE complexes (Rebeck *et al.*, 1995). LRP expression is significantly increased in neurons surrounding amyloid plaque deposition in AD (Poirier *et al.*, 1995). LRP co-localize with senile plaques in AD brains (Poirier *et al.*, 1995).

The LDLR or apoB,E receptor recognizes apoB and apoE. The LDLR is a glycoprotein with a molecular weight of 160kDa encoded by a 45kb gene of 18 exons and 17 introns (Sudhof *et al.*, 1985). The positively charged domain of apoE between residues 140 and 150 interact with the negatively charged amino acids of the LDLR (Wilson *et al.*, 1991). Binding of lipoproteins to LDLR is calcium-dependent and is inhibited by proteases (Brown *et al.*, 1986).

$ApoE_2$, E_3 and E_4 bind to hepatic receptors with different avidity and thus it is quite possible that altered binding of apoE-containing HDL-like particles may play a role in AD. ApoE-rich HDL may be involved in transport of apoE, which is synthesized by degenerating nerve cells by a receptor mediated system (Stewart *et al.*, 1998).

Human brain cell membranes contain receptors, which bind a specific sub-fraction of HDL containing apoE, but do not bind lipoprotein particles deficient in apoE. Both the LDL and the LRP receptors may be responsible for the uptake of physiological lipoprotein ligands such as apoE-rich HDL to provide cholesterol to regenerating nerve cells (Stewart *et al.*, 1998).

3.1.5 Population differences in apolipoproteinE phenotypes

$ApoE_3$ is the most common isoform of apolipoprotein and the $apoE_3$ allele has the highest frequency in all human populations, including the Khoi San in which it is significantly lower than in Caucasians and Asians. The $apoE_4$ frequency in the Khoi San was the highest reported for any human ethnic group. $ApoE_2$ has the lowest frequency in the Khoi San and in Caucasians (Hallman *et al.*, 1991). E_2/E_2 occurs in ± 1% of America's population (Cassel *et al.*, 1984). Ten per cent of Bushmen are $apoE_4$ homozygotes and more than 50% are $apoE_4$ heterozygotes. Overall, 60% of Bushmen carry at least one $apoE_4$ allele, which is similar in New Guineans (Kamboh *et al.*, 1990). High frequencies for $apoE_4$ has also been found in Australian Aborigines (Kamboh *et al.*, 1991) and in Black Africans (Nigerians and Sudanese) (Kamboh *et al.*, 1990; Kamboh *et al.*, 1991). Furthermore, in Black Africans the frequency of the $apoE_4$ allele was found to be 48,0% compared to 20,8% in South African Caucasians (Loktionov *et al.*, 1999). In the Northern Hemisphere, Finns and Inuit from Greenland also have high $apoE_4$ frequencies (Ehnholm *et al.*, 1986; De Knijff *et al.*, 1992). $ApoE_4$ has a 20% frequency in the general population (Utermann *et al.*, 1980; Zannis & Breslow, 1981; Utermann *et al.*, 1982).

The allele distribution pattern is compatible with the theory that $apoE_4$ is the ancestral allele as it is present at high frequencies in "old" populations such as the Khoi San (Sandholzer *et al.*, 1995).

Bushmen have among the lowest cholesterol levels in the world and their cholesterol concentrations are only moderately affected by the $apoE_4$ allele. This supports Utermann's hypothesis that a significant effect of the $apoE_4$ allele is present in cases of high fat diets and high total cholesterol of some populations (Utermann, 1987), but not in populations such as the Bushmen with low total cholesterol (Sandholzer *et al.*, 1995).

In the Bushmen studied, it seems as though the apoE polymorphism has no effect on lipid metabolism, which adds to the idea that the apoE allele's effect on cholesterol levels depend on the environmental factors such as nutrition or diet (Sandholzer *et al.*, 1995).

The association of apoE_4 with AD and atherosclerosis in North American Caucasians, Europeans and Japanese, indicate that it is a disease susceptibility gene which is independent of ethnic background. Bushmen are probably protected from atherosclerosis due to their diet. If Western Caucasian population risk figures apply to Bushmen, they should theoretically have a high risk for developing atherosclerosis and AD if they are exposed to modern civilization (Sandholzer *et al.*, 1995).

ApoE_3/E_3, the predominant phenotype, is degraded more rapidly than apoE_2/E_2 by the human hepatoma derived cell line, HepG2. This is also characteristic in Type III hyperlipoproteinaemia, which suggests the diminished catabolism of E_2/E_2 by intact livers is due to lack of recognition by hepatocytes. The distinction between apoE_3/E_3 and apoE_2/E_2 isoforms is due to the presence of a HepG2 cell-surface receptor, which binds apoE_3/E_3 with greater affinity than apoE_2/E_2. Diminished catabolism of apoE_2/E_2 compared to apoE_3/E_3 by human liver-derived cells is reported (Cassel *et al.*, 1984).

Each isoform is specified by one of three alleles, E_2, E_3, E_4, at a single genetic locus. The three alleles determine the six apoE phenotypes namely: E_2/E_2, E_3/E_3, E_4/E_4, E_4/E_3, E_4/E_2 and E_3/E_2 (Cassel *et al.*, 1984). The three major isoforms of human apoE heterogeneity is due to the differences in the primary structure, which involves a Cys-Arg interchange. ApoE_4 has no Cys, apoE_3 has one Cys and apoE_2 has two Cys. There are two other rare variants of apoE_2. ApoE_2* (Arg 145-Cys) and apoE_2** (Lysine 146-Glutamine). All three apoE_2 variants are functionally defective as they all have reduced binding activity to LDL (apoB,E) receptors. All three apoE_2 variants have the same charge and therefore can not be distinguished by isoelectric focusing (IEF), but only by SDS-PAGE (Cassel *et al.*, 1984). The apoE_2 mutant is more severely defective in binding (< 2% of normal binding compared to apoE_3) than apoE_2* and apoE_2** (40-50% of normal binding) (Cassel *et al.*, 1984).

The positive charge on Arg 158 in apoE_3/E_3 is important for normal receptor binding on cell surfaces because when a positive charge is added to Cys 158 in apo$E_2/_2$, the binding activity is largely restored. The apoE_3 protein has one more positive charge than the E_2 protein and one less positive charge than the E_4 isoform. These charge differences are due to variations in the primary structure that involves Cys-Arg exchanges at two different

locations. The apoE molecule contains a series of amphiphatic helices which, just as with apoA and apoC are involved in the lipid-binding characteristics of the molecule (Cassel *et al.*, 1984).

The wild-type form of apoE is $apoE_3$ (Yamamura *et al.*, 1984 (b)). Gene frequencies of apoE alleles of healthy Japanese individuals were found to be: $E_4 = 0,090$; $E_3 = 0,845$ and $E_2 = 0,065$ and this is comparable to th~ German as well as the American populations (Yamamura *et al.*, 1984 (b)). Whilst gene frequencies in Germany were reported as: $E_4 = 0,150$; $E_3 = 0,773$ and $E_2 = 0,077$ (Utermann *et al.*, 1980 & 1982). In America gene frequencies were reported to be: $E_4 = 0,110$; $E_3 = 0,720$ and $E_2 = 0,170$ (Zannis & Breslow, 1981). Therefore, it was initially thought that there are no striking differences in apoE allele frequencies in different populations (Yamamura *et al.*, 1984 (b)).

ApoE-Bethesda is also associated with type III hyperlipoproteinaemia (Gregg *et al.*, 1983). Similarly an $apoE_5$ and then later an apoE-Suita phenotype were identified in hyperlipidaemic- and in atherosclerotic patients. $ApoE_5$ and apoE-Suita isoprotein have isoelectric points more basic than $apoE_3$ (which is the parent type) by two and four units of charge respectively. ApoE-Suita isoprotein has the same molecular weight as ordinary apoE-isoproteins, but the molecular weight of $apoE_5$ isoprotein is $1,5 \times 10^3 - 2,0 \times 10^3$ lower than other apoE isoproteins by SDS-PAGE (Yamamura *et al.*, 1984 (a)). The prevalence of these two newly discovered $apoE_5$ and apoE-Suita mutants in atherosclerotic and hyperlipidaemic subjects might be limited to the Japanese population as it has not been found in any Western country (Yamamura *et al.*, 1984 (b)).

The special domain which contains residues 139 - 146 of an apoE polypeptide chain is important for binding to apoB, E receptors and $apoE_2$ mutants with amino acid mutations in this region are defective in their receptor binding (Innerarity *et al.*, 1983; Weisgraber *et al.*, 1982). The newly discovered $apoE_5$ and apoE-Suita mutants are not associated with β-VLDL or cholesterol-rich remnant retention, therefore their receptor binding activity was preserved (Yamamura *et al.*, 1984 (b)).

It is well known that E_2 and E_4 are dysfunctional isoforms of apoE in their binding to receptors and catabolism (Mahley & Innerarity, 1983; Boerwinkle & Utermann, 1988). The increased frequency of these two isoforms in retinitis pigmentosis (RP) might have significance in the pathogenesis of the disease (Huq *et al.*, 1993).

$ApoE_2$ has about 1% of the affinity for the LDLR compared to $apoE_3$ and $apoE_4$ *in vitro*, which results in type III hyperlipoproteinaemia in some $apoE_2$ homozygotes (Poirier *et al.*, 1995). The $apoE_2$ from hypo-, normo-, and hypercholesterolemic subjects with $apoE_2/E_2$ phenotype all have defective receptor binding, but in addition, other factors are necessary to manifest in type III familial hyperlipoproteinaemia (Cassel *et al.*, 1984). $ApoE_2$ has only slightly lower heparin affinity than does $apoE_3$ or $apoE_4$. Other apoE properties such as binding to cell surface heparan-sulfate proteoglycans are important for apoE function in lipoprotein transport (Poirier *et al.*, 1995). $ApoE_4$ has normal affinity for LDLR as well as for heparin but is functionally different from $apoE_3$ as it has a higher affinity for VLDL (Poirier *et al.*, 1995).

Three isoforms of apoE are detected by IEF. $ApoE_3$ is most common and $apoE_2$ and $apoE_4$ are designated according to their isoelectric point (pI) relative to the E_3 isoform (Poirier *et al.*, 1995). On SDS-PAGE gels, the E_2 isoform moves slower than E_3 and E_4 due to its higher molecular weight (Huq *et al.*, 1993).

3.1.6 Role of lipids in nephrotic syndrome

Secretion of lipoproteins consists of a wide range of density classes such as: VLDL, IDL, LDL and HDL (Marsh, 1974). VLDL consists of B100 as well as B48 (the truncated B100 contained in chylomicrons) and production of both forms is increased in nephrosis (Sparks & Marsh, 1981), however, the plasma levels of free fatty acids are not increased (Garber *et al.*, 1984). Increased VLDL, IDL and LDL production appears to be driven by increased apoB synthesis and secretion in the early stages of nephrosis. Decreased VLDL catabolism promotes the sustained increase of VLDL levels (Marsh, 1996).

Lipid abnormalities are common in ESRD where abnormal lipid metabolism may contribute to the progression of renal disease. Lipoprotein catabolism varies according to

the apoE phenotype and the frequency of apoE alleles is different in various races (Oda *et al.*, 1999).

Hyperlipidaemia may contribute to progression of renal dysfunction (Oda & Keane, 1995). Very few clinical studies in humans have been done to determine the genetic pathogenesis of progressive renal dysfunction in relation to abnormalities of lipids. It has been reported that apoE plays an important role in lipoprotein metabolism and the catabolism of lipoproteins vary according to apoE phenotype (Sherill *et al.*, 1980).

The three isoproteins E_2; E_3 and E_4 differ in their lipid-modulating effect and the polymorphism affects the morbidity of atherosclerosis in the general population. ApoE_4 is associated with high concentrations of apoB and low-density lipoprotein cholesterol implicated in atherosclerosis and coronary heart disease (Utermann, 1987). The apoE polymorphism may also modulate serum levels of cholesterol and the susceptibility to atherosclerotic vascular disease in haemodialysis patients (Imura *et al.*, 1999).

The phenotype distribution, as well as the allele frequency, of haemodialysis patients is similar to those found in controls. It is not sure whether apoE polymorphism influences the prevalence of atherosclerosis in dialysis patients (Imura *et al.*, 1999).

ApoE polymorphism modulates cholesterol metabolism in patients on dialysis, but have little association with atherosclerosis in these patients (Imura *et al.*, 1999). Patients on haemodialysis are at high risk for atherosclerotic vascular disease. Cardiovascular disease is the major cause of death in this case (Becker *et al.*, 1997).

Hypercholesterolaemia promotes atherosclerosis and might be a risk factor for chronic progressive kidney disease (Moorhead *et al.*, 1982).

In NS, the most striking feature is the quantitative abnormalities in the metabolism of lipoproteins. Triglycerides, phospholipids, total serum cholesterol and apoB, -C and -E are all increased (Joven *et al.*, 1990).

Lipids occur in the kidney in a wide range of renal disease. In FSGS, foam cell formation and lipid deposition are almost always present (Schönholzer *et al.*, 1992). It was hypothesized that lipid accumulation may injure glomerular cells and predispose for glomerulosclerosis (Moorhead *et al.*, 1982). Glomerular epithelial cells are abundant in the glomerulus. Glomerular epithelial cells are directly exposed to high concentration of lipoproteins in many renal diseases accompanied by NS. Prolonged circulation and exposure of renal and extrarenal tissue to lipoproteins can cause atherosclerosis and glomerulosclerosis, as often found in nephrotic renal diseases. Although glomerular epithelial cells have low LDL-receptor activity, they do exhibit receptor-mediated lipoprotein uptake and high affinity for β-VLDL (Krämer *et al.*, 1993).

Hyperlipidaemia is a common characteristic of NS and is included in the definition of NS (Wheeler *et al.*, 1989; Stenvinkel *et al.*, 1993). Increased synthesis as well as decreased clearance of lipoproteins contribute to hyperlipidemia characterized by increased total and LDL cholesterol levels, with normal or decreased HDL cholesterol (Appel *et al.*, 1985; Stenvinkel *et al.*, 1993). As early as 1969, an increased incidence of coronary heart disease has been reported in NS patients (Berlyne *et al.*, 1969).

Infants with CNF have growth retardation and extremely high levels of cholesterol. Patients with CNF show marked elevation of triglycerides in all lipoproteins especially in the VLDL fraction. The increase in serum cholesterol is mainly attributable to an increase of cholesterol in triglyceride- rich particles (chylomicrons, VLDL and IDL) (Antikainen *et al.*, 1992).

Multiple mechanisms lead to lipid abnormalities in CNF patients. Stimulated hepatic lipoprotein synthesis, impaired conversion of VLDL and IDL to LDL, compositional changes, urinary loss of low-molecular apoproteins and reduced LPL activity all play a role and increase the risk of arteriosclerosis in CNF patients (Antikainen *et al.*, 1992). CNF is an incurable but rare autosomal recessively inherited disease (Norio, 1966). Characteristic features of CNF are selective proteinuria, which starts in utero, a large placenta and NS from birth (Hallman *et al.*, 1967).

Any NS is associated with lipid abnormalities, which seem to correlate with the severity of the hypoproteinaemia (Appel *et al.*, 1985; Antikainen *et al.*, 1992).

The increased content of apoE in VLDL in diabetic patients may be of particular importance, due to the role of apoE in the uptake of lipoproteins by the liver receptor. This function of apoE is mostly reserved to its less acidic isoforms, especially $apoE_3$ (Weisweiler *et al.*, 1983).

In a group of diabetic and control individuals, it was found that there is a shift in diabetic patients to the more acidic isoforms $apoE_2$ and $apoE_4$. This can be explained by the increase of the sialic acid content of apoE. There could be a defect in desialation of apoE in diabetic patients. This observation may be associated with development of premature atherosclerosis in diabetes mellitus (Weisweiler *et al.*, 1983).

Immunologic or metabolic insults may cause deposition of lipids in renal glomeruli in advanced glomerular lesions (Olson & Heptinstall, 1988). Glomerular lipid accumulation is implicated in progression of glomerular sclerosis (Diamond & Karnovsky, 1988). NS patients accumulate lipids in glomeruli and renal interstitium (Gröne *et al.*, 1990).

Foam cells can be seen in sclerotic areas, in young patients with NS, with minimal change glomerulopathy with focal and segmental sclerosis. Lipid droplets were found in endothelial-, mesangial-, visceral epithelial cells, subendothelial- and subepithelial parts of the GBM. In terms of volume, podocytes (visceral epithelial cells) are the most abundant cell type of the glomerulus (Gröne *et al.*, 1990).

Mesangial cells have phagocytic functions and can take up lipoproteins by a non-saturable, receptor independent mechanism and under pathological conditions may accumulate lipids like macrophages do in atherosclerotic plaques. It was found that these cells take up lipoproteins in a similar way to smooth muscle cells by a receptor mediated pathway (Warrman *et al.*, 1989).

Urine of nephrotic humans contain lipoprotein-like particles such as apoproteins A-I; A-II, C-II and C-III as well as HDL-like particles. Loss of apoprotein C-II is associated with low lipoprotein lipase activity in human nephrosis. Nephrosis is associated with accelerated atherosclerotic coronary disease (Shafrir *et al.*, 1990).

Two of the enzymes involved in regulation of lipoprotein transport in humans are LPL and hepatic lipase. They remove triglycerides from the core of lipoprotein particles and initiate their change into smaller, denser particles. Hepatic lipase is mainly located in the liver but small quantities are present in other organs. LPL is found in all extrahepatic tissue especially skeletal muscle and adipose tissue (Packard & Shepherd, 1993; Olivecrona *et al.*, 1993). Macrophages, adrenal cells, ovarian cells and some neuronal cells are other minor sites of LPL synthesis (Olivecrona *et al.*, 1993).

Changes in LPL activity are associated with parallel changes in fatty acid uptake by tissue from plasma lipoproteins. LPL acts at vascular endothelial cells, although it is produced by other cells, for transfer to binding sites at the endothelium. (Olivecrona *et al.*, 1993).

Human glomerular epithelial cells take up apoB-100 containing lipoprotein, that is LDL as well as apo B,E containing lipoproteins. The affinity for uptake of B,E containing lipoproteins is much higher than for LDL (Gröne *et al.*, 1990).

Renal lipiodosis can be seen in human glomerular disease. Cholesterol and phospholipids make up the bulk of these lipid deposits. NS patients accumulate lipids in glomeruli and renal interstitium VLDL and chylomicron remnants accumulate in the plasma of these patients. These lipoproteins have an abnormal lipid-protein composition (Gröne *et al.*, 1990).

Visceral epithelial cells of human glomeruli synthesize basement membrane components and are also involved in genesis of sclerotic foci. These cells accumulate lipids in glomerular disease. In kidneys free of disease, visceral epithelial cells rest on the semi-permeable GBM and are surrounded by plasma ultrafiltrate without lipoproteins. In glomerular proteinuric states, high molecular weight proteins and lipoproteins are filtered and interact with these visceral epithelial cells (Gröne *et al.*, 1990).

In various renal diseases, apoE containing triglyceride-rich lipoproteins such as VLDL, β-VLDL and chylomicron remnants accumulate in these patients plasma. ApoE serves as a ligand for their cellular uptake (Gröne *et al.*, 1990).

In glomerular proteinuria, the acid uptake of apoE-containing lipoproteins could cause lipid accumulation in visceral epithelium. Intracellular lipid deposition is increased even further by a significant reduction in plasma lecithin: cholesterol acyl transferace activity in the nephrotic state. ApoB- and apoE-containing lipoproteins with β-electrophoretic mobility accumulate in glomeruli and this contributes to sclerosis in NS (Gröne *et al.*, 1990).

3.1.6.1 ApolipoproteinE phenotypes in lipid regulation

Six different apoE phenotypes are distinguished and their frequencies determined in normolipidaemia, hypertriglyceridaemia, hypercholesterolaemia and mixed hyperlipidaemia. In the normolipidemic group the frequencies were found to be as follows: $E_3/_3 = 62,2\%$; $E_4/_4 = 2,2\%$; $E_2/_2 = 0.9\%$; $E_4/_3 = 19,9\%$; $E_3/_2 = 11,7\%$ and $E_4/_2 = 2,9\%$. $E_2/_2$ occurred in 2,5% of hypertriglyceridaemic persons and 5,0% in mixed hyperlipidaemic persons. $E_4/_4$ occurred in 5,0% of hypercholesterolaemic patients. These results indicate that $E_2/_2$ and $E_4/_4$ homozygosity predispose to hyperlipidaemia (Assman *et al.*, 1984).

3.1.7 Role of apolipoproteins in nephrotic syndrome

Proteinuria, hypoalbuminaemia, oedema and hyperlipidaemia characterize NS. It is possible that low plasma oncotic pressure leads to hyperlipidaemia (Marsh, 1996). High plasma lipid levels from any primary cause exacerbate renal damage (Diamond & Karnovsky, 1992). Long-standing NS is associated with an increased incidence of coronary heart disease (Ordoñez *et al.*, 1993).

Proteinuria is followed by hypoalbuminaemia, which decreases oncotic (plasma colloid osmotic) pressure, which leads to oedema. Regulation of extracellular fluid volume in

nephrosis is complex, especially with respect to sodium retention. Hypoalbuminaemia alone is not responsible for nephrotic oedema in human nephrosis (Koomans *et al.*, 1986). It was suggested that an osmoreceptor exists which responds to changes in oncotic pressure by secreting a factor similar to the atrial natriuretic peptide found in rats, which is secreted in response to pressure change in the heart's atrium. This factor acts on hepatocytes with the result that there is an increase in the transcription of most genes responsible for synthesis of plasma proteins, which includes apolipoproteins and this all results in hyperlipidaemia (Marsh, 1996).

In kidney disease, accumulation of lipoproteins is pronounced. In nephrosis in humans, apoE-rich VLDL and their remnants show an increased cholesterol and triglyceride content. These lipoproteins are taken up by a receptor-mediated pathway into the glomerular visceral epithelial cells. In glomerular proteinuric states, the active taking up of apoE-containing lipoproteins lead to lipid accumulation in the visceral epithelium. ApoB- and apoE-containing lipoproteins accumulate in glomeruli and could contribute to sclerosis in NS (Gröne *et al.*, 1990). Excretion of plasma protein constituents diminishes with increasing molecular weight in NS (Shafrir *et al.*, 1990).

ApoE gene polymorphisms result in three main alleles namely E_2; E_3 and E_4, which modulate lipid metabolism (Wilson *et al.*, 1994). Several studies have shown that apoE_2 has a lipid-lowering effect and apoE_4 has a lipid-increasing effect because of the difference in their lipoprotein receptor affinity (Davignon *et al.*, 1988; Asami *et al.*, 1999). ApoE_2 has a low affinity for cellular receptors (Innerarity *et al.*, 1987). ApoE_3 and apoE_4 show adequate binding to LDLR, but apoE_2 is defective in its binding to the receptors (Yamamura *et al.*, 1992).

ApoE_3 has an affinity for apoB,E receptor on the cell surface, and this is important in the cholesterol metabolism regulation (Mahley, 1982). ApoE_2 lacks this affinity (Weisgraber *et al.*, 1982; Schneider *et al.*, 1981).

ApoE phenotypes were determined in patients with glomerulonephritis and in patients with ESRD (Oda *et al.*, 1999). Allele frequency of apoE_2; E_3 and E_4 in the glomerulonephritis patients was similar to that found in the normal control population. The

glomerulonephritis patients with proteinuria had a higher $apoE_2$ allele frequency as well as $apoE_4$ and a lower allele frequency of $apoE_3$ than controls (Oda *et al.*, 1999).

ESRD patients had a higher allele frequency of $apoE_2$ and a lower $apoE_4$ allele frequency than controls. A high prevalence of NS was found in proteinuric glomerulonephritis patients with the $apoE_2$ phenotype. The high frequency of $apoE_2$ in Japanese ESRD patients suggests that $apoE_2$ possibly genetically predispose to ESRD in the Japanese population (Oda *et al.*, 1999).

3.1.8 Pathophysiology of apolipoproteinE phenotypes

On average, heterozygotes as well as homozygotes for $apoE_4$ have increased cholesterol with a concomitant increased risk for coronary heart disease (Davignon *et al.*, 1988).

Hyperlipoproteinaemia is a major risk factor for atherosclerosis. The frequency of $apoE_4$ was higher in patients with acute myocardial infarction and $apoE_2$ was lower, than in controls. $ApoE_4$ is a positive risk factor and $ApoE_2$ a negative risk factor for atherosclerosis development. It could be that the action of the apoE isoprotein is one of the mechanisms of atherosclerosis. The effect of apoE isoform on serum lipoproteins is evident in childhood already (Yamamura *et al.*, 1992).

Individuals with the $E_3/_2$ phenotype had decreased LDL levels and increased VLDL levels. Individuals with the $E_4/_3$ phenotype have increased LDL levels in their serum (Yamamura *et al.*, 1992).

$ApoE_2$ also have reduced binding activity to remnant receptors which together with the impaired binding to LDL-receptors would lead to a low conversion of VLDL remnants to LDL and also to decreased transportation of chylomicron remnants to the liver. This results in up-regulation of hepatic LDLR. Therefore individuals with $apoE_2$ have reduced LDL concentrations in their plasma (Yamamura *et al.*, 1992).

Abnormal apoE components such as $apoE_5$ and apoE-Suita are high in hyperlipidaemic patients and in patients with ischaemic heart disease (7:127). These components were not

found in 100 healthy individuals. Five of the seven patients with abnormal apoE had overt atherosclerosis, which suggests that these kinds of apolipoprotein mutations are related to atherosclerosis development (Yamamura *et al.*, 1984 (b)).

The existence of $apoE_5$ was found in atherosclerotic diseased patients. The prevalence of $apoE_5$ and apoE-Suita is as much as 5% in atherosclerotic individuals, and in individuals with hyperlipidaemia, but was not found in a group of 100 healthy individuals (Yamamura *et al.*, 1984 (b)).

A four fold increase in E_2/E_2 incidence and an eight fold increase in E_4/E_4 incidence were reported in a 100 Scottish RP families compared to a control Scottish population (Huq *et al.*, 1993). Hereditary retinal degeneration disease, is often accompanied by abnormal levels of cholesterol or polyunsaturated fatty acids. John, (1987) reported a ten fold increased frequency of the E_2/E_2 phenotype in German RP patients compared to the average German population. The data suggest that the effect on lipid metabolism caused by $apoE_2$ may play a role in the pathogenesis of RP in these particular patients (Huq *et al.*, 1993).

RP may be polygenic or multifactorial in RP patients homozygous for either E_2 or E_4. These patients would inherit the RP gene but the disease would only be expressed if they also inherit a dysfunctional apoE phenotype such as E_2/E_2 or E_4/E_4. This is consistent with the E_2/E_2 and E_4/E_4 occurrence in simplex cases of RP (Huq *et al.*, 1993).

Type III familial hyperlipoproteinaemia patients are phenotypically homozygous for $apoE_2$ (Cassel *et al.*, 1984). Type III hyperlipoproteinaemia appears in individuals homozygous for $apoE_2$ or in those where apoE is completely absent when they have another factor or nutritional factors, which lead to combined hyperlipidaemia (Yamamura *et al.*, 1984 (b)).

The abnormal protein specified by the E_2 allele of some type III familial hyperlipoproteinaemia patients has a markedly reduced ability to bind to LDLR. In these patients, accumulation of remnant lipoproteins in plasma may be due to the reduced ability of $apoE_2/E_2$ to bind hepatic receptors (Cassel *et al.*, 1984).

By immunostaining, several diverse proteins apart from βA, have been identified as being associated with senile plaques in AD. These proteins include proteinases such as trypsin, proteinase inhibitors such as α_2M and α_1-antichymotrypsin, complement, apoE, lactoferrin and heparan sulfate (Rebeck *et al.*, 1995).

Recently a strong genetic association was found between sporadic-; late-onset familial- and early-onset AD and the inheritance of the E_4 allele of the apoE gene (Strittmatter *et al.*, 1993; Rebeck *et al.*, 1995; Poirier *et al.*, 1995). This led to the suggestion that $apoE_4$ protein is a risk factor for developing AD (Rebeck *et al.*, 1995; Holtzman *et al.*, 1995). $ApoE_4$ homozygosity is not sufficient or necessary to cause AD but is an additional risk to those prone to the disease due to the presence of other factors in their genome and environment (Sandholzer *et al.*, 1995). The E_4 allele frequency is increased two- to three-fold in AD, while E_2 allele presence seems to correspond to a longer life span and possibly a neuroprotective effect (Holtzman *et al.*, 1995). The occurrence of the E_2 allele of the apoE gene is rare in AD, which suggests that it is a negative risk factor (Rebeck *et al.*, 1995). The well-established association between $apoE_4$ and AD is not population specific (Poirier *et al.*, 1995). Higher expression of $apoE_4$ opposed to $apoE_3$ occurs in brains of $apoE_4$ positive AD patients, but not in their age- and genotype-matched controls (Blacker *et al.*, 1998).

The differences in $apoE_2$, E_3 and E_4 binding to βA binding or transport can underlie the genetic association of apoE alleles with AD. Individuals with $apoE_4$ alleles are susceptible to senile plaque formation and AD. Understanding of AD neuropathology can also depend on the biological consequences of recruitment of apoE and LRP-related molecules to senile plaques (Rebeck *et al.*, 1995).

ApoE may play a role in the central nervous system processes of synaptic maintenance and proliferation of new dendrites, which are normal processes in the aging brain. This is particularly relevant to AD where there is both synaptic degeneration and loss of compensatory dendritic outgrowth (Holtzman *et al.*, 1995).

In the central nervous system, apoE is expressed by astrocytes so that apoE is a major apolipoprotein in brain and cerebrospinal fluid. There are two known cell-surface

receptors, which can bind and initiate endocytosis of apoE-containing lipoproteins namely the LDLR and the LRP receptor. LRP is a multifunctional cell-surface receptor that bind and endocytose many distinct ligands (Holtzman *et al.*, 1995).

Alpha$_2$M is a LRP ligand abundant in serum and it is a pan-proteinase inhibitor. In the brain α_2M and LRP are upregulated during injury and are localized in senile plaques in AD. Alpha$_2$M binds tightly to the βA peptide which is the major component of βA, and attenuates fibrillogenesis and neurotoxicity of βA. Alpha$_2$M also mediates degradation of βA and its clearance via endocytosis through LRP (Blacker *et al.*, 1998).

It is not clear whether α_2M acts alone or in conjunction with other genes to cause an increased risk for AD (Blacker *et al.*, 1998). Increased apoE$_4$ levels may cause an increased risk for AD by interfering with α_2M-mediated clearance and/or degradation of βA by competing with α_2M for either βA or for LRP (Blacker *et al.*, 1998). ApoE has been reported to inhibit α_2M-mediated degradation of βA (Blacker *et al.*, 1998).

3.1.9 Aim

The aim of this part of the study was to determine the apolipoprotein E genotypes in white and black South African children with FSGS or MLNS/(MCNS) and to look for an association of genotype with FSGS or MLNS/(MCNS).

3.2 Materials and Methods

3.2.1 Materials

The following materials were obtained from Promega (Madison, WI): dNTP's, Taq DNA polymerase, Cfo 1 restriction enzyme and proteinase K. The oligonucleotide primers used to detect the apoE polymorphisms were synthesized by Genosys Biotechnologies Inc (Woodlands, Tx). The DNA molecular weight markers were supplied by BioVentures Inc (Murfreesboro, TN). The 2 ml eppendorf tubes were supplied by Treff Laboratories (Switzerland); the PCR tubes were from Laboratory and Scientific Equipment Co (Johannesburg). Double-distilled water was obtained from SABAX (Johannesburg); all other reagents used to prepare buffers and solutions were of analytical grade (Merck, Darmstadt, Germany).

3.2.2 Subjects

Twenty-three black (Bantu-speaking) NS patients, (13 girls), 16 white (Caucasoid) NS patients, (4 girls) and 4 mixed race, (1 girl), aged 2-18 years were screened for apoE genotyping. Both the white and the black control group allele frequencies were taken from Loktionov *et al.*, (1999).

The NS patients were recruited from the Paediatric Nephrology Clinic of the Johannesburg hospital and were diagnosed with either FSGS or MLNS/(MCNS).

3.2.3 Methodology

DNA extraction

The PCR method requires a source of either DNA or RNA for amplification. The PCR technique is tolerant of impurities in the nucleic acid sample being amplified. De-proteinization of the sample is required to remove extraneous proteases, nucleases and phosphatases that might destroy reactants crucial for the amplification reaction (Bloch, 1991).

A DNA extraction method developed by Higuchi (1989) to release DNA from nucleated blood cells was modified and used in this study. This method allows the direct addition of cells to a PCR-compatible solution containing nonionic detergents, which lyses cell membranes. This method releases DNA from approximately $3x10^5$ nucleated cells. A number of washing steps remove haemoglobin, RNA and cytoplasmic DNA released from blood cells, which might prevent efficient amplification. The resulting pellet containing nuclear DNA is resuspended in a proteinase K detergent solution. This method yields approximately 20 µg of nuclear DNA/1 ml of whole blood (Higuchi, 1989).

DNA extraction protocol

Five milliliters of venous blood was collected from all subjects by venupuncture into EDTA coated vacutainer tubes. DNA was extracted from this blood using the following modification of the Higuchi (1989) method.

1. Mix 1000 µl EDTA-containing blood with 1000 µl lysis buffer (0,32 M sucrose; 10 mM Tris-HCl, pH 7,5; 5 mM $MgCl_2$; 1% Triton X) (Appendix A), in a 2 ml eppendorf tube.
2. Centrifuge at 6000xg for 15 seconds.
3. Carefully pour the supernatant off, add 1000 µl lysis buffer and resuspend the pellet by gently pipetting up and down.
4. Repeat steps 2-3 twice or thrice, but definitely no more than four times, as more washes result in DNA degradation.
5. Centrifuge at 6000xg for 15 seconds.
6. Remove the supernatant and carefully resuspend pellet in 300 µl of PCR storage buffer (50 mM KCl; 10 mM Tris-HCl, pH 8,3; 2,5 mM $MgCl_2$; 0,1 mg/ml gelatin; 0,45% Nonidet P40; 0,45% Tween 20) (Appendix A). Add 10 µl proteinase K (10 mg/ml).
7. Incubate at 56°C in a water bath for 1-3 hours.
8. Inactivate the proteinase K by boiling the samples at 95°C for 10 minutes.

Twenty-five microliter of this lysate is equivalent to 1 µg of genomic DNA. The integrity of this extracted DNA was determined by electrophoresis in 0,7% (w/v) agarose gels in 1x

TBE buffer (0,089 M Tris-borate; 0,002 M EDTA, pH 8,2) (Appendix A) at 100 Volts ror 20-30 minutes.

3.2.4 Detection of the apolipoproteinE polymorphism

The prevalence of apoE_2; -E_3 and -E_4 allelic variations within four exons of the gene at position 19q13.2, was determined using PCR and restriction enzyme analysis with the Cfo 1 restriction enzyme. The following primers, as published by Wenham *et al.*, (1991), were used in this study:

Upstream 5' forward apoE1 primer:

5' TCCAAGGAGCTGCAGGCGGCGCA 3' (23 mer).

Downstream 3' reverse apoE2 primer:

5' ACAGAATTCGCCCCGGCCTGGTACACTGCCA 3' (31 mer).

These two primers amplify a 227bp region of DNA, spanning the two apoE polymorphic sites located in codons 112 and 158 containing the polymorphic nucleotides 3745 and 3883.

The Cfo restriction enzyme recognizes and cuts the sequence: 5' NNNGCG / CNNN 3'. The Cfo 1 restriction enzyme gives rise to various combinations of four fragments from six possible apoE genotypes. These four fragments are 48bp; 72bp; 81bp and 91bp long. The 48bp fragments are easily recognisable on the polyacrylamide gel but they do not photograph well. A genotype of E_2/E_4 is indicated by fragment sizes: 48bp; 72bp; 81bp and 91bp and an E_3/E_4 genotype by fragments 48bp; 72bp and 91bp only (Figure 3.2).

Briefly, the target sequence was amplified in a 25 µl reaction volume containing 200 ng genomic DNA (as determined by spectrophotometric reading); 25 pmol of each primer; 2,5 µl dimethyl sulphoxide (DMSO); 0,5 units of Taq DNA polymerase; 0,2 mM each of dATP; dCTP; dGTP and dTTP; 10 mM Tris-HCl buffer pH 8,3. Negative PCR controls contained all the reagents except genomic DNA. The reaction mixture containing all the components except the Taq DNA polymerase enzyme, was heated to 94°C for 5 minutes in a Hybaid DNA thermal cycler to denature the DNA (Hybaid, UK). Following a cooling step of 65°C sample temperature, 0,5 units of Taq DNA polymerase was added and then 40

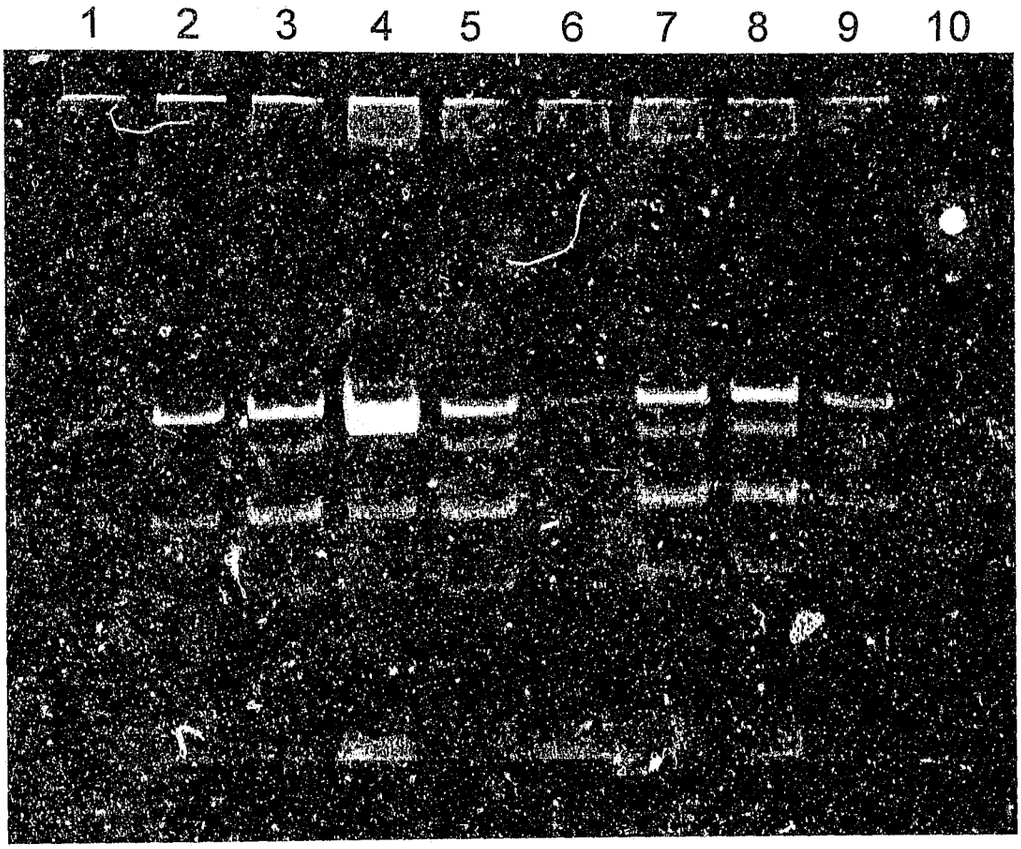


Figure 3.2
Polyacrylamide gel electrophoresis of apoE, showing different size fragments.
Lanes 3, 5, 7, 8 and 9 show the apo E_2/E_4 genotype
Lane 4 shows the apo E_2/E_3 genotype.

cycles of annealing at 65°C for 0,5 minutes; extension at 70°C for 1,5 minutes; denaturation at 94°C for 0,5 minutes and a final extension at 70°C for 10 minutes followed.

Enzyme restriction

Twenty-two microliter of the amplification product was digested with 24 units of Cfo 1 restriction enzyme for at least three hours at 37°C. Ten microliter of restricted DNA sample was mixed with 8 µl of loading (Appendix A) buffer and subjected to electrophoresis for 18 hours at 100 Volts on a 20% polyacrylamide gel (Appendix A). The gel was viewed and photographed under ultraviolet light of wavelength 302 nm, after staining with ethidium bromide (10 mg/1ml stock solution of which 10 µl is used in 100 ml buffer).

3.2.5 Statistical analysis

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

The apoE allelic genotypes were grouped as follows, to make sensible statistical analysis possible:

E_3/E_3 as "good", indicating the normal receptor binding ability of $apoE_3$.

E_3/E_4 and E_2/E_3 as "moderate", indicating the defective receptor binding of $apoE_4$ and $apoE_2$ as not severe, due to the combination with the normal receptor binding ability of $apoE_3$.

E_4/E_4; E_2/E_2 and E_4/E_2 as "severe", indicating the defective receptor binding ability of both $apoE_4$ and $apoE_2$ alleles in combination.

The Fisher exact test was used to determine the apoE allele frequency distribution in FSGS patients of all races compared to MLNS/(MCNS) patients of all races.

The Fisher exact test was used to determine the frequency distribution of FSGS compared to MLNS/(MCNS) in black and white NS patients.

The Fisher exact test was used to determine the frequency distribution of the apoE genotypes in black control individuals compared to black NS patients, and in white control individuals compared to white NS patients.

The Fisher exact test was used to determine the apoE allele frequency in black and white NS patients.

3.3 Results

Table 3.1 shows the frequency distribution of apoE alleles in FSGS patients compared to MLNS/(MCNS) patients of all races (percentages included to facilitate interpretation).

Comparison of the frequency distribution of apoE alleles in FSGS and MLNS/(MCNS) patients by the Fisher exact test showed no statistical significance in the E_2 allele ($p = 0,412$); the E_3 allele ($p = 0,375$) or the E_4 allele ($p = 0,525$).

Table 3.1 Frequency distribution of apoE alleles in FSGS patients of all races compared to MLNS/(MCNS) patients of all races.

Alleles	FSGS patients	MLNS/(MCNS) patients
E_2	7 (15%)	4 (10%)
E_3	25 (52%)	22 (58%)
E_4	16 (33%)	12 (32%)
Total	48 (100%)	38 (100%)

Fisher exact test: E_2 $p = 0,412$; E_3 $p = 0,375$ and E_4 $p = 0,525$.

Table 3.2 shows the frequency distribution of FSGS and MLNS/(MCNS) in black and white patients (percentages included to facilitate interpretation).

When comparing the frequency distribution of FSGS with the frequency distribution of MLNS/(MCNS) in blacks, a highly significant result is obtained (Fisher exact est: $p < 0,001$), as FSGS is more prevalent, while MLNS/(MCNS) is rare, in the black NS patients.

When comparing the frequency distribution of FSGS with the frequency distribution of MLNS/(MCNS) in whites, a highly significant result is obtained (Fisher exact test: $p = 0,003$), as MLNS/(MCNS) is more prevalent, while FSGS is rare, in the white NS patients.

Table 3.2 Frequency distribution of FSGS compared to MLNS/(MCNS) in black and white nephrotic syndrome patients.

	FSGS	MLNS/(MCNS)	Total
Blacks	18 (78%)	5 (22%)	23
Whites	4 (25%)	12 (75%)	16
Total	22	17	39

Fisher exact test: Blacks $p < 0,001$; whites $p = 0,003$.

Table 3.3 shows the frequency distribution of apoE genotypes in black control individuals* and black nephrotic (FSGS and MLNS/(MCNS)) patients (percentages were included to facilitate interpretation).

Comparison of apoE genotypes between black control individuals and black NS patients by the Fisher exact test showed significance for the good apoE genotypes ($p = 0,097$), but no significance for the moderate ($p = 0,328$) and severe ($p = 0,510$) genotypes.

Table 3.3 Frequency distribution of apoE genotypes in black control individuals* and in black nephrotic syndrome patients.

	E_3/E_3	E_3/E_4 and E_2/E_3	E_4/E_4; E_2/E_2 and E_4/E_2	
	(Good)	(Moderate)	(Severe)	Total
Black Controls*	30 (30%)	54 (54%)	16 (16%)	100
Black NS Patients	3 (13%)	15 (65%)	5 (22%)	23
Total	33	69	21	123

* Population taken from Loktionov *et al.*, (1999).

Fisher exact test: Good $p = 0,097$; moderate $p = 0,328$ and severe $p = 0,510$.

Table 3.4 shows the frequency distribution of apoE genotypes in white control individuals* and white NS (FSGS and MLNS/(MCNS)) patients (percentages included to facilitate interpretation).

Comparison of the good and moderate apoE genotypes between white control individuals and white NS patients by the Fisher exact test, showed a highly significant difference ($p = 0,003$ and $p = 0,002$ respectively). The p-value for the severe apoE genotypes was not significant ($p = 0,581$) by the Fisher exact test.

Table 3.4 Frequency distribution of apoE genotypes in white control individuals* and in white nephrotic syndrome patients.

	E_3/E_3	E_3/E_4 and E_2/E_3	E_4/E_4; E_2/E_2 and E_4/E_2	
	(Good)	(Moderate)	(Severe)	Total
White Controls*	74 (69%)	31 (29%)	2 (2%)	107
White NS Patients	5 (31%)	11 (69%)	0 (0%)	16
Total	79	42	2	123

*Population taken from Loktionov *et al.*, (1999).

Fisher exact test: Good $p = 0,003$; moderate $p = 0,002$ and severe $p = 0,581$.

Table 3.5 shows the frequency distribution of apoE alleles in black NS patients (including 4 mixed race) compared to white NS patients.

Comparison of apoE alleles in black and white NS patients by the Fisher exact test, showed a significant difference for the $apoE_2$ allele ($p = 0,035$). There was no statistical difference between the two groups by the Fisher exact test, for $apoE_3$ and $apoE_4$ ($p = 0,088$ and $p = 0,518$ respectively).

Table 3.5 Frequency distribution of apoE alleles in black (including 4 mixed race), and white nephrotic syndrome patients.

Alleles	Black patients	White patients
E_2	10 (19%)	1 (3%)
E_3	26 (48%)	21 (66%)
E_4	18 (33%)	10 (31%)
Total	54 (100%)	32 (100%)

Fisher exact test: E_2 $p = 0,035$; E_3 $p = 0,088$ and E_4 $p = 0,518$.

3.4 Discussion

$ApoE_3$ occurs in the highest frequency in all population groups (Hallman *et al.*, 1991). $ApoE_2$ occurs at the lowest frequency in most population groups especially in Caucasians (Hallman *et al.*, 1991). $ApoE_4$ occurs at a high frequency in the Bushmen and the New Guineans (Kamboh *et al.*, 1990), in the Australian Aborigines (Kamboh *et al.*, 1991) and in the Black Africans (Nigerians and Sudanese) (Kamboh *et al.*, 1990; Kamboh *et al.*, 1991). In a recent study, the $apoE_4$ allele was found to be associated with coronary heart disease and was present in 48% of Black South Africans compared to 20,8% of Caucasians ($p < 0,0001$) (Loktionov *et al.*, 1999). High $apoE_4$ frequencies have also been found in the Finns and in the Inuit from Greenland (Ehnholm *et al.*, 1986) whilst the $apoE_4$ frequency in the general population is approximately 20% (Uterman *et al.*, 1980; Zannis & Breslow, 1981; Uterman *et al.*, 1982).

The three isoproteins vary in their lipid-modulatory effect (Utermann, 1987) and catabolism of lipoproteins (Sherill *et al.*, 1980). Both $apoE_2$ and $apoE_4$ are dysfunctional isoforms of apoE, both in their ability to bind to receptors and in their catabolism processes (Mahley *et al.*, 1983; Boerwinkle & Utermann, 1988). $ApoE_2$ has only approximately one percent of the affinity to the LDL-receptor *in vitro*, compared to $apoE_3$ and $apoE_4$ (Poirier *et al.*, 1995). $ApoE_4$ has normal LDL-receptor affinity, but is functionally different from $apoE_3$ as it has a high affinity for VLDL (Poirier *et al.*, 1995). $ApoE_2$ is not degraded as rapidly as $apoE_3$ (Cassel *et al.*, 1984), so that $apoE_2$ is present for much longer than $apoE_3$ which binds efficiently to its receptor and is rapidly removed from circulation. $ApoE_2$ is defective in its binding to the LDL-receptor (Poirier *et al.*, 1995), due to the Cys at position 158 instead of an Arg as in $apoE_3$ (Davignon *et al.*, 1988; Rall *et al.*, 1982). ApoE in general, but especially $apoE_3$ which is less acidic, facilitates the uptake of lipoproteins by the liver receptors (Weisweiler *et al.*, 1983). Triglycerides, phospholipids, total serum cholesterol and apoB, -C, and -E are all increased in NS (Joven *et al.*, 1990). Glomerular epithelial cells have a high affinity for β-VLDL and are exposed to high concentrations of lipoproteins in many renal diseases. Prolonged exposure of renal and extra renal tissue to lipoproteins, can cause glomerulosclerosis which is often present in NS (Krämer *et al.*, 1993). Lipids accumulate in glomeruli and in the renal interstitium of NS patients (Gröne *et al.*, 1990). Stimulated hepatic lipoprotein synthesis, impaired conversion of VLDL to

IDL to LDL, compositional changes, urinary loss of low-molecular weight apoproteins as well as reduced LPL activity, play a role in the increased arteriosclerosis risk of NS patients especially in those with the CNF type (Antikainen *et al.*, 1992).

Nephrosis is associated with accelerated atherosclerotic coronary disease (Shafrir *et al.*, 1990). The production of VLDL is increased in nephrosis (Sparks & Marsh, 1981) and IDL and LDL production are also increased in nephrosis due to the increase in apoB synthesis in early nephrosis. Decreased VLDL catabolism also increase the VLDL levels (Marsh, 1996). ApoE is important in lipoprotein metabolism and when this process is abnormal in any way, it may contribute to the progression of renal disease (Oda *et al.*, 1999). Hyperlipidaemia is common in NS and may contribute to the progression of renal dysfunction (Oda & Keane, 1995).

In disease free kidneys, the visceral epithelial cells rest on a semi-permeable GBM and are surrounded by a plasma ultrafiltrate without lipoproteins. In glomerular proteinuric states, high molecular weight proteins and lipoproteins are filtered and therefore interact with visceral epithelial cells (Gröne *et al.*, 1990). ApoE-containing lipoproteins with β-electrophoretic mobility accumulate in the glomeruli and this contributes to sclerosis in NS patients (Gröne *et al.*, 1990).

The prevalence of the apoE_4/E_4 and -E_2/E_2 genotypes is increased in hyperlipidaemic persons (Assman *et al.*, 1984). ApoE_2 is defective in its binding to receptors (Yamamura *et al.*, 1992) as it lacks an affinity for apoB,E receptors (Weisgraber *et al.*, 1982). Allele frequency for apoE_2, -E_3 and –E_4 are the same in patients with glomerulonephritis than in the normal population (Oda *et al.*, 1999), but when proteinuria is involved, glomerulonephritis patients had a higher E_2 and E_4 allele frequency and a lower E_3 frequency, than control individuals (Oda *et al.*, 1999). Hetero- as well as homozygotes for apoE_4 have increased cholesterol levels with an additional increased risk of developing coronary heart disease (Davignon *et al.*, 1988). ApoE polymorphisms occur with a high prevalence in hyperlipidaemic patients (Yamamura *et al.*, 1984 (b)).

Of further interest are the raised α_2M levels (as determined by EBC) in NS children patients, as it is known that children have higher α_2M levels than adults. It has been shown

that in the presence of activated α_2M, the binding of apoE to the LDL-receptor is reduced (Stewart *et al.*, 1998). The combination of increased α_2M and deficient apoE alleles might lead to increased deposition of triglycerides in various tissues. Furthermore, when α_2M binds to LRP, the complex is taken up and degraded in lysosomes and no signaling cascade is triggered, but when α_2M binds to the α_2M-signaling receptor, a cascade is activated which regulates cell proliferation (Howard *et al.*, 1996 (a)).

An increased deposition of triglycerides and α_2M-TGF-β_1 complex binding to LRP could result in proliferation of various cells in the kidney matrix, resulting in matrix proliferation. ApoE-rich lipoproteins accumulate in the plasma of these patients with FSGS and results in visceral epithelial cells being exposed to high concentrations of lipoprotein. The avid apoE-containing lipoprotein uptake, contribute to glomerular lipid accumulation and sclerosis in the kidney (Gröne *et al.*, 1990).

In the present study, no statistically significant difference was found in the $apoE_2$ ($p = 0{,}412$); the $apoE_3$ ($p = 0{,}375$) or the $apoE_4$ ($p = 0{,}525$) allele frequency distribution between FSGS patients and MLNS/(MCNS) patients using the Fisher exact test. This finding prompted further investigation to possibly find a racial difference between the prevalence of FSGS and MLNS/(MCNS), as well as a racial difference in the frequency distribution of the three apoE alleles.

A highly significant result was obtained when comparing the frequency distribution of FSGS to MLNS/(MCNS) in black patients ($p < 0{,}001$), as FSGS is more prevalent in black individuals with NS than in white individuals with NS (Thomson *et al.*, 1997). A highly significant result was also obtained when comparing the frequency distribution of FSGS to MLNS/(MCNS) in white patients ($p = 0{,}003$), as MLNS/(MCNS) is more prevalent in white individuals with NS than in black individuals with NS (Thomson *et al.*, 1997).

The E_3/E_3 genotype was significantly decreased in both black ($p = 0{,}097$) and white ($p = 0{,}003$) NS patients compared to black and white control individuals. This result confirms the finding of Oda *et al.*, (1999), that when proteinuria is present, the $apoE_3$ allele frequency is decreased. These findings indicate that the apolipoprotein E may not be

efficient in lipid metabolism in these patients, as $apoE_3$ has normal lipid binding ability compared to $apoE_2$ and $apoE_4$.

In the present study, the $apoE_2$ allele was significantly increased in black patients (including four mixed race) ($p = 0,035$) of whom the majority presented with FSGS (20 out of 27), compared to white patients of whom the majority presented with MLNS/(MCNS) (12 out of 16). This confirms Oda *et al.*, (1999), who found that when proteinuria is present, $apoE_2$ and $apoE_4$ allele frequencies are increased. The E_4/E_4 genotype was found to be significantly raised in black South Africans (9%) compared to Caucasians (1,9%) (Oda *et al.*, 1999). The present study has shown a significant increase in the $apoE_2$ allele frequency in black South African patients in whom FSGS (renal scarring) is more prevalent compared to the white South African patients in whom MLNS/(MCNS) (non-scarring) is more prevalent. Due to the small sample size, it was not possible to statistically determine the frequency distribution of the various apoE genotypes in black or white patients with FSGS, compared to black or white patients with MLNS/(MCNS).

In conclusion, the present study has shown that a combination of high α_2M levels and a decrease in $apoE_3$ allele frequency, as well as an increase in the $apoE_2$ allele frequency may result in more severe disease as seen in this syndrome.

CHAPTER 4: PROTEIN S IN NEPHROTIC SYNDROME

4.1 Introduction to protein S

4.1.1 Structure of the protein S gene

The two protein S genes are located on chromosome 3 with conserved restriction sites, suggesting a high degree of homology (Ploos van Amstel *et al.*, 1988; Yamazaki *et al.*, 1995).

The active protein S gene, also called the α-gene or PROS1, consists of 14 introns and 15 exons, on chromosome 3 near the centromere in a DNA region of more than 80kb (Walker, 1992; Leroy-Matheron *et al.*, 1998). The analysis of protein S is complicated by the existance of a 55kb pseudogene, also called the β-gene or PROS2, which does not code for an active protein, as it consists of many frame shifts; multiple base changes and stop codons (Schmidel *et al.*, 1990; Leroy-Matheron *et al.*, 1998). The protein S α-gene and protein S β-gene span approximately 1,200 nucleotides. There is a high degree of homology (~97%) with deviations caused by small insertions, deletions and point mutations. The protein S β-gene has more mutations than the protein S α-gene (Ploos van Amstel *et al.*, 1988). The 5' end of the PROS1 gene has strong homology with other vitamin K-dependent proteins. Exon 1 encodes the signal peptide, exon 2 the pro-peptide and glutamic acid (Gla) domain, exon 3 a domain with high aromatic amino acid content, exon 4 a thrombin sensitive loop and exons 5 to 8, four epidermal growth factor (EGF)-like domains. The 3' end of the PROS 1 gene is different to all other known coagulation proteins as the last seven exons, exon 9 to 15, encode a sex-hormone-binding globulin-homologous domain (Leroy-Matheron *et al.*, 1998). Human protein S deficiency is inherited as an autosomal dominant trait (Marchetti *et al.*, 1993; Krachmalnicoff *et al.*, 1990). Hereditary deficiencies of protein C and protein S, together account for 10-15% of patients with familial thrombophilia (Ploos van Amstel *et al.*, 1989). A risk for developing thrombotic disease exists when protein S is deficient (Marchetti *et al.*, 1993; Krachmalnicoff *et al.*, 1990).

The two protein S alleles are designated positive (+) for the 271bp allele, not cut by the restriction enzyme Bst XI and negative (-) for the 235bp allele cut by Bst XI. Allele frequencies were estimated in 36 unrelated Italian and in 30 Black African subjects to be 0,47 for the 271bp (+) allele with sequence CCG and 0,52 for the 235bp (-) allele with sequence CCA (Marchetti *et al.*, 1993). An allele frequency of 0,48 and 0,52 were found respectively for these same alleles in 28 unrelated European Caucasians (Diepstraten *et al.*, 1991).

It was previously shown that in a family with hereditary protein S deficiency, the reduced plasma protein S level is associated with a partial deletion in the locus of protein S (Ploos van Amstel *et al.*, 1988).

The protein S gene polymorphism in codon Pro 626 was detected by allele specific hybridization by Diepstraten in 1991. Marchetti improved this method in 1993 by restriction analysis with Bst XI of the 3' coding region of exon 15 (Walker, 1992).

4.1.2 Discovery of protein S function

In the early 1970's, a new vitamin K-dependent protein, designated protein C, was purified (Stenflo, 1976). Proteases such as trypsin or thrombin could convert it to a serine protease which inhibited blood coagulation through the proteolysis of Factor Va, with Factor VIII also being a substrate (Figure 4.1). The active forms of Factors V and VIII were found to be better substrates than the circulating forms. Proteolysis of Factors V and VIII was shown to dependent upon the presence of phospholipid vesicles and calcium ions (Walker, 1992). In 1980 it was suggested that activated protein C needed a cofactor protein for expression of anticoagulant activity, in the same way as protease derived from vitamin K-dependent proteins, Factors Xa, IXa and VIIa required cofactor proteins for maximum activity expression. Early experiments showed that plasma could enhance the rate of inactivation of Factor Va by activated protein C. Protein S was revealed to be the factor present in the plasma, causing this enhancement. The anticoagulant activity of activated protein C was also proved to be dependent upon protein S (Walker, 1980). This demonstrated the relationship between the two activities and also the function of protein S (Walker, 1992).

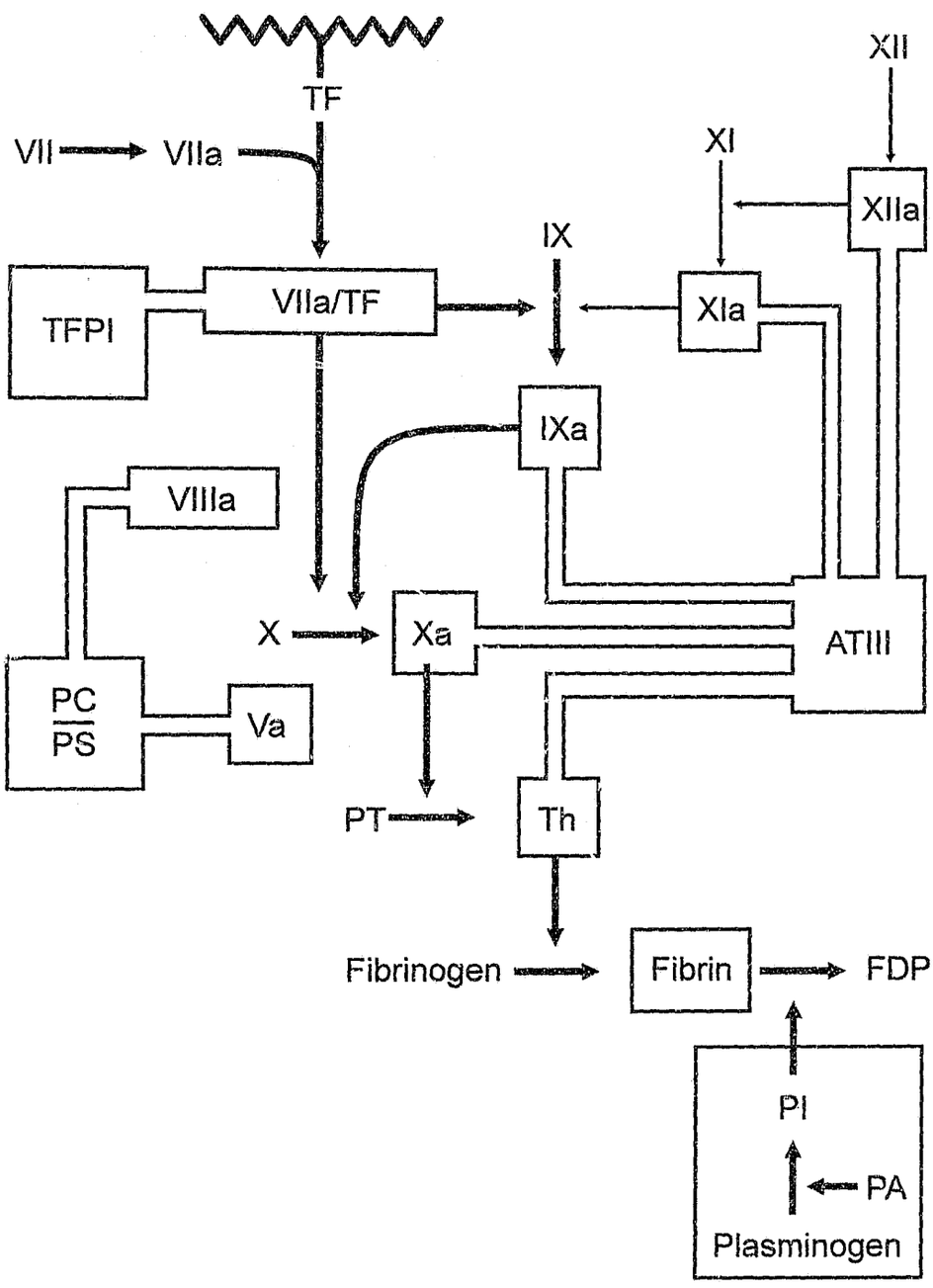


Figure 4.1 Anti-thrombotic pathways showing the interaction between factors VIII, V and X; and protein S and protein C (Schafer, 1994).

4.1.3 Structural properties of protein S

Protein S is a single chain (Krachmalnicoff *et al.*, 1990) vitamin K-dependent glycoprotein discovered in Seattle by Di Scipio and Davie as a side product in purification of other vitamin K-dependent proteins (Di Scipio & Davie, 1979). At the time of discovery, no function was assigned to this protein and it was arbitrarily called protein S, after the city of discovery (Lundwall *et al.*, 1986). A high degree of homology with other vitamin K-dependent proteins has been noticed. The protein S sequence consists of well-defined domains with the amino-terminal containing a Gla domain consisting of ten γ-carboxyglutamic acid residues (Di Scipio & Davie, 1979).

The Gla region is followed by a thrombin sensitive region, which is a disulfide-linked loop that converts a single-chain protein S into a two-chain protein upon cleavage by thrombin. The thrombin-sensitive region is followed by four repeats of an EGF-like region containing two unusual amino acids. In the first EGF domain a β-hydroxyaspartic acid residue is found (Fernlund & Stenflo, 1983) and each of the other three domains contain a β-hydroxyasparagin (Stenflo *et al.*, 1987). A region that has some homology with steroid-binding proteins follows the EGF region. In other vitamin K-dependent proteins, a protease domain follows the EGF region. The steroid-binding region of protein S makes up roughly two thirds of the protein S mass and contains only two disulfide bonds (Walker, 1992).

Like other vitamin K-dependent proteins, protein S has calcium-binding sites since a calcium interaction with protein S is very important for its function. The first calcium-binding site is in the Gla region which is essential in the interaction between phospholipid-containing membranes and protein S (Walker, 1986). The second calcium-binding site is in the three EGF-like domains (Dahlback *et al.*, 1990) and occupation of these calcium-binding sites cause a conformational change in the protein (Dahlback *et al.*, 1990). The significance of the function of these binding sites is not known, but they may be involved in the interaction between activated protein C and protein S (Walker, 1992).

4.1.4 Functional properties of protein S

Protein C is the vitamin K-dependent zymogen of a potent anticoagulant serine protease. The thrombin-thrombomodulin complex activates this zymogen on endothelial surfaces. Once activated, maximum enzymatic activity is only obtained in the presence of stoichiometric quantities of the protein S cofactor. Protein S, also a vitamin K-dependent protein circulates in a free and in a C4b-binding protein (C4b-bp) form. Only the free protein S form serves as cofactor of activated protein S. The enzyme cofactor complex exerts its anticoagulant activity by proteolytically degrading the activated procoagulant Factors Va and VIIIa and also by accelerating fibrinolysis possibly by decreasing the activity of the plasminogen activator inhibitor (Ploos van Amstel *et al.*, 1989).

Protein S is a cofactor of the protein C system, a natural anticoagulant mechanism. Activated protein C inactivates Factors Va and VIIIa in the presence of protein S, and thereby reduces thrombin generation (Leroy-Matheron *et al.*, 1998; Krachmalnicoff *et al.*, 1990). Protein S can directly inhibit prothrombin activation and the prothrombin complex formation on phospholipid surfaces, can be inhibited by protein S binding to Factors Va and Xa (Leroy-Matheron *et al.*, 1998).

Membrane binding of protein S is essential for expression of its cofactor activity (Walker, 1981; Walker, 1992). Upon chemical conversion of γ-carboxyglutamic acid residue to γ-methylene glutamic acid, the resulting protein lacks cofactor activity and can not bind to phospholipid vesicles (Walker, 1986). Upon thrombin cleavage of protein S into a two-chain protein, the resulting protein has no cofactor activity, is not able to bind to membranes and has decreased calcium binding (Walker, 1984). The inactivation rate by thrombin on protein S is calcium ion sensitive as calcium inhibits inactivation (Sugo *et al.*, 1986). All of this suggests that the protein S interaction with membranes is essential in activity expression of protein S. When phosphatidylserine is low in concentration in the phospholipid vesicles containing phosphatidylcholine and phosphatidylserine, protein S has a greater effect on enhancement of Factor Va inactivation (Walker, 1992).

Activated protein C cleaves three or four bonds in the Factor VIII molecule. The more protein C present, the more enhanced the inactivation rate of Factor VIII and also the rate

of cleavage of the bonds (Walker *et al.*, 1987). Therefore the kinetic effect of protein S increases the rate of cleavage of all bonds catalyzed by activated protein C and does not cause a preferential cleavage of a specific type of bond (Walker, 1992). In human plasma, protein S has a major effect on the anticoagulant activity of activated protein C. Factor Xa has a protective effect on Factor Va from inactivation by protein C. Protein S can however reverse this protection. Therefore it is proposed that the modulation of protection of Factor Va by activated protein C, is the main function of protein S (Solymoss *et al.*, 1988).

Coagulation activity of activated protein C is species specific so that activated protein C from one species is a poor anticoagulant in another species (Marciniak, 1970). Protein S is also a cofactor of activated protein C in the profibrinolytic action of activated protein C. When blood is depleted of protein S, the fibrinolytic activity of activated protein C is reduced (De Fouw *et al.*, 1986).

4.1.5 Interaction of cells and protein S

Hepatocytes, endothelial cells and the megaloblastic cell line, MEG-01, produce protein S (Schmidel *et al.*, 1990). Protein S is secreted as well as synthesized by vascular endothelial cells (Stern *et al.*, 1986). Protein S is stored in megakaryocytes and in platelets (Schwarz *et al.*, 1985) where it represents about 2,5% of total blood protein S. Protein S is released from the storage α-granules, upon thrombin activation of platelets (Schwarz *et al*, 1985). After release, protein S circulates in plasma in two forms, which are kept in dynamic equilibrium (Krachmalnicoff *et al.*, 1990). In platelets as well as endothelial cells, protein S presence promotes inactivation of Factor Va by activated protein C (Stern *et al.*, 1986).

4.1.6 Protein S activity regulation

Sixty percent of total protein S is bound to C4b-bp, which co-migrates with protein S on gel filtration (Dahlback, 1986). Only free protein S serves as cofactor of activated protein C and thus accelerate coagulation Factors Va and VIIIa inactivation (Ploos van Amstel *et al.*, 1988). Protein S is no longer able to act as cofactor for activated protein C once it is bound to C4b-bp (Dahlback, 1986). Binding of protein S with C4b-bp is regulated by the

mass action law, and this interaction has been established to have a high affinity of Kd-70 mM (Krachmalnicoff *et al.*, 1990).

C4b-bp is a large multimeric protein with a molecular weight of $5,7x10^5$ Da (Hillarp *et al.*, 1988; Yamazaki *et al.*, 1995) and is a component of the complement system (Ploos van Amstel *et al.*, 1988). C4b-bp consists of eight polypeptide chains, of which seven are identical α-chains and one is a non-identical β-chain. The β-chain is important for the protein S interaction with C4b-bp at an one to one ratio (Hillarp *et al.*, 1988; Yamazaki *et al.*, 1995). Many forms of C4b-bp exist, but not all bind to protein S, however, if the β-chain is present, protein S can be bound (Hillarp *et al.*, 1989). Almost all β chain-containing C4b-bp (C4b-bp β+) in plasma is in complex with protein S because of their extremely high affinity (Yamazaki *et al.*, 1995). Therefore, free protein S is due to a molar excess of protein S in relation to C4b-bp β+, and the distribution of free and bound protein S depends on the C4b-bp β+ concentration. In case of a heterozygous quantitative protein S deficiency, there is no molar excess of protein S in relation to C4b-bp β+ (Yamazaki *et al.*, 1995). Protein S can be inhibited from binding to C4b-bp by peptides from the carboxy-terminal disulfide-linked loop of protein S (Walker, 1989). A peptide with sequence GVQLDLDEAI in particular, inhibits protein S binding to C4b-bp. This peptide also increases the free protein S plasma amount and enhance the anticoagulant activity of activated protein C in plasma (Weinstein & Walker, 1990).

4.1.7 Detection of protein S defects

Diagnosis of protein C and protein S deficiency rely on measurement of plasma levels. Protein S deficiency rely on plasma total protein S antigen levels and the previous finding of the association of half normal plasma concentrations and thrombotic risk in families with hereditary thrombophilia. Similarly in families with hereditary thrombophilia, the risk of developing thrombosis is related to plasma protein C levels of 50% of normal (Ploos van Amstel *et al.*, 1989).

The International Society on Thrombosis and Haemostasis (ISTH) standardization subcommittee, defines three types of protein S deficiency, on the basis of total protein S levels, free protein S levels and activated protein C cofactor activity:

Type I deficiency is characterized by low total and free protein S antigen levels.
Type II deficiency is characterized by normal total and free protein S antigen levels, and low activated protein C cofactor activity.
Type III deficiency is characterized by selectively reduced levels of free protein S antigen and activity (Leroy-Matheron *et al.*, 1998).

It has been reported that Type 1 and III are two phenotypic expressions of the same genetic defect (Leroy-Matheron *et al.*, 1998).

Theoretically, functional assays for protein S should be preferred to immunoassays because of the ability to detect dysfunctional protein S, but its use is hampered by poor standardization and susceptibility to artifacts (Krachmalnicoff *et al.*, 1990).

4.1.7.1 Antigenic methods

Commercial kits are available for detection of and determining the amount of free protein S by enzyme-linked immunosorbent assay (ELISA) (Krachmalnicoff *et al.*, 1990), radioimmunoassay (RIA) quantitative immunoblotting (Walker, 1992) and crossed immunoelectrophoresis (Edson *et al.*, 1990).

Two forms of protein S exist in plasma: free and bound to C4b-bp (Walker, 1992). In human plasma, only the free form of protein S has activated protein C-cofactor activity, the other ± 60% of protein S is bound to the complement component, C4b-bp (Walker, 1992; Leroy-Matheron *et al.*, 1998). Therefore it is assumed that changes in free protein S levels are related to thrombosis risk (Walker, 1992).

Total protein S antigen can be detected immunologically when the complexed form is dissociated from C4b-bp by longer electrophoresis time, using room temperature or by conditions favouring dissociation (Krachmalnicoff *et al.*, 1990).

4.1.7.2 Functional methods

In ELISA or RIA tests, dissociation can be achieved more consistently by diluting samples and by prolonged incubation with antibodies (Krachmalnicoff *et al.*, 1990). Protein S is the cofactor for activated protein C in the inactivation process of Factors V and VIII. Therefore functional assays either show the ability to enhance inactivation rate of these cofactors or to increase the anticoagulation effect of activated protein C in protein S deficient plasma. Commercial kits and deficient plasmas are available for the functional assay (Walker, 1992).

4.1.8 Normal protein S levels in newborns and adults

Using an enzyme immunoassay, total protein S antigen in general was found in percentages ranging from 53% to 152% with a mean of 106% (Krachmalnicoff *et al.*, 1990).

Normal levels of protein S have been determined at approximately 24 µg/ml of plasma, which equals 100% in most studies. Protein S levels are lower in women than in men but there is a lot of variation. Free protein S range from 50 - 130% in women and from 81 - 133% in men. A 100% free value refers to the amount of protein S in a pool of normal plasma after polyethylene glycol precipitation. The median functional activity of 82% has been determined in 46 adults. By immunological methods, newborns have low total protein S levels compared to adults (23% of adult level). When functional protein S levels are determined in newborns, it is found to be 74% of the adult level (Walker, 1992). This is because C4b-bp levels are very low or undetectable in the newborn. Therefore most of the protein S is in the free form and functions as cofactor to activated protein C. At 6 months of age, premature as well as full-term infants reach protein S levels of adults (Andrew *et al.*, 1987; Andrew *et al.*, 1988).

Discrepancies exist between functional and antigenic protein S levels. Due to decreased levels of C4b-bp, antigenic levels can be low while functional levels are near normal. In cases of oral anticoagulant treatment, or when a mutation exists that does not alter protein expression, low functional levels are detected although total and free antigenic levels are

normal. Relying on either the functional assay or the antigenic assay could result in missing several potential defects or abnormal conditions (Walker, 1992).

4.1.9 Inherited defects in protein S structure and function

Protein S deficiency occurs in families where it is inherited together with thrombosis in an autosomal-dominant fashion. Many types of inherited protein S deficiency have been observed (Walker, 1992).

Type I protein S deficiency is defined by low to very low free protein S levels while the total levels are normal and near normal. Type II is defined by both low levels of free protein S, as well as low levels of total protein S. Type III is not very common and is defined as having normal free and total protein S levels, but the free protein S is non-functional (Walker, 1992).

Types I and II deficiencies are not genetically distinct defects. Both types I and II protein S deficiencies have been found in many families with inherited thrombosis associated with protein S deficiency. This suggests that other factors are involved that influence plasma levels. (Walker, 1992).

Cases of protein S associated thrombosis have been reported in children as young as three years of age (Israels & Seshisa, 1987). There is correlation between severity of onset of thrombosis and heterogeneity. There is no relationship between plasma protein S levels in heterozygous protein S deficiency and age of onset of thrombosis (Dix *et al.*, 1988).

A protein S variant named protein S-Heerlen, is not associated with thrombosis. In this variant serine 460 is replaced with proline. This variant has normal functional properties with an allele frequency of 0,52% in the general population (Bertina *et al.*, 1990).

4.1.10 Acquired defects in protein S structure and function

Several cases of thromboembolic disease are not predisposed to thrombosis by a genetic factor. Often low protein S is acquired (Walker, 1992).

Of all the identifiable anticoagulant proteins, protein S is the most common cause of thrombotic disease, although in an Israeli population, antithrombin III was found to be the most common cause of thrombosis, followed by protein C and then by protein S. This observation might be due to differences in expression of the disease in different population groups (Walker, 1992).

In general the occurrence of heterozygous protein S deficiency among young unrelated patients with thrombosis is approximately 5%, followed by 4% for protein C and 3% for antithrombin III (Walker, 1992).

4.1.11 Protein S levels and nephrotic syndrome

Thrombotic disease and many coagulant abnormalities are common in patients with NS where total protein S levels are increased in NS while free protein S levels are lowered (Allon *et al.*, 1989). Free protein S levels can also be increased or unchanged in NS. These differences may be due to patients of different population groups (Walker, 1992).

In the USA a frequency of 3-5% thromboembolic events was found in children with NS. The pathogenesis of clotting abnormalities remain poorly understood in these patients. A particular child with NS developed severe deep vein thrombosis (DVT) and pulmonary embolism secondary to acquired protein S deficiency was reported to have markedly decreased C4b-bp plasma levels. Till 1991 this was the only reported paediatric case of NS, which is complicated by thromboembolism in which a circulating anticoagulant has been implicated (Garbrecht *et al.*, 1991).

Many abnormalities in the levels of procoagulant and anticoagulant proteins have been described in nephrotic children, including elevations of α_2M; Factors II; VIII and X, deficiencies of antithrombin III and plasminogen (Garbrecht *et al.*, 1991). Lupus anticoagulants (LAC) are associated with arterial and venous thrombotic events but has not been described in paediatric nephrotic patients (Garbrecht *et al.*, 1991).

Events predisposing to hypercoagulation in nephrotic children are poorly understood. It includes factors favouring the increase of some circulating procoagulant factors, the loss of

some anticoagulant factors, as well as platelet activation. Some clotting factors are lost through the abnormally permeable glomerular membrane into the urine. Decreased activity of Factors IX; XI and XII, increased Factors I; II. VII; VIII and X; increased α_2M, protein C and antithrombin III plasma levels have been described in NS. Some studies report plasminogenuria and antithrombinuria associated with lowered plasma levels of these proteins. One particular NS patient had normal levels of Factors II, V and antithrombin III; plasminogen and protein C, and undetectable levels of Factors VIII; IX and XI. LAC in this patient was not associated with any of the known causes of circulating anticoagulants, therefore the authors speculated that the components of his disease represent part of the spectrum of a primary antiphospholipid syndrome (Garbrecht *et al.*, 1991). This patient most likely had an acquired protein S deficiency, which predisposed him to development of a primary thrombotic event. This patient had very low levels of free and total protein S and of the C4b-bp. The low C4b-bp levels would have been expected to result in a redistribution of protein S from the bound to active free form. The decrease in total protein S makes the contribution of redistribution difficult to assess (Garbrecht *et al.*, 1991).

It has been shown that LAC may interfere with phospholipid-mediated protein C thrombomodulin interaction, causing a protein C activation decrease. Modulation of protein S activity by LAC has also been suggested on the basis of two patients with antiphospholipid syndrome who had protein S deficiency and LAC. LAC may interact with phospholipids of the vascular endothelium, which results in a decrease in prostaglandin I_2 production, which enhances platelet adhesion to endothelial cells (Garbrecht *et al.*, 1991).

4.1.12 Protein S in thrombosis and nephrotic syndrome

Heterozygotes for a deficiency of protein C or -S are at risk at a young age already, to develop venous thromboembolic disease, due to a haemostatic balance shift favouring insoluble fibrin formation (Ploos van Amstel *et al.*, 1988). Some protein S deficient persons develop thrombotic events and others not. After the age of 15, the numberof patients with thrombotic events increase (Engesser *et al.*, 1987).

Thromboembolic complications are enhanced in cases of hereditary deficiencies of coagulation inhibitors such as anti-thrombin-III, protein C and protein S. More recently, acquired deficiencies of protein S, reaching the extent realized by hereditary protein S deficiencies, were described in many diseases in which thromboembolic complications frequently occur (Kemkes-Matthes, 1992). Protein S deficiency is an autosomal dominant trait which in heterozygous subjects cause these individuals to be at risk of recurrent thromboembolic disease in adulthood. In patients with very low protein S levels, thrombotic complications can occur early and be very severe (Leroy-Matheron *et al.*, 1998). Acquired protein S deficiency is one of the causes of thromboembolic complications that occur in NS; acute phase reactions; malignancy; pregnancy and oral contraceptive usage (Kemkes-Matthes, 1992).

After activation by thrombin/thrombomodulin, protein C is the major inhibitor of coagulation. Activated protein C degrades Factors Va and VIIIa (Odegaard *et al.*, 1987; Walker *et al*, 1987) and posseses profibrinolytic properties (Sakata *et al.*, 1986). Only about 40% of total protein S is in the free form and acts as cofactor for activated protein C concerning degradation of Factors Va and VIIIa (Kemkes-Matthes, 1992). The other 60% is bound to C4b-bp and has no function in the coagulation cascade (Hillarp *et al.*, 1989). C4b-bp is a regulatory protein involved in the complement cascade and acts as an acute phase reactant (Saeki *et al.*, 1989). It is well known that NS patients have an increased risk of suffering from thromboembolic complications. Acquired protein S deficiency have been described in NS (Kemkes-Matthes, 1992).

Low free protein S levels (69% ± 27% of normal) are found in NS patients, despite elevated total protein S antigen levels (139% ± 42% of normal). Urinary loss of free protein S and increased C4b-bp of up to 170% ± 52% of normal, lead to a shift from free to bound protein S, and result in a pronounced decrease of free protein S. In NS, thrombotic risk has two causes: (i) low molecular weight free protein S loss in urine and (ii) increased high molecular weight C4b-bp bound protein S. These two causes result in a pronounced drop in free protein S (Kemkes-Matthes, 1992).

An increase of C4b-bp in acute phase reactions, induce a decrease in free protein S which possibly leads to thromboembolic events. Regulation of protein S levels depend on many different mechanisms (Kemkes-Matthes, 1992).

4.1.13 Protein S in kidney transplantation

C4b-bp is an acute phase reactant, which in part regulates free protein S levels. It is known that free protein S levels drop during inflammation, therefore the question arises whether protein S levels drop during transplantation. Patients who had undergone kidney transplantation has decreased free protein S levels during transplant rejection (Tsuchida *et al.*, 1990). It is not known whether this decrease is due to the inflammatory response and increased C4b-bp levels or due to elevated proteinuria due to renal failure, as total protein S levels were not determined (Walker, 1992).

The pathobiology of protein S is poorly understood in comparison with other areas of medical biology. It is clear though that patients with low protein S levels are at risk of developing thrombosis. The mechanisms that regulate the amount of protein S bound to C4b-bp is not clear either. Genetics of protein S is confusing especially when type I and II protein S deficiency exist in the same family and they appear interchangeable (Walker, 1992).

Thrombosis is common in NS patients. Approximately 30% of nephrotic patients develop deep vein thrombosis of the extremities or in renal veins (Liach, 1985; Bernard, 1988). A number of factors are involved in these homeostatic disturbances, such as platelet aggregability (Figure 4.2), increased procoagulant factors in plasma (Lieberman *et al.*, 1968), low anti-thrombin-III plasma concentration (Kaufman *et al.*, 1978) and depression of fibrinolysis (Thomson *et al.*, 1987). Deficiency of free protein S as cofactor for activated protein C is implicated as a risk factor for thromboembolism in adults with NS (Tsuchida *et al.*, 1990).

Procoagulant activity might be increased due to glomerular injury in nephrotic patients and therefore increase the thrombotic tendency in these patients. Activation of intraglomerular system homeostasis ensues via increased thrombin formation. Low anti-thrombin-III

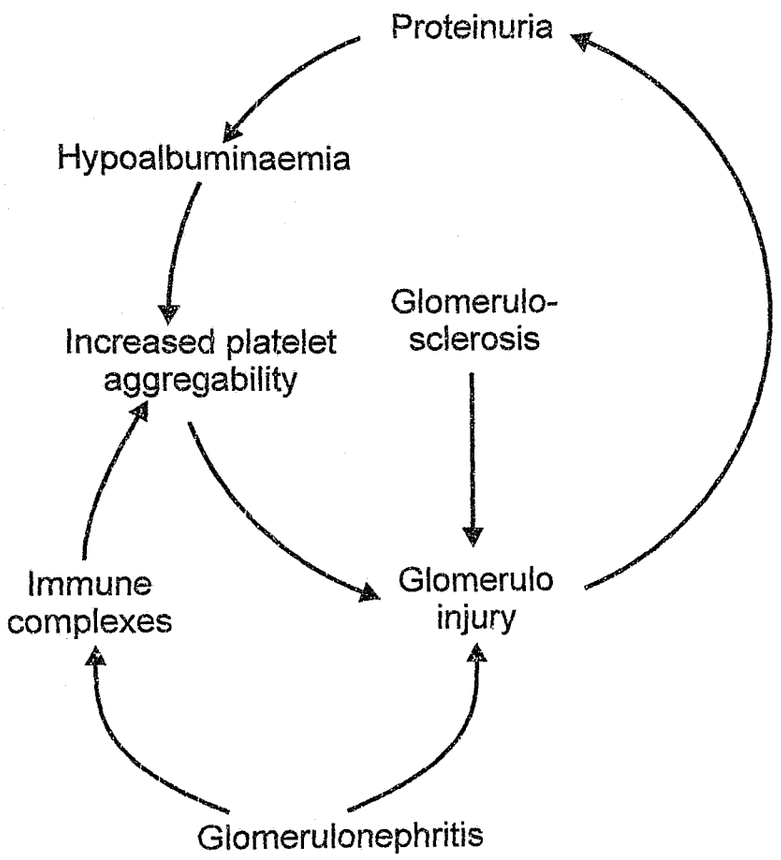


Figure 4.2 Diagram illustrating the state of hypercoagulability in NS patients (Cameron, 1990).

levels in NS may be due to intravascular consumption as well as to urinary loss (Chen *et al.*, 1993). Selective urinary loss of free protein S may cause a decrease in protein S activity (Vigano-D'Angelo *et al.*, 1987).

In children, hypoalbuminaemia is more pronounced and this might be related to the more severe thromboembolic complications in children, although the incidence of coagulation abnormalities in children is low (Mehls *et al.*, 1987; Marsh *et al*, 1991).

High protein C concentrations might have a protective effect against thrombotic complications associated with anti-thrombin-III deficiency in adults (Yermiahu *et al.*, 1996). Protein C concentration and activity as well as total and free protein S levels are increased in adult nephrotic patients (Liach, 1985; Bernard, 1988). Normal protein C and protein S antigens were found in paediatric patient plasma although they were excreted in urine. A kinetic study of the production and excretion of protein C and S may shed some light on the discrepancy between paediatric and adult plasma levels in the NS patient (Yermiahu *et al.*, 1996).

The protein C anticoagulant pathway is active on the surface of quiescent endothelial cells and plays an important role in haemostasis prevention (Esmon, 1987). Protein C requires two key cofactors namely thrombomodulin and protein S. Thrombomodulin on the endothelial cell surface, is a high affinity receptor for thrombin. Thrombin - thrombomodulin complex is an activator of circulating protein C (Tsuchida *et al.*, 1990).

For many years, fibrin deposition has been recognized as a histologic accompaniment of human renal allograft rejection (Kincaid-Smith, 1967). Fibrin formation is a common feature of various inflammatory reactions. Fibrin deposition (based on *in vitro* studies) occur through cytokine induced macrophage- and endothelial- activation and expression of tissue factor procoagulant activity as well as concomitant down regulation of the protein C/protein S/thrombomodulin anticoagulant pathway. Only scant data is available concerning protein C/protein S levels in acute kidney rejection (Tsuchida *et al.*, 1990).

Serial blood samples taken from post-transplantation patients in the first month after transplantation, indicated that two to three days after surgery, protein C and free protein S

levels return to normal. With onset of acute rejection suspected by increased creatinine and confirmed by renal biopsy, protein C and free protein S levels drop to 30% - 50% of the normal range (Tsuchida *et al.*, 1990).

In paediatric NS patients, there is a relationship between free protein S excretion and thromboembolic complications (Hanevoled *et al.*, 1994). This latter statement is surrounded by controversy, as paediatric NS patients followed for one year, had no clinical evidence of thromboembolism. The mechanism involved in thromboembolism in children is unrelated to protein S and protein C loss in urine. In another study, no evidence of a high incidence of thromboembolic disease was found in adult nephrotic patients. This all adds to the controversy in the literature (Yermiahu *et al.*, 1996).

Increased incidence of thromboembolic complications, in adult nephrotic patients, is due to increased Factor IX, Factor VII, Factor VIII, Factor V, fibrinogen, thrombocytosis and also increased platelet reactivity. Reduced protein S functional levels reduced protein C activity and acquired anti-thrombin-III deficiency also play a role. Although increased protein C and protein S antigenicity were identified in urine of five out of 14 children and in seven out of 12 children respectively, no thromboembolic phenomena were found in these children (Yermiahu *et al.*, 1996).

4.1.14 Protein S mutations

Till 1989 no association between protein S deficiency and genetic defects in the protein S gene had been described. A partial protein S gene deletion was found in a family with hereditary protein S deficiency. Only heterozygotes for the gene defect had recurrent thrombosis, which indicated that familial thrombophilia might be the direct result of a protein S gene defect (Ploos van Amstel *et al.*, 1989).

One "proband" had gross alterations in the protein S gene. Using restriction fragments covering various parts of the coding region of human protein S copy DNA, indicated that a large middle portion of the protein S gene has been deleted (Ploos van Amstel *et al.*, 1989).

Thrombosis only occurred in family members heterozygous for the protein S gene defect. This provides further evidence that protein S deficiency and familial thrombosis are casually related (Ploos van Amstel *et al.*, 1989).

The protein S gene defect can be used as a genetic marker for hereditary protein S deficiency, thereby supporting the diagnosis based on plasma total protein S antigen levels (Ploos van Amstel *et al.*, 1989).

A 50-year-old Japanese man with recurrent venous thrombosis was found to have hereditary protein S deficiency. Total protein S antigen in plasma was reduced by half in this patient as well as in his two sons. Upon DNA sequencing, it was found that a novel nonsense mutation, TAG for glutamine (Gln) 522, instead of the usual CAG in exon 14 of the protein S gene occurred (Yamazaki *et al.*, 1995).

A dimorphism for Pro 626 (CCA/CCG) i.e. 626 A/G distinguished by the restriction enzyme, Bst XI (CCANNNNN/NTG) digest was investigated. The Bst XI site in exon 15 (626 A allele) results in cleavage of the PCR product by Bst XI digestion, but a fragment with the 626 G allele would not be cut by Bst XI (Yamazaki *et al.*, 1995).

Sequencing showed that only the protein S α-gene was amplified by PCR, but not the protein S β-gene, except for exon 3 as there is no known difference between the protein S α-gene and the protein S β-gene sequence of exon 3. There is a stop codon in exon 14. In exon 14 of the protein S α-gene, a single point mutation from C- to T- at nucleotide 1833 was detected. Patients were heterozygous for this mutation where the CAG codon coding for Gln 522 was altered to a TAG stop codon (Yamazaki *et al.*, 1995).

Ninety mutations of which two large deletions, have been identified in the α-gene, as casual defects. Most are point mutations causing nonsense or missense changes in coding or splice site sequences. Frame shifts and premature stop codons are caused by micro insertions or deletions. Most of these genetic defects co-segregate with the Type 1 protein S deficiency. Few mutations cause Type II protein S deficiency (Leroy-Matheron *et al.*, 1998).

The single point mutation in exon 13 of the α-gene, 460 TCC → CCC which leads to the replacement of Ser 460 by Pro, called the Heerlen polymorphism, is frequently associated with Type III protein S deficiency. Type II protein S deficiency is not common. Only five mutations have been identified in patients with normal protein S concentrations and low activated protein C cofactor activity. All five of these mutations are located in the amino-terminal part of protein S, which is homologous to that of other vitamin K-dependent proteins and encodes the domains interacting with activated protein C. Two of these five mutations are located in the pro-peptide at position -1 and -2. The first case of type II protein S deficiency due to a splice site mutation resulting in two alternative splice transcripts, lacking either exon 5 or both 5 and 6 has been described. It is probably a truncated protein lacking EGF-1 (Leroy-Matheron *et al.*, 1998).

Until recently, all mutations in patients with Type II protein S deficiency were missense mutations. The first α-gene splice site mutation associated with hereditary Type II protein S deficiency has been found. Previously described splice site mutations in the α-gene were associated with a quantitative deficiency, but this mutation is associated with a qualitative deficiency. The synthesis of a truncated protein is suspected, because the exon skipping observed in the messenger RNA transcript led to conservation of an open reading frame. The skipped exon encoded EGF-1. Plasma phenotypes established using monoclonal antibodies are consistent with the presence of a truncated circulating protein S, which lacks EGF-1. This finding proofs that the EGF-1 domain is not an absolute requirement for the correct folding; the stability and the secretion of protein S (Leroy-Matheron *et al.*, 1998).

4.1.15 Aim

The aim of this part of the study was to determine the frequency distribution of the two protein S alleles in white and black children with NS and in white and black control individuals.

4.2 Materials and Methods

4.2.1 Materials

The following materials were obtained from Promega (Madison,WI): Agarose; dNTP's; Taq DNA Polymerase; Bst XI restriction enzyme and proteinase K. The oligonucleotide primers that were used to detect the protein S polymorphism were synthesized by Genosys Biotechnologies Inc (Woodlands,Tx); the molecular weight markers were supplied by BioVentures Inc (Murfreesboro, TN). The 2ml eppendorf tubes were supplied by Treff Laboratories (Switzerland); the PCR tubes were from Laboratory and Scientific Equipment Co (Johannesburg); double-distilled water was obtained from SABAX (Johannesburg); all other reagents used for buffers were of analytical grade (Merck, Darmstadt, Germany).

4.2.2 Subjects

Thirty black (Bantu-speaking) NS patients (17 girls), 25 white (Caucasoid) NS patients (12 girls), 18 black control individuals (9 girls) and 22 white control individuals 10 girls) were screened for protein S genotyping. All individuals were of the age range 2-18 years.

The nephrotic syndrome patients were recruited from the Paediatric Nephrology Clinic of the Johannesburg hospital and were diagnosed with either the FSGS or the MLNS/(MCNS) form of nephrotic syndrome. The control individuals were recruited from the Asthma and Respiratory Clinic at the Johannesburg hospital and were free from any renal disease.

4.2.3 Methodology

DNA Extraction

See section 3.2.3 for the Higuchi, (1989) method.

Optimization of PCR and DNA restriction

In order to optimize the PCR as well as the restriction of the DNA to yield the best possible results, three different sets of primers were tested.

First set of protein S gene primers

On the first attempt to amplify exon 15 of the protein S gene, containing the mutated region, the method described by Marchetti *et al.*, (1993) was used (Marchetti *et al.*, 1993). These authors mentioned that they used the protein S sequence as described by Lundwall and co-workers, (1986), to design the protein S primers (Lundwall *et al.*, 1986). When comparing the sequence published by Lundwall *et al.*, (1986) to the sequence published by Marchetti *et al.*, (1993), a single base pair mismatch, possibly a typing error, was noticed (an extra – C – at position 2401) in the publication by Marchetti *et al.*, (1993). The mismatch was only noticed after the primers for the study were already obtained. The mismatch occurred in the downstream primer (PS3) used by Marchetti and co-workers, (1986), in the RT-PCR. The first set of primers used in this study to detect the mutant protein S allele was exactly the same as described by Marchetti *et al.*, (1993), including the extra mismatched – G – base in the downstream primer PS3, so that the primers used were as follows:

Upstream primer PS2: 5' CATAATGATATTAGAGCTCAC 3' (21 mer).

Downstream primer PS3: 5' TTCTTAGATAGCAAGAGAAGT 3' (21 mer) (The - G – represents the mismatched – C – of the sequence, as for the downstream primer the opposite bases are used and the sequence of bases is reversed). (Figure 4.3 indicates the exact primer positions).

The melting temperatures of both the primers were 52°C and this temperature was thus used as the annealing temperature. The PCR cycles were as follows: 96°C for 10 minutes to denature the DNA in the Hybaid PCR cycler (Hybaid, UK), 52°C to add the Taq DNA polymerase and 72°C for 2 minutes as an extension period, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute, with a final extension step of 72°C for 5 minutes.

Following amplification, the PCR products were resolved by electrophoresis in 1% (w/v) agarose gels, stained with ethidium bromide and viewed under 302 nm ultraviolet transillumination.

Upon restriction with 5 units of Bst XI restriction enzyme (Appendix B) at 50°C for 1 hour (Section 2.4.6), the DNA yielded three fragments. An individual heterozygous for the protein S mutation, had one fragment of 271bp, which had not been cut by Bst XI, and one fragment of 235bp which was cut by Bst XI and were visible on the 4% (w/v) agarose gel electrophoresis stained with ethidium bromide. The residual 35bp fragment was not visible upon ultraviolet transillumination at 302 nm. A homozygous individual, had either one 271bp Bst XI uncut (-) fragment or one 235bp Bst XI cut (+) fragment with a not visible 35bp fragment.

Second set of protein S gene primers

As the uncut (mutant (-)) fragment and the cut (normal (+)) fragment were separated by only 35bp, it was often difficult to interpret the results accurately. Therefore, the uptream primer was re-designed and moved further away from the Bst XI cutting site in order to generate larger fragments. At the same time the downstream primer was moved 5bp closer to the Bst XI cutting site as it seemed a better position which could improve primer annealing. The newly designed primers were as follows:
Upstream primer PS1: 5' AGTGCCACACCAGTGAATGCC 3' (21 mer).
Downstream primer PS2: 5' CACTATTCTTA - ATAGCAAGAG 3' (21 mer) (in this case the mismatch – G – was not included as indicated by the – space in the primer sequence). (Figure 4.3 indicates the exact primer positions).

These two primers had different melting temperatures. The upstream primer, PS1 had a melting temperature of 65°C and the downstream primer, PS2 had a melting temperature of 55°C. Therefore, the annealing temperature was initially tested at 60°C but this resulted in the formation of non-specific fragments. The annealing temperature was increased by 1°C up to 64°C at which clear bright bands without any non-specific binding were obtained when electrophoresed on 1% (w/v) agarose gels, stained with ethidium bromide and viewed under 302 nm ultraviolet transillumination.

Theoretically, these two newly designed primers should have given rise to three larger fragments upon restriction enzyme digestion with 5 units of Bst XI (Appendix B), which should have increased visibility and eased interpretation of results on a 4% (w/v) agarose

gel. An individual heterozygous for the mutant protein S gene should have yielded one fragment of 365bp, another fragment of 240bp and a third fragment of 125bp. An individual homozygous for the protein S mutant (-) gene should have yielded one fragment of 365bp only. An individual homozygous for the normal (+) gene which is cut by Bst XI, should have yielded two fragments of 240bp and 125bp. This was not the case, as the Bst XI restriction enzyme was incapable of digesting the longer DNA sequence at 50°C for 1 hour. Increasing the time period of restriction enzyme digestion to two, three, and even four hours respectively, did not generate results. Increasing the concentration of Bst XI added to the PCR generated DNA, to 10 units, 20 units or 40 units did not alter the situation either. Therefore no genotyping results were obtained using the two newly designed primers.

Third set of protein S gene primers

The upstream primer (PS2) described and designed by Marchetti *et al.*, (1993), and used at first in this study, gave clear bands and Bst XI was able to cut the PCR generated DNA. Therefore, this primer was tested again in combination with the newly designed downstream PS2 primer, without the mismatched base. The primer sequences were as follows:

Upstream PS2 (1st attempt): 5' CATAATGATATTAGAGCTCAC 3' (21 mer).

Downstream PS2 (2nd attempt): 5' CACTATTCTTA - ATAGCAAGAG 3' (21 mer) (no – G – mismatch as indicated by the – space in the primer sequence). (Figure 4.3 indicates the exact primer positions).

This combination of upstream- and downstream primers proved to be ideal, as the Bst XI enzyme was able to restrict the smaller PCR product DNA generated by these two primers, and clear, bright bands resulted after a purification step (Section 2.4.5), although the fragments were still close in size. When restricted DNA was subjected to electrophoresis on a 2% (w/v) agarose gel (Appendix B), instead of a 4% (w/v) agarose gel as used previously, fragments were easily distinguishable upon ethidium bromide staining and ultraviolet transillumination at 302 nm. On a less dense 2% (w/v) agarose gel (Appendix B), fragment separation improved. This lead to the detection of the protein S polymorphism as described in section 4.2.4.

Figure 4.3 Nucleotide sequence of cDNA coding for human protein S and its corresponding predicted amino acid sequence. Primers used in 1st, 2nd and 3rd attempt as well as Bst XI recognition site indicated in corresponding positions.

Ala Met Lys Ala Lys Val Ala Thr Tyr Leu Gly Gly Leu Pro Asp Val Pro Phe Se. Ala Thr Pro Val Asn Ala Phe Tyr Asn Gly Cys Met Glu Val Asn Ile 603
GCA ATG AAA GCA AAA GTG GCC ACA TAC CTG GGT GGC CTT CCA GAT GTT CCA TTC AGT GCC ACA CCA GTG AAT GCC TTT TAT AAT GGC TGC ATG GAA GTG AAT ATT 2100
Upstream PS 2 2nd

Asn Gly Val Gln Leu Asp Leu Asp Glu Ale Ile Ser Lys His Asn Asp Ile Arg Ala His Ser Cys Pro Ser Val Trp Lys Lys Thr Lys Asn Ser
AAT GGT GTA CAG TTG GAT CTG GAT GAA GCC ATT TCT AAA CAT AAT GAT ATT AGA GCT CAC TCA TGT CCA TCA GT/T TGG AAA AAG ACA AAG AAT TCT TAA GGC ATC 2205
Upstream PS 2 1st + 3rd Bst XI recognition site

TTT TCT CTG CIT ATA ATA CCT TTT CCT TGT GTG TAA TTA TAC TTA TGT TTC AAT AAC AGC TGA AGG GTT TTA TTT ACA ATG TGC AGT CTT TGA TTA TTT TGT GGT 2310
CCT TTC CTG GGA TTT TTA AAA GGT CCT TTG TCA AGG AAA AAA TTC TGT TGT GAT ATA AAT CAC AGT AAA GAA ATT CTT /ACT T*CT CTT GCT ATCT AAG AA//T AGT G*TA 2415

mismatched base

/ to / Downstream PS 3 1st (opposite bases; reversed and including mismatch)
5' TT CTT AGAT AGC AAG AGA AGT3'

* to * Downstream PS 2 2nd + 3rd (opposite bases; reversed and without mismatch)
5' C ACT ATT CTT AAT AGC AAG AG 3'

4.2.4 Detection of the protein S polymorphism

The prevalence of the protein S mutation within exon 15 of the α-protein S gene was determined by using PCR and the Bst XI restriction enzyme. The following primers were designed and used as described in the above optimization section, to amplify the specific region containing the mutation:
Upstream 5' forward primer PS2 (1st): 5' CATAATGATATTAGAGCTCAC 3' (21 mer).
Downstream 5' reverse primer PS2 (2nd, 3rd): 5' CACTATTCTTAATAGCAAGAG 3' (21 mer). (Figure 4.3 indicates the exact primer positions).

These two primers amplify the region of DNA spanning the protein S mutation. The Bst XI restriction enzyme (Appendix B) recognizes the sequence: CCATCAGT / TTGG, so that an individual heterozygous for the protein S mutation, yielded one cut 240bp fragment (+ allele) and one uncut 275bp fragment (- allele). An individual homozygous for the presence of the protein S mutation, yielded only one fragment of size 275bp, as neither of the (–) alleles were cut by Bst XI. An individual homozygous for the absence of the mutation in both (+) alleles, yielded one fragment of size 240bp as both alleles were cut by Bst XI (the 35bp fragment is too small to be visualized on a gel).

Briefly, the target sequence was amplified in a 50 µl reaction volume containing 0,4 µg genomic DNA; 0,2 mM each of dCTP; dGTP; dTTP and dATP; 10 mM Tris-HCl buffer pH 8,3; 25 pmol of each of the two primers PS2 (1st) upstream and PS2 (2nd, 3rd) downstream. This mixture was overlaid with 50 µl mineral oil to prevent evaporation. Negative PCR controls contained all the reagents except genomic DNA. The reaction mixture containing all the components except Taq DNA polymerase, was heated to 95°C for 5 minutes to denature the DNA in a Hybaid thermal cycler (Hybaid, UK). Samples were cooled to 60°C and 1 unit of Taq DNA polymerase was added, followed by 40 cycles of: 95°C denaturation for 1 minute; 60°C annealing for 2 minutes and extension at 72°C for 2 minutes. The procedure was completed by a last 72°C extension for 10 minutes.

Following amplification, the PCR products were resolved by electrophoresis in 1% (w/v) agarose gels, stained with ethidium bromide and viewed under 302nm transillumination.

4.2.5 Purification of DNA PCR product

To enable the restriction enzyme to restrict the PCR product, the DNA needed to be purified by an ethanol precipitation step done as follows: 50 µl PCR product; a tenth volume (5 µl) 3 M NaAc pH 5,75; 2,5 volumes (125 µl) 100% ethanol. This mixture was placed at -70°C for 30 minutes and then centrifuged at 10 000xg at 4°C for 30 minutes. The resulting pellet was washed once with 2,5 volumes (125 µl) 70% ethanol for 30 minutes at 4°C by centrifugation at 10 000xg. The pellet was air dried and reconstituted in 50 µl double-distilled water.

4.2.6 DNA restriction

The PCR products were restricted with Bst XI as follows: One µg/µl PCR product was digested with 5 units of Bst XI in the presence of 0,1 mg/ml acetylated bovine serum albumin (BSA) and the specific supplied and recommended restriction enzyme buffer, to improve the quality and efficiency of the enzyme in restricting impure DNA. The volume was made up to 20 µl with double-distilled water and overlaid with 10 µl mineral oil to prevent evaporation. Restriction was allowed to take place for 1 hour at 60°C.

Ten microlitre of the restricted sample was mixed with 8 µl loading buffer (Appendix A) and 15 µl of this mixture was then loaded into the wells of a 2% (w/v) agarose gel (Appendix B), containing 1 µg/ml ethidium bromide. The gel was subjected to 80 Volts for 1 hour and then visualized and photographed under ultraviolet illumination of 302 nm wavelength (Figure 4.4).

4.2.7 Statistical analysis

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

The protein S genotypes were grouped into three different allelic combinations to make sensible statistical analysis possible, namely:

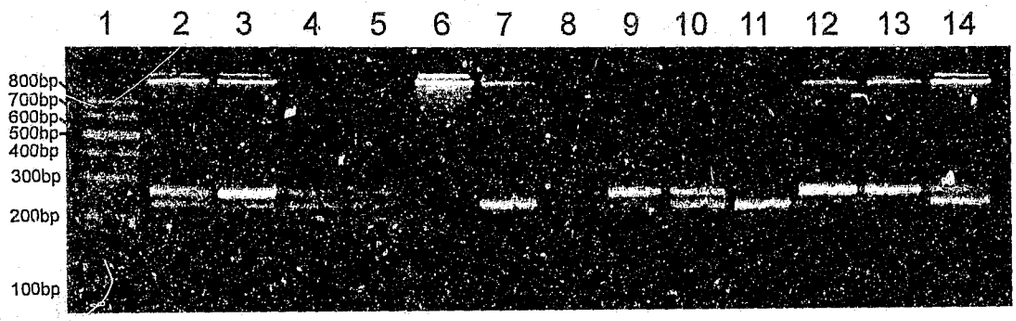


Figure 4.4
Agarose gel electrophoresis showing the two fragments obtained for the two protein S alleles.
Lane 1: 100bp molecular weight marker.
Lanes 2, 3, 4, 5, 9, 10 and 14: protein S heterozygous genotype.
Lanes 7 and 11: Protein S homozygous (+ allele) genotype.
Lanes 12 and 13: Protein S homozygous (- allele) genotype.

+ + to indicate that both protein S alleles are cut with the Bst XI restriction enzyme.
+ - to indicate that one protein S allele is cut with the Bst XI restriction enzyme, but the other allele not.
- - to indicate that neither of the two protein S alleles are cut with the Bst XI restriction enzyme.

The Fisher exact test was used to determine the frequency distribution of the protein S alleles in black and white nephrotic syndrome patients, the frequency distribution of the protein S alleles in black and white control individuals and the frequency distribution of the protein S alleles in black and white individuals in general.

4.3 Results

Table 4.1 indicates the frequency distribution of the protein S alleles in black and white nephrotic syndrome patients (percentages were included to facilitate interpretation).

There was a highly significant difference in the frequency of the + and – alleles between black and white nephrotic syndrome (FSGS and MLNS/(MCNS)) patients, as the + allele is more prevalent in the black nephrotic syndrome group of patients than in the white nephrotic syndrome group of patients.

Table 4.1 Frequency distribution of + and – alleles of protein S in black and white nephrotic syndrome patients.

	++	+ -	- -	Total
Black NS Patients	16 (53%)	12 (40%)	2 (7%)	30
White NS Patients	5 (20%)	13 (52%)	7 (28%)	25
Total	21	25	9	55

Fisher exact test: (++) p = 0,011; (+ -) p = 0,268 and (- -) p = 0,038.

Table 4.2 indicates the frequency distribution of the + and the – alleles of protein S in black and white control individuals (percentages were included to facilitate interpretation).

There was a highly significant difference in the frequency distribution of the + allele of protein S between black and white control individuals, as the + allele is more prevalent in the black population.

Table 4.2 Frequency distribution of the + and – protein S alleles in black and white control individuals.

	++	+ -	- -	Total
Black Controls	13 (72%)	3 (17%)	2 (11%)	18
White Controls	7 (32%)	7 (32%)	8 (36%)	22
Total	20	10	10	40

Fisher exact test: (++) p = 0,012; (+ -) p = 0,233; (- -) p = 0,069.

Table 4.3 indicates the frequency distribution of the + and – alleles of protein S in all black and white individuals (percentages were included to facilitate interpretation).

There was a highly significant difference in the frequency of the + and – alleles of protein S between blacks (control- and patient individuals) and whites (control- and patient individuals), as the + allele is more prevalent in the black population in general.

Table 4.3 Frequency distribution of the + and – alleles of protein S in all black and all white individuals.

	++	+ -	- -	Total
Blacks	29 (61%)	15 (31%)	4 (8%)	48
Whites	13 (28%)	20 (43%)	15 (32%)	48
Total	42	35	19	96

Fisher exact test: (++) p = 0,001; (+ -) p = 0,176; (- -) p = 0,004.

4.4 Discussion

Proteins lost in urine include albumin and proteins involved in the modulation of the coagulation cascade such as protein S and AT III. Thromboembolic complications of NS have been well described, beginning with Addis in 1948 who referred to frequent occurrence of venous thrombosis during the degenerative state of glomerulo-nephritis. Thromboembolic events are observed in 19-70% of European children with NS (Hoyer *et al.*, 1986). Abnormalities in levels of procoagulants and anticoagulant proteins such as protein S (Mehls *et al.*, 1987), as well as increased α_2M (Thaler *et al.*, 1978) have been described in NS children. Venous thrombosis is a well recognized complication of the hypercoaguable state, but thrombosis of the arterial system is much more unusual and mainly reported in nephrotic children (Siddiqi *et al.*, 1997). NS is associated with venous thrombosis and a rate of 19-70% has been reported in adult NS patients. The thrombotic phenomena include renal vein thrombosis; DVT of the leg; pulmonary embolism; venous thrombosis in other sites and arterial thrombosis. The most common is renal vein thrombosis, but DVT has been reported in 2-27% of adults (Hoyer *et al.*, 1986). In children, incidence of venous thrombosis was reported as 2% in one study (Mehls *et al.*, 1987). Venous thrombosis in NS is considered a "normal" complication with a multifactorial background (Petäjä *et al.*, 1995).

NS result from glomerular disease caused by changes in GBM, leading to alterations in the filtration process. The result is a marked filtration of albumin and other intermediate-sized proteins into Bowman's space (Siddiqi *et al.*, 1997).

NS patients who develop venous or arterial thrombosis are in a hypercoaguable state of which the pathogenesis is multifactorial. Deficiency in coagulation inhibitors, AT III and protein S increase the risk of thrombosis in patients with NS. The hypercoagulability state is contributed to by increased blood and plasma viscosity caused by haemoconcentration as well as elevated fibrinogen levels aggravated by use of diuretics. Steroids used to treat NS increase the concentration of clotting Factor VIII and shorten prothrombin and partial thromboplastin times. Thrombocytosis, platelet aggregation and adhesiveness associated with NS contribute to development of thromboembolic complications. Lowered fibrinolytic activity associated with hypertriglyceridaemia and lowered plasma

plasminogen correlate with the extent of proteinuria. NS is characterized by a profound change in concentration and turnover of plasma proteins including ones involved in the coagulation cascade. Factors IX; XI and XII are decreased due to urinary loss. Procoagulant Factors II; V; VII; VIII; X and XIII are increased, correlating with serum albumin reduction (Siddiqi *et al.*, 1997).

AT III plasma concentration is decreased in NS as a result of increased GBM filtration. In cases of AT III deficiency, a 50-70% lifetime risk exists of developing thrombotic complications. Low levels of AT III occur in up to 80% of nephrotic patients. Thrombosis also occur in nephrotic patients with normal AT III levels, which indicates that although it is a major determinant of plasma antithrombin activity, it is not the only one as other regulatory proteins of the coagulation cascade also play a role (Siddiqi *et al.*, 1997).

Once activation by thrombin occurred, activated protein C acts as a major inhibitor of blood coagulation as it proteolytically degrades Factors Va and VIIIa and thus inhibits coagulation. Protein S serves as a cofactor for protein C. Forty percent of protein S is unbound and active while 60% is bound to C4b-bp and does not participate in the coagulation cascade. Acquired protein S deficiency occurs in NS and other clinical conditions (Siddiqi *et al.*, 1997). Low free protein S levels has been reported in nephrotic patients, despite having elevated total protein S antigen levels (Vigano-D'Angelo *et al.*, 1987). Lowered free protein S levels in NS can be attributed to low-molecular-weight free protein S loss in urine and also to increased high molecular weight C4b-bp bound to protein S, resulting in a state of hypercoaguability. Protein C activity is usually normal in NS patients, or even elevated (Siddiqi *et al.*, 1997).

Predictions regarding clinical severity might be possible on the basis of genotype in family members (Formstone *et al.*, 1996). Some patients may be clinically symptomatic while protein S and C levels are in the normal range. This could be due to another lesion in as yet unidentified genes, acting as risk factors, or thrombosis may have been triggered by acquired deficiency of an as yet unidentified anticoagulant factor (Formstone *et al.*, 1996).

A study by Vigano-D' Angelo *et al.*, (1987) showed that mean functional protein S activity in nephrotic patients is 69% ± 53% (2SD) ($p<0,001$) which is significantly lower than

protein S levels in controls 105% ± 38%. Immuno-electrophoretic analysis indicated reduced levels of free protein S antigen in nephrotic patients: 90% ± 38% ($p<0,05$) compared to controls 112% ± 40% ($p<0,05$). C4b-bp levels were found to be significantly increased in nephrotic patients: 170% ± 108% compared with controls: 114% ± 65%, ($p<0,001$) (Vigano-D'Angelo *et al.*, 1987). It could be because C4b-bp levels increase, there is a shift in protein S to the bound form according to the law of mass action or that there is urinary loss of free protein S (Vigano-D'Angelo *et al.*, 1987). A deficiency of protein S is associated with thrombosis (Heeb *et al.*, 1993; Kemkes-Matthes, 1992). A polymorphism in the protein S gene was found to be associated with symptomatic protein S deficiency in an Italian family (Marchetti *et al.*, 1993). The mutated allele was designated + to indicate that it is cut by BstXI and the wild type was designated – to indicate that BstXI does not cut. In the present study, there was a positive association of the protein S + allele with the black NS patients compared to the white NS patients ($p = 0,011$), as the + allele is more prevalent in the black patients. A significant difference was also found in the present study, between the frequency distribution of the – allele in the black and white NS patients ($p = 0,038$), as the – allele is more prevalent in the white patients. This is particularly interesting as the + allele has been linked to thrombosis in an Italian family (Marchetti *et al.*, 1993). Although the same study reported no difference in allele frequency between black and white populations (0,52 and 0,47 respectively), the present study showed a difference. This protein S polymorphism being more prevalent in the black population, could indicate a predisposition to thrombosis incidence (Klatsky *et al.*, 1991; Alter, 1994). Halkas *et al.*, (1998), found functionally deficient variants of $\alpha_1$anti-trypsin in black NS patients,and this proteinase inhibitor can also inhibit thrombin.

Selective urinary loss of free protein S is a unique feature of nephrotic syndrome and is probably the decisive factor in causing reduced levels of free protein S in plasma. Other factors such as immobility in patients with oedema, are important in determining which patients will develop thrombosis (Vigano-D'Angelo *et al.*, 1987).

When observing total protein S and -C antigen levels, the conclusion could be made that protein S and -C deficiency does not occur in NS patients and are therefore not a risk factor for developing thrombosis. But, in some patients the unbound, active protein S fraction is reduced and affects the activity of functional protein S. Decreased free protein S levels in

itself explains reduced protein S activity only in some patients, as there is a discrepancy between anticoagulant activity and antigenic levels of free protein S, because the specific activity of free protein S is markedly reduced in many patients. A general defect in processing vitamin K-dependent proteins may exist as the reduction of protein C specific activity is similar to the protein C level in nephrotic patients (31% compared with 26%). Decreased specific activity of protein S and reduction of free protein S are both responsible for the low protein S activity in nephrotic patients (Vigano-D'Angelo *et al.*, 1987).

Speculation is that because protein S is vitamin K-dependent, ineffective carboxylation of gamma-carboxyglutamic acid residues of vitamin K-dependent factors could occur in nephrotic patients as a result of the abnormally high turnover of these proteins (Vigano-D'Angelo *et al.*, 1987).

Protein S has been shown to be polymorphic and point mutations were identified in 15 individuals, of which 8 were affected. One mutation where the insertion of a -T- in codon 25 is present has been described in a Dutch pedigree. The other mutations are located in exons that code for the protein S domain, which is homologous to steroid hormone binding globulins. These include amino acid replacements such as in one person with 340Gly→Val; two with 467Val→Gly and four frame shift mutations. The frame shift mutations are due to either one base pair deletion in codon 561 (deletion of T) and in codon 267 (deletion of G), or one base pair insertion in codon 565 (insertion of T) and in codon 578 (insertion of C) (Gómez *et al.*, 1995).

Inflammation is often associated with onset of disseminated intravascular coagulation and more patients experience their first thrombosis after an infection. This is due to the fact that the immune mediator IL-1 initiate tissue factor formation on endothelial cells and induce the cells to form leukocyte-binding sites on the cell surface. When the endothelium is exposed to endotoxins, IL-1 or TNF, thrombomodulin function and the capacity of the endothelial cell surface to bind protein S and stimulate Factor Va inactivation are lost (Esmon, 1987).

A significant difference was found in the black and white control individuals as the protein S ++ genotype is more prevalent in the black population (p = 0,012) and the - - genotype is more prevalent in the white population (p = 0,069).

In the present study, a significant difference was found in the frequency distribution of the protein S ++ and - - genotypes in all black (p = 0,001) and in all white (p = 0,004) individuals, as individuals homozygous for the + allele are more prevalent in the black population, and individuals homozygous for the – allele are more prevalent in the white population. A higher percentage white individuals are heterozygous for the protein S alleles than black individuals.

In conclusion, the present study shows that black NS patients as well as black control individuals have a higher frequency of the protein S + allele than white NS patients and white control individuals. This together with the higher prevalence of a functionally defective $\alpha_1$anti-trypsin deficient variance, could result in a higher predisposition to thrombosis in black NS patients than in white NS patients.

5.1 CONCLUSIONS

The factors investigated in this study have revealed interesting differences. The LRP is quite central to the pathophysiology of the syndrome and acts as a receptor for α_2M proteinase complexes; apoE and tPA (Krieger & Herz, 1994).

This study has shown that apoE genotypes were different in FSGS patients. The $apoE_2$, which does not bind lipids as efficiently to the LRP, was found to be linked to black patients with FSGS and might represent a risk factor. Furthermore, increased levels of α_2M would bind to the LRP and disturb the binding of apoE.

Cytokines such as IL-1 and TNF are known to be produced in the inflammatory process and are also known to bind to α_2M. These complexes are mitogenic and result in cell proliferation. Furthermore, these cytokines produce alterations in the normal anti-coagulant state leading to procoagulant expression, followed by thrombin formation and fibrin deposition.

The protein S + allele, found at a high frequency in the black population, might also be a predisposing factor to increased coagulation in the black patients. Taken all together, these findings could explain why the disease is more severe in black patients.

Appendix A

Lysis buffer

0,32 M Sucrose	10,9 g
10 mM Tris base	0,121 g
5 mM $MgCl_2$	0,101 g
1% Triton X	1 ml

Dissolve the sucrose, Tris base and Mg Cl_2 in 90 ml double-distilled water and adjust the pH to 7,5 with HCl. Add 1 ml Triton X and make up to a final volume of 100 ml with double-distilled water. Autoclave, aliquot and store at +4°C or at -20°C for long term storage.

PCR storage buffer

50 mM KCl	0,375 g
2,5 mM $MgCl_2$	0,050 g
10 mM Tris base	0,121 g
0,1 mg/ml Gelatin	0,010 g
0,45% Nonidet P40	0,450 ml
0,45% Tween 20	0,450 ml

Dissolve the KCl, $MgCl_2$, Tris base and gelatin in 90 ml double-distilled water, adjust the pH to 8,3 with HCl. Add the Nonidet P40 and Tween 20 and make up to a final volume of 100 ml with double-distilled water. Autoclave, aliquot and store at +4°C or at -20°C for long term storage.

Loading buffer

0,25% Bromophenol Blue
0,25% Xylene Cyanol
15% Ficol type 400 (Pharmacia, Uppsala, Sweden)

Mix all three reagents together and make up to a final volume of 100 ml with double-distilled water.

15% Polyacrylamide gel

4 ml Double-distilled water
1 ml 10x TBE buffer
5 ml Acrylamide stock solution

Mix these three reagents together and when ready to pour the gel, add 20 µl TEMED and 16 µl of 0,1% ammonium persulphate.

10x TBE buffer (10 M)

Tris HCl	108 g
Boric Acid	55 g
0,5 M EDTA pH 8,0 with HCl	40 ml

Mix the three reagents, adjust pH to 8,2 with HCl and make up to 1liter with double-distilled water. Dilute 1:10 with double-distilled water for a 1,0 M concentration to submerge the 15% polyacrylamide gel for electrophoresis.

Acrylamide stock solution

Acrylamide	29 g
Bis acrylamide	1 g

Mix reagents in a final volume of 100 ml of double-distilled water. Filter just before use. Store at +4°C for no more than one month.

Appendix B

2% (w/v) Agarose gel

0,6% Agarose
24 ml SABAX water
6 ml 5x TBE buffer
Microwave on high till melted
6 µl Ethidium bromide
Mix well by swirling
Cool to approximately 50°C before pouring into gel mold.
Submerge the gel in 0,5x TBE buffer once it is set.

Base pair ladder

Use 5 µl of the 100bp ladder and add 2 µl of the specific supplied dye mixture. Mix well and load 5 µl of this mixture into the first and the last wells of the 2% agarose gel.

10x TBE buffer (10 M)

As described in Appendix B, but for submerging a 2% agarose gel in for electrophoresis instead of the 15% polyacrylamide gel as described in Appendix B.

Bst XI restriction enzyme

Bst XI recognition site: CCANNNNN / NTGG.
The enzyme is supplied as 250 units in 25 µl, which equals 10 units /µl.
The enzyme is supplied with a vial of its specific optimal buffer at a 10x concentrate.
Dilute the 10x concentrate buffer to a 1x concentrate in double-distilled water. Add 25 µl of the diluted 1x concentrate buffer to the 25 µl of supplied Bst XI restriction enzyme. The total volume equals 50 µl of a 1:2 dilution of restriction enzyme. Add 1 µl of the 1:2 dilution (5 units) to 10 µl PCR product, and incubate at the optimal enzyme function temperature of 50°C for 1 hour to achieve DNA restriction.

Appendix B

2% (w/v) Agarose gel

0,6% Agarose
24 ml SABAX water
6 ml 5x TBE buffer
Microwave on high till melted
6 µl Ethidium bromide
Mix well by swirling
Cool to approximately 50°C before pouring into gel mold.
Submerge the gel in 0,5x TBE buffer once it is set.

Base pair ladder

Use 5 µl of the 100bp ladder and add 2 µl of the specific supplied dye mixture. Mix well and load 5 µl of this mixture into the first and the last wells of the 2% agarose gel.

10x TBE buffer (10 M)

As described in Appendix B, but for submerging a 2% agarose gel in for electrophoresis instead of the 15% polyacrylamide gel as described in Appendix B.

Bst XI restriction enzyme

Bst XI recognition site: CCANNNNN / NTGG.
The enzyme is supplied as 250 units in 25 µl, which equals 10 units /µl.
The enzyme is supplied with a vial of its specific optimal buffer at a 10x concentrate.
Dilute the 10x concentrate buffer to a 1x concentrate in double-distilled water. Add 25 µl of the diluted 1x concentrate buffer to the 25 µl of supplied Bst XI restriction enzyme. The total volume equals 50 µl of a 1:2 dilution of restriction enzyme. Add 1 µl of the 1:2 dilution (5 units) to 10 µl PCR product, and incubate at the optimal enzyme function temperature of 50°C for 1 hour to achieve DNA restriction.

REFERENCES:

Allon, M., Soffer, O., Evatt, B., Hixon, G. and Wideman, C.S. (1989). Protein S and C antigen levels in proteinuric patients : Dependence on type of glomerular pathology. *Ann J Hematol,* vol. 31, pp. 96-101.

Alter, M. (1994). Black-White Differences in Stroke Frequency: Challenges for Research. *Neuroepidemiology,* vol. 13, pp. 301-307.

Andrew, M., Paes, B., Milner, R., Johnston, M., Mitchell, L., Tollefsen, D.M. and Powers, P. (1987). Development of the human coagulation system in the full-term infant. *Blood,* vol. 70, pp. 165-172.

Andrew, M., Paes, B., Milner, R., Johnston, M., Mitchell, L., Tollefsen, D.M., Castle, V. and Powers, P. (1988). Development of the human coagulation system in the healthy premature infant. *Blood,* vol. 72, pp. 1651-1657.

Antikainen, M., Holmberg, C. and Taskinen, M-R. (1992). Growth, serum lipoproteins and apoproteins in infants with congenital nephrosis. *Clin Nephrol,* vol. 38, no. 5, pp. 254-263.

Appel, G.B., Blum, C.B., Chien, S., Kunis, C.L. and Appel, A.S. (1985). The hyperlipidemia of nephrotic syndrome : Relation to plasma albumin concentration, oncotic pressure and viscosity. *N Eng J Med,* vol. 312, pp. 1544-1548.

Asami, T., Ohsawa, S., Tomisawa, S., Hashimoto, K., Toyabe, S. and Sakai, K. (1992). Glomerular Deposition of α_2-Macroglobulin in a Child with Steroid Refractory Nephrotic Syndrome. *Nephron,* vol. 61, pp. 211-213.

Asami, T., Ciomartan, T., Hayakawa, H., Uchiyama, M. and Tomisawa, S. (1999). Apolipoprotein E ε 4 allele and nephrotic glomerular diseases in children. *Pediatr Nephrol,* vol. 13, pp. 233-236.

Assmann, G., Schmitz, G., Menzel, H-J. and Schulte, H. (1984). Apolipoprotein E Polymorphism and Hyperlipidermia. *Clin Chem,* vol. 30, no. 5, pp. 641-643.

Bachem, M.G., Schüftan, G., Schirrmacher, P. and Gressner, A.M. (1994). Feedback Mechanisms between α_2M and TGF β_1 Reduce Extracellular Matrix Synthesis of Liver Fat-Storing Cells. *Ann NY Acad Sci,* vol. 737, pp. 421-424.

Bakir, A., Bazilinksi, N., Rhee, H., Ainis, H. and Dunea, G.G. (1989). Focal segmental glomerulosclerosis : A common entity in nephrotic black adults. *Arch Intern Med,* vol. 149, pp. 1802-1804.

Bakir, A.A., Share, D.S., Levy, P.S., Arruda, L.A.L. and Dunea, G. (1996). Focal segmental glomerulosclerosis in adult African Americans. *Clin Nephrol,* vol. 46, no. 5, pp. 306-311.

Barnett, H.L., Edelmann, C.M. (Jr), Greifer, I., Goldsmith, D.I., Spitzer, A., *et al.* (1984). Minimal Change Nephrotic Syndrome in Children : Deaths During the First 5 to 15 Years' Observation. *Pediatrics*, vol. 73, no. 4, pp. 497-501.

Barrett, A.J. and Starkey, P.M. (1973). The Interaction of α_2-Macroglobulin with Proteinases. Characteristics and Specificity of the Reaction, and a Hypothesis Concerning its Molecular Mechanism. *Biochem J*, vol. 133, pp. 709-724.

Barrett, A.J., Brown, M.A. and Sayers, C.A. (1979). The Electrophoretically "Slow" and "Fast" Forms of the α_2-Macroglobulin Molecule. *Biochem J*, vol. 181, pp. 401-418.

Barrett, T.G., Taylor, C.M. and Milford, D.V. (1995). Characteristic phenotype in congenital nephrotic syndrome. *Eur J Pediatr*, vol. 154, pp. 239-240.

Becker, N.B., Himmelfarb, J., Henrich, W.L. and Hakim, R. (1997). Reassessing the cardiac risk profile in chronic hemodialysis patients: A hypothesis on the role of oxidant stress and other non-traditional cardiac risk factors. *J Am Soc Nephrol*, vol. 8, pp. 475-486.

Berlyne, G.M. and Mallick, N.P. (1969). Ischaemic heart disease as a complication of nephrotic syndrome. *Lancet*, vol. 2, pp. 399-400.

Bernard, D.B. (1988). Extrarenal complications of the nephrotic syndrome. *Kidney Int*, vol. 33, pp. 1184-1202.

Bernard, D.B., Alexander, E.A., Couser, W.G. and Levinsky, N.G. (1978). Renal sodium retention during volume expansion in experimental nephrotic syndrome. *Kidney Int*, vol. 14, no. 5, pp. 478-485.

Bertina, R.M., Ploos van Amstel, H.K., Van Wijngaarden, A., Coenen, J., Leemhuis, M.P., Deutz-Terlouw, P.P., van der Linden, I.K. and Reitsma, P.H. (1990). Heerlen Polymorphism of PS, an Immunologic Polymorphism Due to Dimorphism of Residue 460. *Blood*, vol. 76, no. 3, pp. 538-548.

Birkenmeier, G., Osman, A.A., Kopperschläger, G. and Mothes, T. (1997). Epitope mapping by screening of phage display libraries of a monoclonal antibody directed against the receptor binding domain of human α_2-macroglobulin. *FEBS Letters*, vol. 416, pp. 193-196.

Birkenmeier, G., Schuke, H.L., Briest, S. and Koppenschläger, G. (1994). Determination of Total and Transformed α_2-Macroglobulin by a Monoclonal Antibody Immunosorption Assay in Patients with Different Diseases. *Ann NY Acad Sci*, vol. 737, pp. 425-427.

Blacker, D., Wilcox, M.A., Laird, N.M., Rodes, L., Horvath, S.M., Go, R.C.P., Perry, R., Watson, B. Jr., Bassett, S.S., McInnis, M.G., Albert, M.S., Hyman, B.T. and Tanzi, R.E. (1998). Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nature Genetics*, vol. 19, pp. 357-360.

Bloch, W. (1991). A biochemical perspective of the polymerase chain reaction. *Biochemistry*, vol. 30, no. 11, pp. 2735-2747.

Boerwinkle, E. and Utermann, G. (1988). Simultaneous effects of the apolipoprotein E polymorhism on apolipoprotein E, apolipoprotein B, and cholesterol metabolism. *Am J Hum Genet*, vol. 42, pp. 104-112.

Bonner, J.C. (1994). Regulation of Platelet - Derived Growth Factor (PDGF) and Alveolar Macrophage-Derived PDGF by α_2-Macroglobulin. *Ann NY Acad Sci*, vol. 737, pp. 324-338.

Border, W.A. and Ruoslahti, E. (1992). Transforming Frowth Factor-β in Disease: The Dark Side of Tissue Rapeir. *J Clin Invest*, vol. 90, pp. 1 -7.

Border, W.A., Noble, N.A., Yamamoto, T., Harper, J.R., Yamaguchi, Y., Pierschebacher, M.D. and Ruoslahti, E. (1992). Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. *Nature*, vol. 360, pp. 361-364.

Borth, W. (1994). α_2-Macroglobulin. A Multifunctional Binding and Targeting Protein with Possible Roles in Immunity and Autoimmunity. *Ann NY Acid Sci*, vol. 737, pp. 267-272.

Brown, M.S. and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. *Science*, vol. 232, pp. 34-37.

Bu, G. and Rennke, S. (1996). Receptor-associated protein is a folding chaperone for low density lipoprotein receptor-related protein. *J Biol Chem*, vol. 271, pp. 22218-22224.

Bu, G. and Schwartz, A.L. (1998). RAP, a novel type of ER chaperone. *Trends Cell Biol*, vol. 8, pp. 272-276.

Bu, G., Sun, Y., Schwartz, A.L. and Holtzman, D.M. (1998). Nerve Growth Factor Induces Rapid Increases in Functional Cell Surface Low Density Lipoprotein Receptor-related Protein. *J Biol Chem*, vol. 273, no. 21, pp. 13359-13365.

Cameron, J.S. (1990). Proteinuria and Progression in Human Glomerular Diseases. *Am J Nephrol*, vol. 10, (suppl. 1), pp. 81-87.

Cassel, D.L., Phillips, M.C., Rostron, P., Rothblat, G.H. and Utermann, G. (1984). The Conformation of Apolipoprotein E Isoforms in Phospholipid Complexes and their Interaction with Human Hep G2 Cells. *Atherosclerosis*, vol. 52, pp. 302-218.

Chappell, D.A., Fry, G.L., Waknitz, M.A., Muhonen, L.E., Pladet, M.W., Iverius, P-H. and Strickland, D.K. (1993). Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor in vitro. *J Biol Chem*, vol. 268, pp. 14168-14175.

Chappell, D.A., Inoue, I., Fry, G.L., Pladet, M.W., Iverius, P-H., Lalouel, J-M. and Strickland, D.K. (1994). The Carboxy-Terminal Domain of Lipoprotein Lipase Induces Cellular Catabolism of Normal Very Low Density Lipoproteins via the Low Density Lipoprotein Receptor-Related Protein/α_2-Macroglobulin Receptor. *Ann NY Acad Sci*, vol. 737, pp. 434-437.

Chaturvedi, S. (1993). Fulminant Cerebral Infarctions With Membranous Nephropathy. *Stroke*, vol. 24, no. 3, pp. 473-475.

Chen, T.Y., Huang, Ch.Ch. and Tsao, Ch.J. (1993). Hemostatic molecular markers in nephrotic syndrome. *Am J Haematol*, vol. 44, pp. 276-279.

Chu, C.T. and Pizzo, S.V. (1994). α_2-Macroglobulin, complement and biologic defence: antigens, growth factors, microbial proteases and receptor ligation. *Lab Invest*, vol. 71, pp. 792-812.

Chu, C.T., Howard, G.C., Misra, U.K. and Pizza, S.V. (1994). α_2-Macroglobulin: A Sensor for Proteolysis. *Ann NY Acad Sci*, vol. 737, pp. 291-307.

Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C. (Jr), Rimmler, J.B., Locke, P.A., Conneally, P.M., Schmader, K.E., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1994). Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nat Genet*, vol. 7, pp. 180-184.

Cullmann, W. and Dick, W.J. (1981). A Chromogenic Assay for Evaluation of α_2-Macroglobulin Level in Serum and Cerebrospinal Fluid. *J Clin Chem Clin Biochem*, vol. 19, pp. 287.

D'Agati, V (1994). The many masks of focal segmental glomerulosclerosis. *Kidney Int*, vol. 46, pp. 1223-1241.

Dahlback, B. (1986). Inhibition of protein Ca cofactor function of human and bovine protein S by C4b-binding protein. *J Biol Chem*, vol. 261, pp. 12022-12027.

Dahlback, B., Hildebrand, B. and Linse, S. (1990). Novel types of very high affinity calcium binding sites in betahydroxyasparagine containing epidermal growth factor-like domains in vitamin K-dependent protein S. *J Biol Chem*, vol. 265, pp. 18481-18489.

D'Amico, G., Gentil, M.G. and Manna, G. (1992). Effect of vegetarian soy diet on hyperlipidaemia in nephrotic syndrome. *Lancet*, vol. 339, pp. 1131-1134.

Das, H.K., McPherson, J., Bruns, G.A.P., Karathanasis, S.K. and Breslow, J.L. (1985). Isolation, characterization and mapping to chromosome 19 of the human apolipoprotein E gene. *J Biol Chem*, vol. 260, pp. 6240-6247.

Davignon, J., Gregg, R.E. and Sing, C.F. (1988). Apolipoprotein E polymorphism and atherosclerosis. *Atherosclerosis*, vol. 8, pp. 1-21.

De Fouw, N.J., Haverkate, F., Bertina, R.M., Koopman, J., Wijngaarden, A. and van Hinsbergh, V.W.M. (1986). The cofactor role of protein S in the acceleration of whole blood clot lysis by activated protein C in vitro. *Blood*, vol. 67, pp. 1189-1195.

De Kijff, P., Johansen, L.G., Rosseneu, M., Frants, R.R., Jespersen, J. and Havekes, L.M. (1992). Lipoprotein profile of a Greenland Inuit population : influence of anthropometric variables, Apo E and A4 polymorphism, and lifestyle. *Arterioscler Thromb*, vol. 12, pp. 1371-1379.

De Sain - Van der Velden, M.G.M., Rabelink, T.J., Reijngoud, D-J., Gadellaa, M.M., Voorbij, H.A.M., Stellard, F. and Kaysen, G.A. [1998(a)]. Plasma α_2-macroglobulin is increased in nephrotic patients as a result of increased synthesis alone. *Kidney Int*, vol. 54, pp. 530-535.

De Sain - Van der Velden, M.G.M., Kaysen, G.A., De Meer, K., Stellaard, F., Voorbij, H.A.M., Reijngoud, D-J., Rabelink, T.J. and Koomans, H.A. [1998(b)]. Proportionate increase of fibrinogen and albumin synthesis in nephrotic patients: Measurements with stable isotopes. *Kidney Int*, vol. 53, pp. 181-188.

De Sain - Van der Velden, M.G.M., Kaysen, G.A., Barrett, H.A., Stellaard, F., Gadellaa, M.M., Voorbij, H.A.M., Reijngoud, D-J. and Rabelink, T.J. [1998(c)]. Increased VLDL in nephrotic patients result from a decreased catablolism while increased LDL results from increased synthesis. *Kidney Int*, vol. 53, pp. 994-1001.

Detwiler, R.K., Falk, R.J., Hogan, S.L. and Jennette, J.C. (1994). Collapsing glomerulopathy : A clinically and pathologically distinct variant of focal segmental glomerulosclerosis. *Kidney Int*, vol. 45, pp. 1416-1424.

Di Scipio, R.G. and Davie, E.W. (1979). Characterization of protein S, a gamma-carboxyglutamic acid containing protein from bovine and human plasma. *Biochem*, vol. 18, pp. 899-904.

Diamond, J.R. and Karnovsky, M.J. (1992). A putative role of hypercholesterolemia in progressive glomerular injury. *Ann Rev Med*, vol. 43, pp. 83-92.

Diamond, J.R. and Karnovsky, U.J. (1988). Focal and segmental glomerulosclerosis : Analogies to artherosclerosis. *Kidney Int*, vol. 33, pp. 917-924.

Diepstraten, C.M., Ploos van Amstel, J.K., Rietsma, P.H. and Bertina, R.M. (1991). A CCA/CCG neutral dimorphism in the codon for Pro 626 of the human protein S gene PS α (PROS 1). *Nucleic Acids Res*, vol. 19, no. 18, pp. 5091.

Dix, J.D., Gaston, L. and Patterson, W. (1988). Protein S deficiency. *Arch Pathol Lab Med*, vol. 112, pp. 108.

Du, Y.S., Ni, B.H., Glinn, M., Dodel, R.C., Bales, K.R., Zhang, Z.Y., Hyslop, P.A. and Paul, S.M. (1997). Alpha 2-Macroglobulin as a beta-amyloid peptide-binding plasma protein. *J Neurochem*, vol. 69, no. 1, pp.299-305.

Dullaart, R.P.F., Gansevoort, R.T., Sluiter, W.J., de Zeeuw, D. and de Jong, P. (1996). The Serum Lathosterol to Cholesterol Ratio, an Index of Cholesterol Synthesis, Is Not Elevated in Patients With Glomerular Proteinuria and Is Not Associated With Improvement of Hyperlipidermia in Response to Anti-proteinuric Treatment. *Metabolism*, vol. 45, no. 6, pp. 723-730.

Edson, J.U., Vogt, J.M. and Huesman, D.A. (1990). Laboratory diagnosis of inherited protein S deficiency. *Am J Clin Pathol*, vol. 94, pp. 176-186.

Ehnholm, C., Lukka, M., Kuusi, T., Nikkilä, E. and Utermann, G. (1986). Apolipoprotein E polymorphism in the Finnish population : gene frequencies and relation to lipoprotein concentrations. *J Lipid Res*, vol. 27, pp. 227-235.

Ellgaard, L., Holtet, T.L., Nielsen, P.R., Etzerodt, M., Gliemann, J. and Thøgersen, H.C. (1997). Dissection of the domain architecture of the α_2 macroglobulin-receptor-associated protein. *Eur J Biochem*, vol. 244, pp. 544-551.

Engesser, L., Broekmans, A.W., Briët, E., Brommer, E.J.P. and Bertina, R.M. (1987). Hereditary Protein S Deficiency: Clinical Manifestations. *Ann Intern Med*, vol. 106, pp. 677-682.

Esmon, C.T. (1987). The Regulation of Natural Anticoagulant Pathways. *Science*, vol. 235, pp. 1348-1352.

Fass, D., Blacklow, S., Kim, P.S. and Berger, J.M. (1997). Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature*, vol. 388, pp. 691-693.

Feinman, R.D. (1994). The Proteinase-Binding Reaction of α_2M. *Ann NY Acad Sci*, vol. 737, pp. 245-266.

Fernlund, P. and Stenflo, J. (1983). Beta-hydroxyaspartic acid in vitamin K-dependent proteins. *J Biol Chem*, vol. 258, pp. 12509-12512.

Formstone, C.J., Hallam, P.J., Tuddenham, E.G.D., Voke, J., Layton, M., Nicolaides, K., Hann, I.M. and Cooper, D.N. (1996). Severe Perinatal Thrombosis in Double and Triple Heterozygous Offspring of a Family Segregating Two Independent Protein S Mutations and a Protein C Mutation. *Blood*, vol. 87, no. 9, pp. 3721-3737.

Frenoy, J-P. and Bourrillon, R. (1974). Studies on the structure of human α_2-M. IV. Analyses of the microheterogeneity by isoelectric focussing. *Biochim Biophys Acta*, vol. 371, pp. 168-176.

Gaillard, M.C. and Kilroe-Smith, T.A. (1987). Determination of Functional Activity of α_1-Protease Inhibitor and α_2-Macroglobulin in Human Plasma Using Elastase. *J Clin Chem Clin Biochem*, vol. 25, pp. 167-172.

Gaillard, M.C., Song, E., Noqueira, C.M. and Kilroe-Smith, T.A. (1995). Elastase binding capacity of α_2-macroglobulin and its association with glucocorticoid concentration in southern African black patients with hepatocellular carcinoma. *Clin Chim Acta*, vol. 240, pp. 179-185.

Gaillard, M.C., von Arb, M., da Costa, A. and Kilroe-Smith, T.A. (1989). Functional and immunological concentrations of α_2-macroglobulin compared in plasma of subjects without α_1-protease inhibitor deficiency. *Clin Chem*, vol. 35, no. 7, pp. 1535-1537.

Gandrille, S., Borgel, D., Ireland, H., Lane, D.A., Simmonds, R., Reitsma, P.H., Mannhalter, C., Pabinger, I., Saito, H., Suzuki, K., Formstone, C., Cooper, D.N., Espinosa, Y., Sala, N., Bernardi, F. and Aiach, M. (1997). Protein S Deficiency : A Database of Mutations. For the Plasma Coagulation Inhibitors Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost*, vol. 77, pp. 1201-1214.

Ganrot, P.O. (1966). Determination of α_2-macroglobulin as trypsin-protease esterase. *Clin Chim Acta*, vol. 14, pp. 493-501.

Garber, D.W., Gottlieb, B.A., Marsh, J.B. and Sparks, C.E. (1984). Catabolism of very low density lipoproteins in experimental nephrosis. *J Clin Invest*, vol. 74, pp. 1375-1383.

Garbrecht, F., Gardner, S., Johnson, V. and Grabowski, E. (1991). Deep Venous Thrombosis in a Child with Nephrotic Syndrome Associated with a Circulating anticoagulant and Acquired Protein S Deficiency. *Am J Ped Hem/Oncol*, vol. 13, no. 3, pp. 330-333.

Garin, E.H., Sausville, P.J. and Richard, G.A. (1983). Plasma prostaglandin E_2 concentration in nephrotic syndrome. *J Pediatr*, vol. 103, pp. 253-256.

Geers, A.B., Koomans, H.A, Boer, P. and Dorhout Mees, E.J. (1984). Plasma and blood volumes in patients with the nephrotic syndrome. *Nephron*, vol. 38, pp. 170-173.

Gettins, P.G.W. and Crews, B.C. (1994). Binding of Epidermal Growth Factor to Human α_2-Macroglobulin. Significance for Cytokine α_2-Macroglobulin Interactions. *Ann NY Acad Sci*, vol. 737, pp. 383-398.

Gherardi, E., Rota, E., Calandra, S., Genova, R. and Tamborino, A. (1977). Relationship among the concentrations of serum lipoproteins and changes in their chemical composition in patients with untreated nephrotic syndrome. *Eur J Clin Invest*, vol. 7, pp. 563-570.

Gitlin, D., Janeway, C.A. and Farr, L.E. (1956). Studies on the metabolism of plasma proteins in the nephrotic syndrome. *J Clin Invest*, vol. 35, pp. 44-56.

Gläser, C., Handschug, K., Ludvik, S., Schell, U., Hehr, A., Küchenhoff, A. and Robenek, H. (1994). The importance of the α_2-MR/LRP for Human Genetics. Investigations of the Receptor Expression in Different Human Blood Cells. *Ann NY Acad Sci*, vol. 737, pp. 447-450.

Gliemann, J., Nykjaner, A., Petersen, C.M., Jørgensen, K.E., Nielsen, M., Andreasen, P.A., Christensen, E.I., Lookene, A., Olivecrona, G. and Moestrup, S.K. (1994). The Multiligand α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor-Related Protein (α_2MR/LRP) Binding and Endocytosis of Fluid Phase and Membrane-Associated Ligands. *Ann NY Acad Sci*, vol. 737, pp. 20-38.

Gómez, E., Poort, S.R., Bertina, R.M. and Reitsma, P.H. (1995). Identification of Eight Point Mutations in Protein S Deficiency Type I - Analysis of 15 Pedigrees. *Thrombosis and Haemostasis*, vol. 73, no. 5, pp. 750-755.

Gonias, S.L., LaMarre, J., Crookstan, K.P., Webb, D.J., Wolf, B.B., Lopes, M.B.S., Moses, H.L. and Hayes, M.A. (1994). α_2-Macroglobulin and the α_2 - Macroglobulin Receptor/LRP. A growth regulatory axis. *Ann NY Acad Sci*, vol. 737, pp. 273-290.

Greengard, J.S., Fernandez, J.A., Radtke, K-P. and Griffin, J.H. (1995). Identification of candidate residues for interaction of protein S with C4b binding protein and activated protein C. *Biochem J*, vol. 305, pp. 397-403.

Gregg, R.E., Ghiselli, G. and Brewer, H.B. (Jr) (1983). Apolipoprotein $_{E\ Bethesda}$: a new variant of apolipoprotein E associated with type III hyperlipoproteinemia. *J Clin Endocrinol Met*, vol. 57, pp. 969-974.

Grishman, E. and Churg, J. (1975). Focal glomerular sclerosis in nephrotic patients : An electron microscopy study of glomerular podocytes. *Kidney Int*, vol. 7, pp. 111-122.

Grøn, H. and Pizzo, S.V. (1998). Nonproteolytic Incorporation of Protein Ligands into Human α_2-Macroglobulin: Implications for the Binding Mechanism of α_2-Macroglobulin. *Biochem*, vol.37, pp. 6009-6014.

Gröne, H-F., Walli, A.K., Gröne, E., Krämer, A., Clemens, M.R. and Seidel, D. (1990). Receptor mediated uptake of apoB and apoE rich lipoproteins by human glomerular epithelial cells. *Kidney Int*, vol. 37, pp. 1449-1459.

Grundy, S.M. and Vega, G.L. (1989). Rational and management of hyperlipidemia of the nephrotic syndrome. *Am J Med*, vol. 87, pp. 3N-11N.

Guyton, A.C. (1991). Formation of Urine by the Kidney : 1. Renal Blood Flow, Glomerular Filtration and Their Control. In *Textbook of Medical Physiology, Philadelphia*: WB Saunders Company, pp. 286-297.

Haas, M., Spargo, B.H. and Coventry, S. (1995). Increasing Incidence of Focal-Segmental Glomerulosclerosis Among Adult Nephropathies : A 20-Year Renal Biopsy Study. *Am J Kidney Diseases*, vol. 26, no. 5, pp. 740-750

Habib, R. (1993). Nephrotic syndrome in the 1st year of life. *Pediatr Nephrol*, vol. 7, pp. 347-353.

Halkas, A.C., Thomson, P.D. and Meyers, K.E.C. (1996). The effect of cyclosporin dosage on renal graft function after 1 to 5 years follow-up (abstract). *Afr J Surg*, vol. 34, pp. 104.

Halkas, A.C., Gaillard, M.C., Thomson, P.D., Green, S.L., Ludewick, H. and Kala, U. (1998). Variants of α_1-proteinase inhibitor in black and white South African patients with focal glomerulosclerosis and minimal change nephrotic syndrome. *J Med Genet*, vol. 35, pp. 6-9.

Hall, P.K. and Roberts, R.C. (1978). Physical and Chemical Properties of Human Plasma α_2-Macroglobulin. *Biochem J*, vol. 171, pp. 27-38.

Hallman, D.M., Boerwinkle, E., Saha, N., Sandholzer, C., Menzel, H., Császár, A. and Utermann, G. (1991). The apolipoprotein E polymorphism : a comparison of allele frequencies and effects in nine populations. *Am J Hum Genet*, vol. 49, pp. 338-349.

Hallman, N., Norio, R. and Kouvalainen, K. (1967). Main features of the congenital nephrotic syndrome. *Acta Paediat Scand Suppl*, vol. 172, pp. 75-78.

Hanevoled, C.D., Lazarchick, J., Constantin, M.A., Hiott, K. and Orak, J.F. (1994). Acquired protein S deficiency in nephrotic syndrome in a pediatric population. *Clin Nephrol*, vol. 5, pp. 351.

Hedfors, El, Kistner, S. and Norberg, R. (1971). Serum levels and urinary excretion of α_2-Macroglobulin in patients with renal disease. *Acta Med Scand*, vol. 190, pp. 347-351.

Heeb, M.J., Mesters, R.M., Tans, G., Rosing, J. and Griffon, J.H. (1993). Binding of Protein S to Factor Va Associated with Inhibition of Prothrombinase that is Independent of Activated Protein C. *J Biol Chem*, vol. 268, no. 4, pp. 2872 - 2877.

Heeb, M.J., Rosing, J., Bakker, H.M., Fernandez, J.A., Tans, G. and Griffin, J.H. (1994). Protein S binds to and inhibits factor Xa. *Proc Natl Acad Sci USA*, vol. 91, pp. 2728-2732.

Herz, J. (1993). The LDL-receptor-related protein : Portrait of a multifunctional receptor. *Curr Opin Lipid*, vol. 4, pp. 107-113.

Herz, J. and Willnow, T.E. (1994). Functions of the LDL Receptor Gene Family. *Ann NY Acad Sci*, vol. 737, pp. 14-19.

Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K. and Brown, M.S. (1991). 39-kDA protein modulates binding of ligands to low-density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J Biol Chem*, vol. 266, pp. 21232-21238.

Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gassepohl, H. and Stanley K.K. (1988). Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. *EMBO J*, vol. 7, pp. 4119-4127.

Higuchi, R. (1989). Rapid, efficient DNA extraction for PCR from cells or blood. *Amplifications*, issue 2, pp. 1-3.

Hillarp, A. and Dahlback, B. (1988). Novel subunit in C4b-binding protein required for protein S binding. *J Biol Chem*, vol. 263, pp. 12759-12764.

Hillarp, A., Hessing, M. and Dahlback, B. (1989). Protein S binding in relation to the subunit composition of human c4b-binding protein. *FEBS Lett*, vol. 259, pp. 53-56.

Holmberg, C., Antikainen, M., Rönnholm, K., Ala-Houhala, M. and Jalanko, H. (1995). Management of congenital nephrotic syndrome of the Finnish type. *Pediatr Nephrol*, vol. 9, pp. 87-93.

Holtzman, D.M., Pitas, R.E., Killridge, J., Nathan, B., Mahley, R.W., Bu, G. and Schwartz, A.L. (1995). Low density lipoprotein receptor-related protein mediates apolipoprotein E-dependent neurite outgrowth in a central nervous system-derived neuronal cell line. *Proc Natl Acad Sci USA*, vol. 92, pp. 9480-9484.

Hopp, L., Gilboa, N., Kurland, G., Weichler, N. and Orchard, T.J. (1994). Acute myocardial infarction in a young boy with nephrotic syndrome: a case report and review of the literature. *Pediatr Nephrol*, vol. 8, pp. 290-294.

Housley, J. (1968). Alpha$_2$-macroglobulin levels in disease in man. *J Clin Path*, vol. 21, pp. 27-31.

Howard, G.C., Misra, U.K., De Camp, D.L. and Pizzo, S.V. [1996(a)]. Altered interaction of Cis-dichlorodiammine platinum (II)-modified alpha 2-macroglobulin (alpha 2M) with the low density lipoprotein receptor-related protein/alpha 2M receptor but not the alpha 2M signaling receptor. *J Clin Invest*, vol. 97, no. 5, pp. 1193-1203.

Howard, G.C., Yamaguchi, Y., Misra, U.K., Gawdi, G., Nelsen, A., De Camp, D.L. and Pizzo, S.V. [1996(b)]. Selective Mutations in Cloned and Expressed α-Macroglobulin Receptor Binding Fragment Alter Binding to Either the α_2-Macroglobulin Signaling Receptor or the Low Density Lipoprotein Receptor-related Protein/α_2-Macroglobulin Receptor. *J Biol Chem*, vol. 271, no. 24, pp. 14105-14111.

Hoyer, P.F., Gonda, S., Barthels, M., Krohn, H.P. and Brodehl, J. (1986). Thromboembolic Complications in Children with Nephrotic Syndrome. Risk and Incidence. *Acta Paediatr Scand*, vol. 75, pp. 804-810.

Hug, L., McLachlan, T., Hammer, H.M., Bedford, D., Packard, C.J., Shepherd, J. and Converse, C.A. (1993). An Increased Incidence of Apolipoprotein E_2/E_2 and E_4/E_4 in Retinitis Pigmentosa. *Lipids*, vol. 28, no. 11, pp. 995-998.

Hyman, B.T., Strickland, D.K. and Rebeck, G.W. (1994). α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor Related Protein. Relationship to Apolipoprotein E and Role in Alzheimer Disease Senile Plaques. *Ann NY Acad Sci*, vol. 737, pp. 88-95.

Ikai, A., Koyano, N., Mitsuda, S. and Shibuya, N. (1994). Modulation of Immune Cell Activities by α_2-Macroglobulin. A Proposed Physiological Role Based on Activation Kinetics and Model Vaccine Studies. *Ann NY Acad Sci*, vol. 737, pp. 339-346.

Imura, T., Kimura, H. and Gejyo, F. (1999). Apolipoprotein E phenotypes in hemodialysis patients. *Kidney Int*, vol. 56, Suppl 71, pp. S245-S247.

Innerarity, T.L., Friedlander, E.J., Rall, S.C. (Jr), Weisgraber, K.H. and Mahley, R.W. (1983). The receptor-binding domain of human apolipoprotein E: binding of apolipoprotein E fragments. *J Biol Chem*, vol. 258, pp. 12341-12347.

Innerarity, T.L., Weisgraber, K.H., Rall, S.C. and Mahley, R.W. (1987). Functional domains on apolipoprotein E and apolipoprotein B. *Acta Med Scand Suppl*, vol. 715, pp. 51-59.

Israels, S.J. and Seshisa, S.S. (1987). Childhood stroke associated with protein C or S deficiency. *J Pediatr*, vol. 111, pp. 562-564.

Jensen, P.E.H. and Stigbrand, T. (1992). Differences in the proteinase inhibition mechanism of human alpha 2-macroglobulin and pregnancy zone protein. *Eur J Biochem*, vol. 210, pp. 1071-1077.

Joven, J., Villabona, C., Vilella, E., Masana, L., Alberti, R. and Valles, M. (1990). Abnormalities of lipoprotein metabolism in patients with nephrotic syndrome. *N Eng J Med*, vol. 323, pp. 579-584.

Kamboh, M.I., Bhatia, K.K. and Ferrel, R.E. (1990). Genetic studies of human apolipoproteins : XII Population genetics of apolipoproteins in Papua New Guinea. *Am J Hum Biol*, vol. 2, pp. 17-23.

Kamboh, M.I., Serjeanston, S.W. and Ferrel, R.E. (1991). Genetic studies of human apolipoproteins: XVIII. Apolipoprotein polymorphism in Australian Aborigenes. *Hum Biol*, vol. 63, pp. 179-186.

Kashyap, M.L., Srivastava, L.S., Hynd, B.A., Brady, D., Perisutti, G., Glueck, C.J. and Gartside, P.S. (1980). Apolipoprotein CII and lipoprotein lipase in human nephrotic syndrome. *Atherosclerosis*, vol. 35, pp. 29-40.

Kaufman, R., Veltkamp, J., Van Tilburg, W.H. and Van Es, L.A. (1978). Acquired antithrombin III deficiency in the nephrotic syndrome. *Am J Med*, vol. 65, pp. 607-613.

Kaysen, G.A. (1991). Hyperlipidemia of nephrotic syndrome. *Kidney Int*, vol. 39, pp. S8-S15.

Kaysen, G.A. (1994). Non-renal complications of the nephrotic syndrome. *Ann Rev Med*, vol. 45, pp. 201-210.

Keane, W.F. and Kasiske, B.L. (1990). Hyperlipidemia in the nephrotic syndrome. *N Eng J Med*, vol. 323, pp. 603-604.

Keane, W.F., St Peter, J.V. and Kasiske, B.L. (1992). Is the aggressive management of hyperlipidemia in nephrotic syndrome mandatory? *Kidney Int*, vol. 42, Suppl. 38, pp. S134-S141.

Kemkes-Matthes, B. (1992). Acquired protein S deficiency. *Clin Investigator*, vol. 70, pp. 529-534.

Kilroe-Smith, T.A. and Gaillard, M.C. (1987). Functional Levels of α_2-Macroglobulin in Plasma in Relation to Emphysema. *J Clin Chem Clin Biochem*, vol. 25, pp. 859-863.

Kilroe-Smith, T.A., Becker, P.J. and Gaillard, M.C. (1991). Comparison of normal subjects and asthma patients with respect to age-related elastase-binding activity of α_2-macroglobulin in plasma. *Clin Chim Acta*, vol. 202, pp. 47-54.

Kilroe-Smith, T.A., Dowdeswell, R.J. and Gaillard, M.C. (1989). Elastase binding capacity of α_2-macroglobulin in plasma of patients with asthma or chronic obstructive pulmonary disease, without α_1-protease inhibitor deficiency. *Clin Chim Acta*, vol. 185, pp. 81-90.

Kincaid-Smith, P. (1967). Histological Diagnosis of Rejection of Renal Homografts in Man. *Lancet*, vol. 2, pp. 849-852.

Klatsky, A.L., Armstrong, M.A. and Friedman, G.D. (1991). Racial Differences in Cerebrovascular Disease Hospitalizations. *Stroke*, vol.22, pp. 299-304.

Koomans, H.A., Geers, A.B., Dorhout Mees, E.J. and Kortlandt, W. (1986). Lowered tissue-fluid oncotic pressure protects the blood volume in the nephrotic syndrome. *Nephron*, vol. 42, pp. 317-322.

Koomans, H.A., Geers, A.B., Meiracker, A.H., Roos, J.C., Boer, P. and Dorhout Mees, E.J. (1984). Effects of plasma volume expansion on renal salt handling in patients with nephrotic syndrome. *Am J Nephrol*, vol. 4, pp. 227-234.

Korbet, S.M. (1995). Management of idiopathic nephrosis in adults, including steroid-resistant nephrosis. *Curr Opinion Nephrol and Hypertension*, vol. 4, pp. 169-176.

Korbet, S.M., Schwartz, M.M. and Lewis, E.J. (1986). The prognosis of focal segmental glomerular sclerosis of adulthood. *Medicine*, vol. 65, pp. 304-311.

Kowal, R.C., Herz, J., Goldstein, J.L., Esser, V. and Brown, M.S. (1989). Low density lipoprotein receptor-related protein mediates uptake of cholesteryl esters from apolipoprotein E-enriched lipoproteins. *Proc Natl Acad Sci USA*, vol. 86, pp. 5810-5814.

Krachmalnicoff, A., Tombesi, S., Valsecchi, C., Albertini, A. and Mannucci, P.M. (1990). A monoclonal antibody to human protein S used as the capture antibody for measuring total protein S by enzyme immunoassay. *Clin Chem*, vol. 36, no. 1, pp. 43-46.

Krämer, A., Nauck, M., Pavenstädt, H., Schwedler, S., Wieland, H., Scholmeyer, P. and Wanner, C. (1993). Receptor-mediated uptake of IDL and LDL from nephrotic patients by glomerular epithelial cells. *Kidney Int*, vol. 44, pp. 1341-1351.

Krieger, M. and Herz, J. (1994). Structures and functions of multiligand lipoprotein receptors : Macrophage scavenger receptors and LDL receptor-related protein (LRP). *Ann Rev Biochem*, vol. 63, pp. 601-637.

Legrès, L.G., Pochon, F., Barray, M., Heinrich, P.C. and Delain, E. (1994). Human α_2-Macroglobulin as a Cytokine-Binding Plasma Protein. A study with rh-Interleukin-1β and rh-Interleukin-6. *Ann NY Acad Sci*, vol. 737, pp. 439-443.

Lerique, B., Moulin, B., Delpéro, C., Purgus, R., Olmer, M. and Boyer, J. (1994). Apolipoprotein E Phenotype and Hyperlipoproteinemia in Nephrotic Syndrome. *Clin Chem*, vol. 40, no. 5, pp. 849-850.

Leroy-Matheron, C., Gouwault-Heilmann, M., Aiach, M. and Gandrille, S. (1998). A Mutation of the Active Protein S Gene Leading to an EGF1-Lacking Protein in a Family With Qualitative (Type II) Deficiency. *Blood*, vol. 91, no. 12, pp. 4608-4615.

Liach, F. (1985). Hypercoagulability, renal vein thrombosis and other thrombotic complications of nephrotic syndrome. *Kidney Int*, vol. 2, pp. 4298-4309.

Lieberman, E., Henser, E., Gilchrist, G.S., Donnel, G.N. and Landing, B.H. (1968). Thrombosis, nephrosis and corticosteroid therapy. *J Pediat*, vol. 73, pp. 320-328.

Loktionov, A., Vorster, H., O'Neill, I.K., Nell, T., Bingham, S.A., Runswick, S.A. and Cummings, J.H. (1999). Apolipoprotein E and methylenetetrahydrofolate reductase genetic polymorphisms in relation to other risk factors for cardiovascular disease in UK Caucasians and Black South Africans. *Atherosclerosis*, vol. 145, pp. 125-135.

Lombard, E.H., Kromberg, J.G.R., Thomson, P.D., Milner, L.S., van Biljon, G. and Jenkins, T. (1989). Autosomal recessive polycystic kidney disease. *S Afr Med J*, vol. 76, pp. 321-323.

Lundwall, A., Dackowski, W., Cohen, E., Shaffer, M., Mahr, A., Dahlback, B., Stenflo, J. and Wydro, R. (1986). Isolation and the sequence of the cDNA for human protein S, a regulator of blood coagulation. *Proc Natl Acad Sci USA*, vol. 83, pp. 6716-6720.

Mahley, R.W. (1982). Atherogenic hyperlipoproteinemia: the cellular and molecular biology of plasma lipoproteins altered by dietary fat and cholesterol. *Med Clin N Am*, vol. 66, pp. 375-402.

Mahley, R.W. (1988). Apolipoprotein E : cholesterol transport protein with expanding role in cell biology. *Science*, vol. 240, pp. 622-630.

Mahley, R.W. and Innerarity, T.L. (1983). Lipoprotein receptors and cholesterol homeostasis. *Biochim Biophys Acta*, vol. 737, pp. 197-222.

Mahley, R.W., Ji, Z-S., Brecht, W.J., Miranda, R.D. and He, D. (1994). Role of Heparan Sulfate Proteoglycans and the LDL Receptor-Related Protein in Remnant Lipoprotein Metabolism. *Ann NY Acad Sci*, vol. 737, pp. 39-52.

Mansfield, L.E., Trygstad, C.W., Ajugwo, R.E. and Heiner, D.C. (1980). Serum concentrations of immunoglobulins E and G and α_2-macroglobulin in childhood renal disease. *J Allergy Clin Immunol*, vol. 66, no. 3, pp. 227-232.

Marchetti, G., Legnani, C., Patracchini, P., Gemmati, D., Ferrati, M., Plareti, G., Coccheri, S. and Bernardi, F. (1993). Study of a protein S gene polymorphism at DNA and mRNA level in a family with symptomatic protein S deficiency. *Br J Haematol*, vol. 85, pp. 173-175.

Marciniak, E. (1970). Coagulation inhibitor elicited by thrombin. *Science*, vol. 170, pp. 452-453.

Marsh, E.E., Biller, J., Adams, H.P. and Kaplan, J.M. (1991). Cerebral infarction in patients with nephrotic syndrome. *Stroke*, vol. 22, pp. 90-93.

Marsh, J.B. (1974). Lipoproteins in a nonrecirculating perfusate of rat liver. *J Lipid Res*, vol. 15, pp. 544-550.

Marsh, J.B. (1996). Lipoprotein Metabolism in Experimental Nephrosis. *P.S.E.B.M.*, vol. 213, pp. 178-186.

Martin, M., Hulley, S., Browner, W., Kuller, L. and Wentworth, D. (1986). Serum cholesterol, blood pressure and mortality : implications from a cohort of 361,662 men. *Lancet*, vol. 2, pp. 934-936.

Maschio, G., Oldrizzi, L., Lugiu, C. and Loschiavo, C. (1989). Serum lipids in patients with chronic renal failure on long-term, protein-restricted diets. *Am J Med*, vol. 87, Suppl 5N, pp. 51N-54N.

McCaffrey, T.A., Falcone, D.J., Borth, W. and Weksler, B.B. (1994). α_2-Macroglobulin/ Transforming Growth Factor-β_1 Interactions. Modulation by Heparin-like Molecules and effect on Vascular Smooth Muscle Cells. *Ann NY Acad Sci*, vol. 737, pp. 368-382.

McGinley, E., Lowe, G.D., Boulton Jones, M., Forbes, C.D. and Prentice, C.R. (1983). Blood viscosity and haemostasis in the nephrotic syndrome. *Thromb Haemost*, vol. 49, pp. 155-157.

Mehls, O., Andrassy, K., Koderisch, J., Hertzog, U. and Ritz, E. (1987). Hemostasis and thromboembolism in children with nephrotic syndrome : Differences from adults. *J Pediatr*, vol. 110, no. 6, pp. 862-867.

Melvin, and Bennett, W. (1991). Management of Nephrotic Syndrome in Childhood. *Drugs*, vol. 42, no. 1, pp. 30-51.

Mermod, J.J., Kruithof, E.K.O., Alouani, S., Quiquerez, A.L. and Sadoul, R. (1997). Apo E Genotype Does Not Affect Plasma tPA and PA1-1 Antigen Levels. *Am J Med Genet (Neuropsychiatric Genetics)*, vol. 74, pp. 172-175.

Moorhead, J.F., Chan, M.K., El-Nahas, M. and Varghese, Z. (1982). Lipid nephrotoxicity in chronic progressive glomerular and tubulointerstitial disease. *Lancet*, vol. 2, pp. 1309-1311.

Moorhead, J.F., Wheeler, D.C. and Fernando, R. (1989). Injury to rat mesangial cells in culture by low density lipoproteins (abstract). *Kidney Int*, vol. 35, pp. 433A.

Mulec, H., Johnson, S-A. and Björck, S. (1990). Relationship between serum cholesterol and diabetic nephropathy. *Lancet*, vol. 1, pp. 1537-1538.

Myburgh, J.A., Meyers, A.M., Botha, J.R., Thomson, P.D., Smit, J.A. and Lakier, R. (1987). Total lymphoid irradiation in clinical-kidney transplantation. *Clin Transplant*, vol. 1, pp. 65-70.

Narita, M., Rudolph, A.E., Miletich, J.P. and Schwartz, A.L. (1998). The Low-Density Lipoprotein Receptor-Related Protein (LRP) Mediates Clearance of Coagulation Factor Xa In Vivo. *Blood*, vol. 91, no. 2, pp. 555-560.

Newton, C.R., Graham, A., Hepitinstall, L.E., et al. (1989). Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res*, vol. 17, pp. 2503-2516.

Nishioka, J. and Suzuki, K. (1990). Inhibition of Cofactor Activity of Protein S by a Complex of Protein S and C4b-binding Protein. *J Biol Chem*, vol. 265, no. 16, pp. 9072-9076.

Norio, R. (1966). Heredity in the nephrotic syndrome. *Ann Paediatr Fenn*, vol. 12, Suppl 27, pp. 1-94.

Nykjaer, A., Bengtsson-Olivecrona, G., Lookene, A., Moestrup, S.K., Petersen, C.M., Weber, W., Beisiegel, U. and Gliemann, J. (1993). The α_2-macroglobulin receptor/low density lipoprotein receptor related protein binds lipoprotein lipase and β-migrating very low density lipoprotein associated with the lipase. *J Biol Chem*, vol. 268, pp. 15048-15055.

Obermoeller, L.M., Chen, Z., Schwartz, A.L. and Bev, G. (1998). Ca^{2+} and Receptor-associated Protein Are Independently Required for Proper Folding and Disulfide Bond Formation of the Low Density Lipoprotein Receptor-related Protein. *J Biol Chem*, vol. 273, no. 35, pp. 22374-22381.

Obermoeller, L.M., Warshawsky, I., Wardell, M.R. and Bu, G. (1997). Differential functions of triplicated repeats suggest two independent roles for the receptor-associated protein as a molecular chaperone. *J Biol Chem*, vol. 272, pp. 10761-10768.

Oda, H. and Keane, W.F. (1995). Lipids in progression of renal disease. *Kidney Int*, vol. 52, Suppl 62, pp. S36-S38.

Oda, H., Yoricka, N., Ueda, C., Kushihata, S. and Yamakido, M. (1999). Apolipoprotein E polymorphism and renal disease. *Kidney Int*, vol. 56, Suppl 71, pp. S25-S27.

Odegaard, B. and Mann, K. (1987). Proteolysis of factor Va by factor Xa and activated protein C. *J Biol Chem*, vol. 262, pp. 11233-11238.

Olaisen, B., Teisberg, P. and Gedde-Dahl, T. (Jr), (1982). The locus for apolipoprotein E (apo E) is linked to the complement component C_3 (C_3) locus on chromosome 19 in man. *Human Genet*, vol. 62, pp. 233-236.

Olivecrona, T., Liu, G., Hultin, M. and Bengtsson-Olivecrona, G. (1993). Regulation of lipoprotein lipase. *Biochem Soc Trans*, vol. 21, pp. 509-512.

Olson, J.L. and Heptinstall, R.H. (1988). Nonimmunologic mechanisms of glomerular injury. *Lab Invest*, vol. 59, pp. 564-578.

Ordoñez, J.D., Hiatt, R., Killebrew, E. and Fireman, B. (1990). The risk of coronary artery disease among patients with the nephrotic syndrome. *Kidney Int*, vol. 37, pp. 243A.

Ordoñez, J.D., Hiatt, R.A., Killebrew, E,J. and Fireman, B.H. (1993). The increased risk of coronary heart disease associated with nephrotic syndrome. *Kidney Int*, vol. 44, pp. 638-642.

Packard, C.J. and Shepherd, J. (1993). Lipoprotein metabolism in lipase deficient states : Studies in primary and secondary hyperlipidaemia. *Biochem Soc Trans*, vol. 21, pp. 503-506.

Paik, Y-K., Chang, D.J., Reardon, C.A., Davies, G.E., Mahley, R.W. and Taylor, J.M. (1985). Nucleotide sequence and structure of the human apolipoprotein E gene. *Proc Natl Acad Sci USA*, vol. 82, pp. 3445-3449.

Pei, Y., Cattran, D., Delmore, T., Katz, A., Lang, A. and Rance, P. (1987). Evidence suggesting under treatment in adults with idiopathic focal segmental glomerular sclerosis. *Am J Med*, vol. 82, pp. 938-944.

Perico, N. and Remuzzi, G. (1993). Edoema of the Nephrotic Syndrome: The Role of the Atrial Peptide System. *Am J Kid Dis*, vol. 22, no. 3, pp. 355-366.

Petäjä, J., Jalanko, H., Holmberg, C., Kinnunen, S. and Syrjälä, M. (1995). Resistance to activated protein C as an underlying cause of recurrent venous thrombosis during relapsing nephrotic syndrome. *J Pediatr*, vol. 127, pp. 103-105.

Ploos van Amstel, H.K., Huisman, M.V., Reitsma, P.H., Wouter ten Gate, J. and Bertina, R.M. (1989). Partial Protein S Gene Deletion in a Family with Hereditary Thrombophilia. *Blood*, vol. 73, no. 2, pp. 479-483.

Ploos van Amstel, H.K., Reitsma, P.H. and Bertina, R.M. (1988). The human protein S locus: identification of the PS α gene as a site of liver protein S messenger RNA synthesis. *Biochem Biophys Res Commun*, vol. 157, no. 3, pp. 1033-1038.

Poirier, J. (1994). ApolipoproteinE in animal models of CNS injury and in Alzheimer's disease. *TINS*, vol. 17, no. 2, pp. 525-530.

Poirier, J., Minnich, A. and Davignon, J. (1995). Apolipoprotein E, Synaptic Plasticity and Alzheimer's Disease. *Ann Med*, vol. 27, pp. 663-670.

Pontier, P.J. and Patel, T.G. (1994). Racial differences in the prevalence and presentation of glomerular disease in adults. *Clin Nephrol*, vol. 42, pp. 79-9\84.

Rabelink, A.J., Hene, R.J., Erkelens, D.W., Joles, J.A. and Koomans, H.A. (1990). Partial remission of nephrotic syndrome in patients on long-term simvastatin. *Lancet*, vol. 335, pp. 1045-1046.

Rall, S.C. (Jr), Weisgraber, K.H. and Mahley, R.W. (1982). Human apolipoprotein E. The complete amino acid sequence. *J Biol Chem*, vol. 257, pp. 4171-4178.

Rao, N.R., Bhat, P.G. and Pattabiraman, T.N. (1984). Estimation of serum alpha 2-macroglobulin based on the esterolytic activity of bound alpha-chymotrypsin. *Biochem Med*, vol. 32, pp. 357-363.

Rapola, J., Sariola, H. and Ekblom, P. (1984). Pathology of fetal congenital nephrosis : Immunohisto-chemical and ultrastructural studies. *Kidney Int*, vol. 25, no. 4, pp. 701-707.

Rebeck, G.W., Hart, S.D., Strickland, D.K. and Hyman, B.T. (1995). Multiple, Diverse Senile Plaque-associated Proteins are Ligands of an Apolipoprotein E Receptor, the α_2-Macroglobulin Receptor/Low-Density-Lipoprotein-Receptor-related Protein. *Ann Neurol*, vol. 37, no. 2, pp. 211-217.

Ridker, P.M., Vaughan, D.E., Stampfer, M.J., Manson, J.E. and Hennekens, C.H. (1993). Endogenous tissue-type plasminogen activator and risk of myocardial infarction. *Lancet*, vol. 341, pp. 1165-1168.

Robertson, S., Isles, C. and More, I. (1995). Glomerular disease made easier: 1 (Review). *Brit J Hosp Med*, vol. 53, no. 6, pp. 261-266.

Robson, Wm. L. M. and Leung, A.K.C. (1993). Nephrotic Syndrome in Childhood. *In Advances in Pediatrics*, vol. 40, pp. 287-323.

Saeki, T., Hirose, S., Nukatsuda, M., Kusunokiy, and Nagasawa, S. (1989). Evidence that C4b-binding protein is an acute phase protein. *Biochem Biophys Res Commun*, vol. 164, pp. 1446-1451.

Sakata, Y., Loskutoff, D.J., Gladson, C.L., Hekman, C.M. and Griffin, J.H. (1986). Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. *Blood*, vol. 68, pp. 1218-1223.

Salomaa, V., Stinson, V., Kark, J.D., Folsom, A.R., Davis, C.E. and Wu, K.K. (1995). Association of fibrinolytic paramaters with early atherosclerosis. The ARIC Study. Atherosclerosis Risk in Communities Study. *Circulation*, vol. 91, pp. 284-290.

Sandholzer, C., Delport, R., Vermaak, H. and Utermann, G. (1995). High frequency of the apo ∈4 allele in Khoi San from South Africa. *Hum Genet*, vol. 95, pp. 46-48.

Savin, V.J., Sharma, R., Sharma, M., McCarthy, E.T., Swan, S.K., Ellis, E., Lovell, H., Warady, B., Gunwar, S., Chonko, A.M., Artero, M. and Vincenti, F. (1996). Circulating Factor Associated With Increased Glomerular Permeability to Albumin in Recurrent Focal Segmental Glomerulosclerosis. *N Engl J Med*, vol. 334, no. 14, pp. 878-883.

Schafer, A.J. (1994). Hypercoagulable status: molecular genetics to clinical practice. *Lancet*, vol. 344, pp. 1739-1742.

Schmidel, D.K., Tatro, A.V., Phelps, L.G., Tomczak, J.A. and Long, G.L. (1990). Organization of the Human Protein S Genes. *Biochem*, vol. 29, pp. 7845-7852.

Schnaper, H.W. (1995). Balance between matrix synthesis and degradation: a determinant of glomerulosclerosis. *Pediatr Nephrol*, vol. 9, pp. 104-111.

Schneider, W.J., Kovanen, P.T., Brown, M.S., Goldstein, J.L., Utermann, G., Weber, W., Havel, R.J., Kotite, L., Kane, J.P., Innerarity, T.L. and Mahley, R.W. (1981). Familial dysbetalipoproteinemia : abnormal binding of mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits and cows. *J Clin Invest*, vol. 68, pp. 1075-1085.

Schönholzer, K.W., Waldron, M. and Magil, A.B. (1992). Intra-glomerular foam cells and human focal glomerulosclerosis. *Nephron*, vol. 62, pp. 130-136.

Schwarz, H.P., Heeb, M.J., Wendel-Drake, J.D. and Griffin, J.H. (1985). Identification and quantitation of protein S in human platelets. *Blood*, vol. 69, pp. 231-237.

Shafrir, E., Levy, E. and Deckelbaum, R. (1990). Urinary Excretion of Apolipoproteins Bound to HDL-Like Particles in Rat Nephrotic Syndrome and their Relation to Plasma HDL. *Nephron*, vol. 56, pp. 24-29.

Shanbhag, V.P., Stigbrand, T. and Jeusen, P.E.H. (1997). The contact zones in humen α_2-macroglobulin. Functional domains important for the regulation of the trapping mechanism. *Eur J Biochem*, vol. 244, pp. 694-699.

Sherill, B.C., Innerarity, T.L. and Mahley, R.W. (1980). Rapid hepatic clearance of the canine lipoproteins containing only the E apoprotein by a high affinity receptor. *J Biol Chem*, vol. 255, pp. 1804-1807.

Siddiqi, F.A., Tepler, J. and Fantini, G.A. (1997). Acquired protein S and antithrombin III deficiency caused by nephrotic syndrome: An unusual cause of graft thrombosis. *J Vasc Surg*, vol. 25, no. 3, pp. 576-580.

Solymoss, S., Tucker, M.M. and Tracy, P.B. (1988). Kinetics of inactivation of membrane-bound factor Va by activated protein C. Protein S modulates factor Xa protection. *J Biol Chem*, vol. 263, pp. 14884-14890.

Sottrup-Jensen, L. (1987). α_2M and related thiol ester plasma proteins. In *Plasma Proteins*. (Putnam. F,W, ed.) pp. 191-291. Academic Press, New York.

Sottrup-Jensen, L., Stetanik, T.M. and Kristensen, T. et al. (1984). Primary structure of human α_2-M. *J Biol Chem*, vol. 259, pp. 8318-8327.

Sparks, C.E. and Marsh, J.B. (1981). Metabolic heterogeneity of apolipoprotein B in the rat. *J Lipid Res*, vol. 22, pp. 519-527.

Srivastava, R.N., Agarwal, R.K., Moudgil, A. and Bhuyan, U.N. (1986). Late resistance to corticosteroids in nephrotic syndrome. *J Pediatr*, vol. 108, pp. 66-70.

Stenflo, J. (1976). A new vitamin K-dependent protein. *J Biol Chem*, vol. 251, pp. 225-263.

Stenflo, J., Lundwall, A. and Dahlback, B. (1987). Beta-hydroxyaspargine in domains homologous to the epidermal growth factor precursor in vitamin K-dependent protein S. *Proc Natl Acad Sci USA*, vol. 84, pp. 368-372.

Stenvinkel, P., Berglund, L., Heimbürger, O., Pettersson, E. and Alvestrand, A. (1993). Lipoprotein (a) in nephrotic syndrome. *Kidney Int*, vol. 44, pp. 1116-1123.

Stern, D.M., Nawroth, P.P., Harris, K. and Esmon, C.T. (1986). Cultured bovine aortic endothelial cells promote activated protein C-protein S-mediated inactivation of factor Va. *J Biol Chem*, vol. 261, pp. 713.

Stewart, J.E., Skinner, E.R. and Best, P.V. (1998). Receptor binding of an apolipoprotein E-rich subfraction of high density lipoprotein to rat and human brain membranes. *Int J Biochem Cell Biol*, vol. 30, pp. 407-415.

Stouffer, G.A., La Marre, J., Gonias, S.L. and Owens, G.K. (1993). Activated α_2-macroglobulin and transforming growth factor-β_1 induce a synergistic smooth muscle cell proliferative response. *J Biol Chem*, vol. 268, pp. 18340-18344.

Strauss, J., Zilleruelo, G., Freundlich, M. and Abitbol, C. (1987). Less commonly recognized features of childhood nephrotic syndrome. *Pediatr Clin North Am*, vol. 34, pp. 591-607.

Strittmatter, W.J., Weisgraber, K.H., Huang, D.Y., Dong, L-M., Salvesen, G.S., Pericak-Vance, M., Schmeckel, D., Saunders, A.M., Goldgaber, D. and Roses, A.D. (1993). Binding of human apolipoprotein E to synthetic amyloid β peptide : isoform-specific effects and implications for late-onset Alzheimer disease. *Proc Natl Acad Sci USA*, vol. 90, pp. 8098-8102.

Sugo, T., Dahlback, B., Holmgren, A. and Stenflo, J. (1986). Calcium binding of bovine protein S. Effect of thrombin cleavage and removal of the gamma carboxyglutamic acid-containing region. *J Biol Chem*, vol. 261, pp. 5116-5120.

Sudhof, T.C., Goldsstein, J.L., Brown, M.S. and Russel, D.W. (1985). The LDL receptor gene: a mosaic of exons shared with different proteins. *Science*, vol. 228, pp. 815-822.

Takeda, A., Matsutani, H., Nümura, F. and Ohgushi, H. (1996). Risk factors for relapse in childhood nephrotic syndrome. *Pediatr Nephrol*, vol. 10, pp. 740-741.

Thabet, M.A.E.H., Salcedo, J.R. and Chan, J.C.M. (1993). Hyperlipidermia in childhood nephrotic syndrome. *Pediatr Nephrol*, vol. 7, pp. 559-566.

Thaler, E., Balzar, E., Kopsa, H. and Pinggera, W.F. (1978). Acquired Antithrombin III Deficiency in Patients with Glomerular Proteinuria. *Haemostasis*, vol. 7, pp. 257-272.

Thomson, C., Forbes, C.D., Prentice, C.R.M. and Kennedy, A.C. (1987). Changes in blood coagulation and fibrinolysis in the nephrotic syndrome. *Clin Nephrol*, vol. 27, pp. 182-188.

Thomson, P.D. (1997). Renal problems in black South African children. *Pediatr Nephrol*, vol. 11, pp. 508-512.

Thomson, P.D. (2000). Glomerulonephritis in children: A South African perspective. *Specialist Medicine*, vol 22, no. 1, pp. 20-32.

Tryggvason, K. (1993). Biochemistry and Genetic Diseases of Glomerular Basement Membrane. *Seminars in Nephrol*, vol. 13, no. 5, pp. 447-456.

Tsuchida, A., Thomson, N.M., Salem, H.H., Atkins, R.C. and Hancock, W.W. (1990). Serial Monitoring Shows Plasma Protein C and Free Protein S Levels Are Decreased During Human Acute Renal Allograft Rejection. *Transplant Proc*, vol. 22, no. 5, pp. 2134-2136.

Tümer, N., Baskan, S., Arcosoy, A., Cavolar, A.O. and Ekim, M. (1989). Zinc metabolism in nephrotic syndrome. *Nephron*, vol. 52, pp. 95.

Usberti, M., Federico, S., Meccariello, S., Cianciaruso, B., Bailetta, M., Pecoraro, C., Sacca, L., Ungaro, B., Pisante, N. and Andreucci, V.E. (1984). Role of plasma vasopressin in the impairment of water excretion in nephrotic syndrome. *Kidney Int*, vol. 25, pp. 422-429.

Utermann, G. (1987). Apolipoprotein E polymorphism in health and disease. *Am Heart J*, vol. 113, pp. 433-440.

Utermann, G., Langenbeck, U., Beisiegel, U. and Weber, W. (1980). Genetics of apolipoprotein E system in man. *Am J Hum Genet*, vol. 32, pp. 339-347.

Utermann, G., Steinmetz, A. and Weber, W. (1982). Genetic control of human apolipoprotein E polymorphism: comparison of one- and two- dimensional techniques of isoprotein analysis. *Hum Genet*, vol. 60, pp. 344-351.

Van Leuven, F., Marynen, P., Sottrup-Jensen, L., Cassiman, J-J. and Van den Berghe, H. (1986). The receptor-binding domain of human alpha 2-macroglobulin. Isolation after limited proteolysis with a bacterial proteinase. *J Biol Chem*, vol. 261, no.24, pp. 11369-11373.

Van Leuven, F., Raymakers, S.L. and Hilliker, C. (1994). Structure of the Human α_2M Receptor Gene. *Ann NY Acad Sci*, vol. 737, pp. 502-503.

Vaziri, N.D., Gonzales, E.C., Shayestehfar, B. and Barton, C.H. (1994). Plasma levels and urinary excretion of fibrinolytic and protease inhibitory proteins in nephrotic syndrome. *J Lab Clin Med*, vol. 124, no. 1, pp. 118-124.

Velosa, J., Holley, K., Torres, V. and Offord, K. (1983). Significance of proteinuria on the outcome of renal function in patients with focal segmental glomerulosclerosis. *Mayo Clin Proc*, vol. 58, pp. 568-577.

Vigano-D'Angelo, S., D'Angelo, A., Kaufman, C.E., Sholer-Esmon, C.T. and Comp, P.C. (1987). Protein S Deficiency Occurs in the Nephrotic Syndrome. *Ann Int Med*, vol. 101, pp. 42-47.

Voet, D. and Voet, J.G. (1) Lipoproteins. In *Biochemistry*. Wiley, New York, pp. 304-310 and pp. 1087-1095.

Walker, F.J. (1980). The regulation of activated protein C by a new protein : A possible function for bovine protein S. *J Biol Chem*, vol. 255, pp. 5521-5524.

Walker, F.J. (1981). Regulation of activated protein C by protein S: The role of phospholipid in factor Va inactivation. *J Biol Chem*, vol. 256, pp. 11128-11131.

Walker, F.J. (1984). Regulation of vitamin K-dependent protein S: Inactivation by thrombin. *J Biol Chem*, vol. 259, pp. 10335-10339.

Walker, F.J. (1986). Properties of chemically modified protein S: Effect of the conversion of gamma-carboxy glutamic acid to gamma-methyleneglutamic acid on functional properties. *Biochem*, vol. 25, pp. 6305-6311.

Walker, F.J. (1989). Characterization of a peptide from the protein S sequence that inhibits the interaction between protein S and C4b-binding site. *J Biol Chem*, vol. 264, pp. 17645-17649.

Walker, F.J. (1992). Protein S and Thrombotic Disease (Minireview) *Proc Soc Exp biol Med*, vol. 200, pp. 285-295.

Walker, F.J., Chavin, S.I. and Fay, P.J. (1987). Inactivation of factor VIII by activated protein C and protein S. *Arch Biochem Biophys*, vol. 252, pp. 322-328.

Wardle, E.N. (1979). Nephrotic syndrome and cardiovascular disease. *Lancet*, vol. 2, pp. 1017.

Wass, V., Jarrett, R., Chilvers, C. and Cameron, J. (1979). Does the nephrotic syndrome increase the risk of cardiovascular disease? *Lancet*, vol. 2, pp. 664-667.

Wassermann, J., Santiago, A., Holthofer, H., Epstein, M. and Schlondorff, D. (1989). Interactins of low density lipoprotein with rat mesangial cells. *Kidney Int*, vol. 35, pp. 439.

Weinstein, R.E. and Walker, F.J. (1990). Enhancement of rabbit protein S anticoagulant cofactor activity in vivo by modulation of the protein S-C4b-binding protein interaction. *J Clin Invest*, vol. 86, pp. 1928-1935.

Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1982). Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J Biol Chem*, vol. 257, pp. 2518-2521.

Weisgraber, K.H., Innerarity, T.L., Harder, K.J., Mahley, R.W., Milne, R.W., Marcel, Y.L. and Sparrow, J.T. (1983). The receptor-binding domain of human apolipoprotein E: monoclonal antibody inhibition of binding. *J Biol Chem*, vol. 258, pp. 12348-12354.

Weisgraber, K.H., Newhouse, Y.M. and Mahley, R.W. (1988). Apolipoprotein E genotyping using polymerase chain reaction and allele-specific oligonucleotide probes. *Biochem Biophys Res Commun*, vol. 157, pp. 1212-1217.

Weisweiler, P., Jüngst, D. and Schwandt, P. (1983). Quantitation of Apolipoprotein E-isoforms in Diabetes Mellitus. *Horm. Metabol Res*, vol. 15, pp. 201.

Wenham, P.R., Newton, C.R. and Price, W.H. (1991). Analysis of Apolipoprotein E Genotypes by the Amplification Refractory Mutation System. *Clin Chem*, vol. 37, no. 2, pp. 241-244.

Wheeler, D.C. and Bernard, D.B. (1994). Lipid Abnormalities in the Nephrotic Syndrome: Causes, Consequences, and Treatment. *Am J Kidney diseases*, vol. 23, no. 3, pp. 331-346.

Wheeler, D.C., Varghese, Z. and Moorhead, J.F. (1989). Hyperlipidemia in nephrotic syndrome. *Am J Nephrol*, vol. 9, Suppl. 1, pp. 78-84.

White, R.H.R. (1973). The familial nephrotic syndrome. I. An European survey. *Clin Nephrol*, vol. 1, pp. 215-219.

Williams, S.E., Kounnas, M.Z., Argraves, K.M., Argraves, W.S. and Strickland, D.K. (1994). The α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor - Related Protein and the Receptor-Associated Protein. An Overview. *Ann NY Acad Sci*, vol. 737, pp. 1-13.

Willnow, T.E., Moehring, J.M., Inocencio, N.M., Moehring, T.J. and Herz, J. [1996(a)]. The low-density-lipoprotein receptor-related protein (LRP) is processed by furin in vivo and in vitro. *Biochem J*, vol. 313, pp. 71-76.

Willnow, T.E., Rohlmann, A., Horton, J., Otani, H., Braun, J.R., Hammer, R.E. and Herz, J. [1996(b)]. RAP, a specialized chaperone, prevents ligand-induced [illegible] retention and degradation of LDL receptor-related endocytic receptors. *EMBO J*, vol. 15, no. 11, pp. 2632-2639.

Wilson, C., Wardell, M.R., Weisgraber, K.H., Mahley, R.W. and Agard, D.A. (1991). 3-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science*, vol. 252, pp. 1817-1822.

Wilson, P.W., Meyers, R.H., Larson, M.G., Ordovas, J.M., Wolf, P.A. and Schaefer, E.J. (1994). Apolipoprotein E alleles, dyslipidemia, and coronary artery heart disease: Framingham Offspring study. *JAMA*, vol. 272, pp. 1666-1671.

Yamamura, T., Li-ming, D. and Yamamoto, A. (1992). Apolipoprotein E Polymorphism and Coronary Heart Disease. *Chinese Med J*, vol. 105, no. 9, pp. 738-741.

Yamamura, T., Yamamoto, A., Hiramori, K. and Nambu, S. [1984(a)]. A new isoform of apolipoprotein E - apo E-5 - associated with hyperlipidemia and atherosclerosis. *Atherosclerosis*, vol. 50, pp. 159-172.

Yamamura, T., Yamamoto, A., Sumiyoshi, T., Hiramori, K., Nishioeda, Y. and Nambu, S. [1984(b)]. New Mutants of Apolipoprotein E Associated with Atherosclerotic Diseases But Not to Type III Hyperlipoproteinemia. *J Clin Invest*, vol. 74, pp. 1229-1237.

Yamazaki, T., Hamaguchi, M., Katsumi, A., Kagami, K., Kojima, T., Takamatsu, J. and Sait Hidehiko. (1995). A Quantitative Protein S Deficiency Associated with a Novel Nonsense Mutation and Markedly Reduced Levels of Mutated mRNA. *Thromb Haemost*, vol. 74, no. 2, pp. 590-595.

Yermiahu, T., Shalev, H., Landau, D. and Dvilansky, A. (1996). Protein C and protein S in pediatric nephrotic patients. *SANGRE*, vol. 41, no. 2, pp. 155-157.

Zannis, V.I. and Breslow, J.L. (1981). Human very low density lipoprotein apolipoprotein E isoprotein polymorphism is explained by genetic variation and posttranslational modification. *Biochem*, vol. 20, pp. 1033-1041.

Zilleruelo, G., Hsia, S., Freundlich, M., Gorman, H. and Strauss, J. (1984). Persistance of serum lipid abnormalities in children with idiopathic nephrotic syndrome. *J Pediatr*, vol. 104, pp. 61-64.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Halkas

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 950612

PROJECT Alpha - 1 Protease inhibitor phenotypes and nephrotic syndrome

Extension also approved. 5/12/2000

INVESTIGATORS Dr A C Halkas

DEPARTMENT Paediatrics, Johannesburg Hospital

DATE CONSIDERED 950526

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 950531

CHAIRMAN .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached wher applicable.

c c Supervisor: Professor P D Thomson
Dept of Paed Nephrology, Johannesburg Hospital

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to t Secretary at Room 10001, 10th Floor, Senate House, Universit

I/we fully understand the conditions under which I am/we a authorized to carry out the abovementioned research and I/ guarantee to ensure compliance with these conditions. Should a departure to be contemplated from the research procedure approved I/we undertake to resubmit the protocol to the Committee

DATE 5/04/95 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES