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# Variants of alpha-1-proteinase inhibitor in black and white South African patients with focal glomerulosclerosis and minimal change nephrotic syndrome

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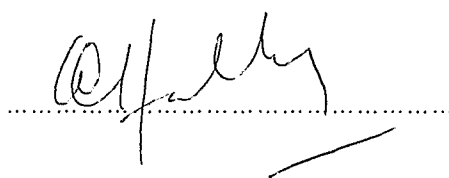
# VARIANTS OF ALPHA-1-PROTEINASE INHIBITOR IN BLACK AND WHITE SOUTH AFRICAN PATIENTS WITH FOCAL GLOMERULOSCLEROSIS AND MINIMAL CHANGE NEPHROTIC SYNDROME

ANDREW CHRISTOS HALKAS

A Research Report submitted to the Faculty of Health Sciences University of the Witwatersrand , Johannesburg in fulfillment of the requirements for the Master of Medicine (Paediatrics)

## DECLARATION

I hereby declare that this dissertation is my own work and has not been submitted or incorporated in another dissertation or thesis for any other degree.



Andrew Christos Halkas

# DEDICATION

To Androula and Christo

# ACKNOWLEDGEMENTS

I wish to thank my supervisor Prof. PD Thomson and Dr Christine Gaillard for their generous guidance and assistance.

## ABSTRACT

The objective of this study was to determine the prevalence and biochemical characteristics of certain alleles of alpha-1-proteinase inhibitor in black, coloured and white South African patients with two common types of pathology causing the nephrotic syndrome.

This was a cross sectional study of black, coloured and white patients with focal glomerulosclerosis (FGS) or minimal change disease (MCNS) and black, coloured and white control individuals.

There was a significant increase in the prevalence of the V allele in black patients with FGS (12%) as compared to black controls (1%) ($p=0.01$). There was also a significant decrease in the M1($Val^{213}$) allele of alpha-1-protease inhibitor in black and coloured patients with FGS (34%) as compared to black and coloured patients with MCNS (62%) ($p=0.04$). An increase in the prevalence of the S allele of alpha-1-proteinase inhibitor was found in white patients with FGS and MCNS (10%) as compared to white controls (2%)

The plasma elastase inhibitory capacity (EIC) associated with the phenotypes (PI) M1($Ala^{213}$) S, M1($Ala^{213}$)V and M1($Ala^{213}$) M1($Ala^{213}$) was significantly decreased as compared to the EIC associated with PI M1($Val^{213}$) M1($Val^{213}$). ($p=0.006$; $p=0.004$ and $p=0.025$ respectively). Twelve of the thirteen patients with FGS and infected with tuberculosis had either the M1($Ala^{213}$) , V or F alleles and required transplantation due to the severity of the disease. All of these patients were either black or coloured..

It is possible that the combination of functionally less efficient alpha-1-proteinase inhibitor and an inflammatory challenge associated with an infection such as tuberculosis could predispose black and coloured nephrotic patients to more aggressive scarring in FGS.

# LIST OF PUBLICATIONS

The following publication has arisen from material included in this research report.

Halkas AC, Gaillard MC, Thomson PD, Green SL, Ludewick H, Kala U. *Variants of alpha-1-proteinase inhibitor in* black and white South African patients with focal glomerulosclerosis and minimal change nephrotic syndrome. J Med Genet 1998; 35 6-9

Presented at the:11th International Congress of Clinical Chemistry London July 1996.

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# CHAPTER 1

## Introduction

Alpha-one-proteinase inhibitor ($\alpha_1$PI) is the major inhibitor of serine proteinases in human plasma. It is a glycoprotein consisting of a polypeptide chain of three hundred and ninety four residues and a carbohydrate content of twelve per cent and shows considerable genetic variation, more than 70 variants had been identified.[1] The inheritance of the allele follows an autosomal codominant pattern. The locus for $\alpha_1$-proteinase inhibitor is on chromosome 14 at 14q32.1 close to the locus for the protease inhibitor $\alpha_1$-antichymotrypsin in a cluster of sequence related genes that includes corticosteroid-binding globulin and protein C inhibitor.

### 1.1.$\alpha_1$-proteinase inhibitor variants

#### 1.1.1 M variant

The most common variant is type M that consists of at least six subtypes. M1($Val^{213}$),M1($Ala^{213}$),M2, M3, M4, AND M5. In serum, all these variants migrate in M region of a standard isoelectric focusing gel (centered at approximately a ph of 4.5) and are thus referred to as the common normal M family variants. When inherited in the homozygous form or

heterozygous form with each other the $\alpha_1$-proteinase inhibitor serum levels are 150 -300mg/dl. They do not all however act as normal inhibitors of neutrophil elastase. Although the M1Ala$^{213}$ alleles have no apparent effect on plasma concentration they result in reduced plasma elastase inhibitory capacity.

#### 1.1.1.1 M1(Val$^{213}$) and M1(Ala$^{213}$)

Among this group the most common variant is M1(Val$^{213}$). ( allele frequency in Caucasians in the United States). The next most common normal variant M1(Ala$^{213}$) was only recognised recently by sequencing at the DNA level[2] since the only difference between M1(Val$^{213}$) and M1(Ala$^{213}$) is a single amino acid that does not result in a difference in the net charge of the protein . The M1(Val$^{213}$) and M1(Ala$^{213}$) protein cannot be identified by isoelectric focusing even with the immobilised ph gradient. However the two genes can easily be identified by restriction fragment length polymorphic analysis since the restriction endonuclease BSTE11 recognises the sequence that defines residue 213 for the M1(Val$^{213}$) variant but does not cut the sequence for this region for M1(Ala$^{213}$) this difference can easily be identified by oligonucleotide analysis.

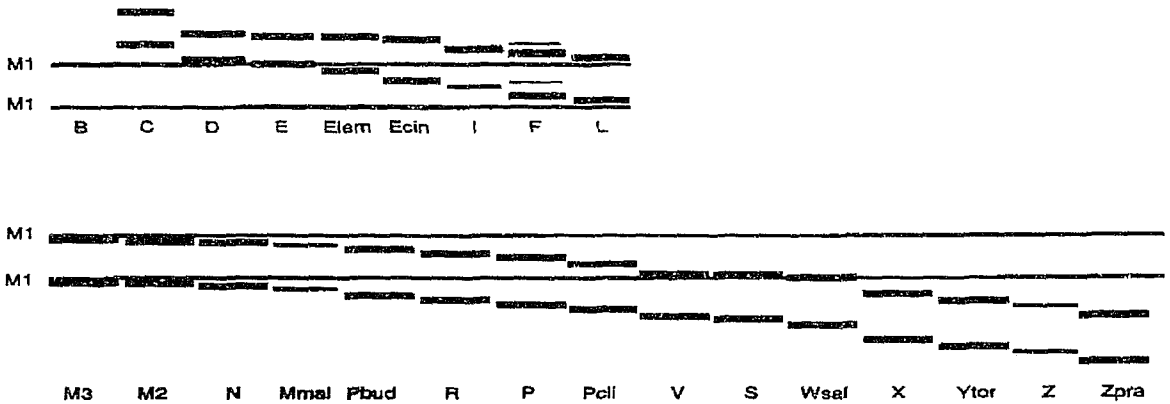


Diagram of selected anodal (top row) and cathodal (bottom row) PI variants as revealed by isoelectric focusing in polyacrylamide gel. The positions of the two major bands of M1 are indicated by the solid lines

### 1.1.1.2 M2 and M3 variants

The other, normal variants are M2 and M3. M3 differs from the M1(Val$^{213}$) sequence by a single base(GAA Glu$^{376}$ in M1(Val$^{213}$) to GAC Asp$^{376}$ in M3 The M2 sequence differs from the M3 sequence by an additional base change (CGT Arg$^{101}$ in M3 to CAT His$^{101}$ in M2. The M2 variant is more common than the M3 variant.

The M2 variant has recently been shown to be associated with asthma[3], and the prevalence of M1 Ala$^{213}$ variant has been found to be significantly increased in black South Africans as compared to whites. This variant was also shown to be associated with reduced plasma elastase inhibitory capacity and has been implicated as a contributing factor to the severity of asthma in black patients[4]. Atopy has been shown to be associated with steroid responsive nephrotic syndrome[5].

### 1.1.2 Z and S variants

The two most important variants of $\alpha_1$-proteinase inhibitor associated with deficient plasma levels are the Z and S variants.

1.1.2.1. Z variant

The Z variant is the classic $\alpha_1$-proteinase inhibitor deficient variant. When inherited in the homozygous form the allele results in serum levels of 15 to 50 micrograms/dl. The sequence of that exon of the Z gene is identical to that of M1 ($Ala^{213}$) except at the codon for residue 342 in exon V (M1 ($Ala^{213}$ GAG $Glu^{342}$ to Z AAG $Lys^{342}$) This causes the protein to migrate in isoelectric focusing gradients towards the cathode .In addition to been present in a reduced amount the Z protein does not function normally as an inhibitor of neutrophil elastase.

In this context, whereas the normal variants have association rate constants for neutrophil elastase in the range of 8 to 12 x $10^6$ $M^{-1}sec^{-1}$, the Z variant has an association rate constant of 3 to 6 x $10^5$ $M^{-1}$.This has profound consequences for the affected person.

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interaction between the reactive center loop of one molecule and the beta sheet of the second[6]. This phenomenon has been called loop-sheet polymerization. The loop-sheet polymerization interferes with the secretion of the molecule from the hepatocytes and macrophages and results in decreased plasma levels of 10-15 per cent of normal. The accumulation of $\alpha_1$- proteinase inhibitor within the hepatocyte is associated with neonatal hepatitis, juvenile cirrhosis[1] and hepatocellular carcinoma[7] and the lack of circulating inhibitor predisposes the Z homozygous patient to early onset panlobar emphysema.

The accumulation and degree of $\alpha_1$- proteinase inhibitor polymerization will depend on its rate of production and temperature. Polymerization is concentration and temperature dependent.

### 1.1.2.2 S variant

The S variant differs from M1 ($Val^{213}$) by the substitution of valine for glutamic acid at the 264 position.The substitution at $glu^{264}$ disrupts the salt bridge with residue 387 rendering the S protein less stable and thus more susceptible to intracellular proteolysis. This mutation

also leads to the formation of the loop sheet polymers although the effect is less marked and only reduces plasma $\alpha_1$-proteinase inhibitor concentrations to 60 per cent of normal.

The S variant is not associated with intracellular accumulation of $\alpha_1$-proteinase inhibitor.

The association of the Z and S $\alpha_1$-proteinase inhibitor deficiency alleles with inflammatory conditions such as membranoproliferative glomerulonephritis[8], rheumatoid arthritis[9] and panniculitis[10], vasculitis and asthma[3] implicate its role as an anti- inflammatory molecule.

### 1.2. $\alpha_1$-proteinase inhibitor gene

The gene for $\alpha_1$- proteinase inhibitor lies in close proximity to the gene for the cortisol binding globulin (CBG) on chromosome 14. The two genes lie so close to each other that the variants of $\alpha_1$-proteinase inhibitor could act as a marker for variants of the CBG gene which play an important role in the inflammatory response[11].

$\alpha_1$- proteinase inhibitor is an acute phase protein and plasma concentrations increase three to four fold due to inflammation or as a result of tissue injury. $\alpha_1$-proteinase inhibitor gene expression is controlled by a combination of 5′ and 3′ regulatory sequences and *trans*-acting nuclear proteins. Basal concentrations are probably maintained by the promoter and neighbouring sequences whereas the response during inflammation more than likely involves the use of enhancer elements. The TATA box is located at the nucleotide position -25 to -20 but no homology for an upstream CAAT consensus has been found. There is a GC rich sequence at -402 to -398, which could be a potential binding site for SPI. However this region is too far removed from the start site to be considered part of a classical promoter module.

The region 300 bp upstream from the transcription site contains two elements essential for transcription. A proximal element located at -137 to -37 appear to direct high-level tissue specific expression. This region is capable of activating other promoters in hepatic cells. It has two domains hepatocyte nuclear factor in one domain of the proximal element. Another protein hepatocyte nuclear factor 2 binds to a site in the second domain of

the proximal element. These transcription factors are believed to be tissue specific and as such are not found in comparable levels in brain or spleen cells.

A second distal enhancing element is located between nucleotide -261 and -210 that is capable of activating transcription but not in the tissue specific manner. This element contains sequences that are found in other plasma protein gene regulatory regions. At the centre of this region is a sequence similar to the core enhancer flanked on one side by an AP1 site and on the other by 11bp identical to that flanking the haptoglobin gene. Within this region there are also sites for the hepatocyte nuclear factors three and four as well as another liver nuclear protein (C/EBP)

Another enhancing element has been found in the 3′ flanking sequence located between 1.2 and 1.3 kb from the end of $\alpha_1$-proteinase inhibitor exon 5. Within this site there is the binding site for AP1 and C/EBP

## 1.3 Proteases and their interaction with $\alpha_1$-proteinase inhibitor

Neutral proteases secreted from monocytes and neutrophils especially elastase ,cathepsin G, collegenase and possibly plasminogen activator are potenty inhibited by $\alpha_1$-proteinase inhibitor not only in the circulation but most importantly in extravascular fluids where its low molecular weight allows it to diffuse . These enzymes are directly responsible for the majority of tissue destruction in inflammatory responses due to their effects on substances such as elastin, structural collagen, basement membrane, fibrin and fibronectin. They generate chemotactic factors from fibrin and complement and produce an enhancing factor of IgG and activate mediators such as complement, kininogen and angiotensinogen to activate lymphocytes and monocytes.

Protease inhibitors in general function by having a reactive center that acts as an ideal substrate. $\alpha_1$ proteinase inhibitor has a Met-Ser sequence at its reactive site that provides an optimal cleavage point for neutrophil elastase. In higher animals it is critical that the

inhibitor should not only complex rapidly with the target protease but also that the resultant complex should remain tightly bound in the circulation until it is catabolized.

### 1.3.1 Mechanism of action

Protease inhibition by a $\alpha_1$-proteinase inhibitor occurs by formation of a tightly bound 1:1 complex between $\alpha_1$-proteinase inhibitor and the target protease, which can be one of the number of serine proteases but is mainly elastase. Specificity is determined by crucial amino acids in the reactor site of $\alpha_1$-proteinase inhibitor. The methionine residue at position 358[12, 13] close to C terminus on the molecule is important for functional activity. The intact inhibitor has a site containing the methionine residue which appears to be exposed on the surface of the molecule in a loop formation, as proposed for the general mechanism for a protease inhibitor[14]. The loop fits precisely the conformation of the reactive site of the target protease.

Proteolytic cleavage at the reactive site causes release of the strand and its subsequent incorporation into a beta sheet[15]. The exposed portion of the reactive site allows ready access oxidation. Methionine can no longer physically complex with elastase and $\alpha_1$- proteinase inhibitor becomes inactive. There is a thousand fold decrease in the association constant for oxidized as compared with native $\alpha_1$-proteinase inhibitor. The oxidization of methionine may be advantageous in some situations for example for allowing local tissue breakdown in areas of inflammation, by a release of oxygen radicals from leukocytes[16].

### 1.3.2 Substrate specificity

The reactive sites of several of the serine protease inhibitors are similar to each other and also to those of low molecular weight plant proteases. Substrate specificity is determined by the composition of the reactive site. The specificity for methionine at amino acid 358 has been proven in a naturally occurring mutant of $\alpha_1$-proteinase inhibitor Pittsburgh in which arginine is substituted for

methionine at the active site[17]. The mutant loses its capacity to inhibit porcine pancreatic elastase and is a highly effective inhibitor of thrombin. The molecule has profound effects on the clotting system resulting in a bleeding diathesis.

It is well-known that the proteinase inhibitor Z in its homozygous form has been associated with the development of early onset emphysema in smokers owing to an inefficient antiproteinase defense mechanism in the lung. Similarly in the kidney serine proteinases released by polymorphonucleocytes can modify the glomerular basement membrane and have been shown to be directly involved in renal pathology as shown by the loss of laminin of the glomerular basement membrane in kidney treated with the elastase and cathepsin G[18]. Proteinases can cleave immunoglobulin G thus liberating the Fc fragment which can augment the lymphocyte response[19].

$\alpha_1$-proteinase inhibitor has an extraordinarily broad range of enzyme inhibitory activity exceeded in this only by $\alpha 2$ macroglobulin whose mechanism of action is unique in allowing it to inhibit the enzymes irrespective of class. It remains a difficult problem to know which of the

interactions of $\alpha_1$-proteinase inhibitor are physiologically important and which are purely in vitro effects. The situation is made more difficult by the fact that $\alpha_1$-proteinase inhibitor is present in such high plasma concentrations that shear quantity may well make up for the lower association constant. Additionally 55 per cent of $\alpha_1$-proteinase inhibitor is distributed in the extravascular compartment and in various secretions where other high molecular weight inhibitors are unable to enter. In such areas enzymes, which are normally preferentially inactivated by other inhibitors in the plasma, may be inhibited by $\alpha_1$-proteinase inhibitor.

## 1.4 $\alpha_1$-proteinase inhibitor and the immune system

Susceptibility to lung and liver destruction in $\alpha_1$-proteinase inhibitor deficiency seems to contribute to susceptibility to other disorders particularly those with inflammatory or immune components.

Membranoproliferative glomerulonephritis has been identified in PIZZ children who have also had liver

disease[8,20]. All the children in whom membranoproliferative glomerulonephritis was identified had severe liver disease suggesting that the kidney abnormality was a consequence of the liver disease. In a study of 246 Swedish PI Z patients 37 per cent showed signs of glomerular renal damage as indicated by constant proteinuria or hematuria[21].

An increase of proteinase inhibitor MZ heterozygotes were reported among patients with severe erosive rheumatoid arthritis[9]. A significantly increased frequency of PI type MZ was found in severely affected British children with juvenile rheumatoid arthritis[22].

Asthma is more prevalent in patients with the proteinase inhibitor MZ or MS phenotype than in other patients[23]. Deficiency types of $\alpha_1$-proteinase inhibitor may lead to more severe lung damage in asthmatics[24]. Anterior and posterior uveitis has been reported to be associated with an increased frequency of Z heterozygotes[24,25].

Panniculitis[10] and membranoproliferative glomerulonephritis[19,20] have occurred in association with proteinase inhibitor ZZ phenotype.

### 1.4.1 Lymphocyte function:

$\alpha_1$-proteinase inhibitor deficient subjects exhibit marked serum mediated enhancement of lymphocyte responsiveness to phytohemagglutinin (PHA) especially at suboptimal doses[26]. The addition of increasing amounts of $\alpha_1$-proteinase inhibitor results in an exponential decrease in the PHA response which reaches a plateau when the $\alpha_1$- proteinase inhibitor level in the serum supplement is increased to 2-4 grams per litre which is the normal physiological range.

The modulating effect of $\alpha_1$-proteinase inhibitor is important up to normal serum levels and only a reduction in $\alpha_1$-proteinase inhibitor levels below this range as occurs in $\alpha_1$-proteinase inhibitor deficient subjects results in increased lymphocyte activation. $\alpha_1$-proteinase inhibitor has a direct effect on adherent cells and no direct action on proliferating T cells. It probably acts by the inhibition of a membrane bound serine esterase, which is already active prior to the addition of PHA. The fact that $\alpha_1$-proteinase inhibitor does not completely inhibit PHA responsiveness but reduces it by 40 to 60 per cent indicates that it inhibits the production of a factor or

factors from adherent cells that enhance but are not absolutely essential for T cell proliferation. This would be consistent with an effect on interleukin one(IL-1) production.

It has been shown that preferential induction of interleukin one receptor antagonist (IL-Ra) by acute phase proteins such as $\alpha_1$-proteinase inhibitor may contribute to the anti-inflammatory effects and provide an important regulatory signal for the acute phase response[27]. Endogenously produced IL-1Ra may play an important role in regulating the effects of extracellular IL-1. IL-1Ra is important in the hosts response to infection or inflammation as a local regulator of the pathophysiological effect of IL-1 .The relative amounts of IL-1 and IL-1Ra in the microenvironment of cells in diseased tissues may influence the resultant degree of inflammation or tissue destruction. IL-Ra has been shown to reduce the severity of sepsis, arthritis, colitis, and other inflammatory processes in several animal models[28].

That these in vitro findings are of clinical relevance is further supported by in vivo studies in which $\alpha_1$-proteinase deficient subjects exhibit exaggerated cell mediated

immunity as manifest by marked acceleration of delayed hypersensitivity responses. Macrophages secrete a number of factors regulating the growth and differentiation of many other cell types. It seems possible that $\alpha_1$-proteinase inhibitor may also inhibit the production of at least some of these factors which could lead to alteration in growth and regulation of cells other than lymphocytes.

$\alpha_1$-proteinase inhibitor is able to modulate T cell proliferation due to its to effect on monocyte function. $\alpha_1$-proteinase inhibitor may also inhibit the serine esterase induced polyclonal activation and enhancement of the B cell function. An attractive but still unproven hypothesis would be that a deficiency of $\alpha_1$-proteinase inhibitor leads to increased T cell activity and polyclonal B cell activation thus leading to abnormalities in immunoregulation which may predispose $\alpha_1$-proteinase inhibitor deficient subjects to autoimmune disease.

Proteases have been shown in a number of studies to affect the immune response. Trypsin and chymotrysin have been shown to act as mitogens on B lymphocytes. Trypsin or

neutophil elastase and cathepsin G can substitute for helper T cells in B cell mitogen assays.

$\alpha_1$-proteinase has been reported to inhibit antibody dependent cell mediated cytotoxicity and natural killer activity.

### 1.4.2.Elastase and the immune system

Neutrophil elastase may impair bacterial opsonofication by cleaving the central component of the classic and alternative pathway C3[29]. Furthermore it may render the influxed neutrophils unable to be stimulated by immune complexes through the cleavage, of immunoglobulin in their hinge region, which deprives opsonic immunoglobulins of the Fc portion. Thus, the contact of complex immunoglobulins with Fc receptors on the neutrophil is prevented. Complement deposition on the $C_H2$ domain of Fc is no more possible.

Evidence that such cleavage actually occurs in vivo comes from the detection of the immunoglobulin fragments in

cystic fibrosis sputa and bronchial secretions. There are additional ways for extracellular neutrophil elastase to render neutrophil quiescent and inactivate the function of its mother cell. One way is to cleave the complement receptor for C3b on the neutrophil cell surface. Again this has not only been demonstrated in vitro but in vivo. In cystic fibrosis CR1 lacking neutrophils have been isolated from sputa. Opsononophagocytosis and killing of P.aeruginosa have been shown to be markedly impaired when neutrophils were pretreated with elastase[30]. It has been shown that CR3 on neutrophils was stable to elastase cleavage but that immuno complex deposited C3BI was labile[31].

$\alpha_1$-proteinase inhibitor modulating T cell-helper function and enhanced T cell helper activity may well predispose to immune disorders while an exaggerated inflammatory response may worsen its severity. Thus the thrust of all available evidence therefore suggests that this generalized hyper-responsiveness in $\alpha_1$-proteinase inhibitor deficiency may explain its many associated diseases.

## 1.5 The role of $\alpha_1$-proteinase inhibitor in the extracellular matrix

Glomerulosclerosis associated with nephrotic syndrome is primarily the result of extracellular matrix synthesis and degradation dysregulation. An imbalance of proteinases and their inhibitors may contribute to the sclerotic lesions that follow glomerular injury[18].

Cytokines and growth factors alter the production of proteases and protease inhibitors, which are important regulators of extracellular matrix(ECM) remodelling and normal cellular proliferation. The proteases include the serine proteases,matrix metalloproteinases and cysteine proteinases (cathepsin).The most important members of the family of serine proteases that are involved in ECM degradation are the plasminogen activators, leukocyte elastase and cathepsin G. Proteinases such as elastase are also effective in degrading type IV collagen of the basement membrane

The serine proteinases and MMPs and their inhibitors interact in a autocrine or paracrine fashion within the glomerulus.

Neutrophil elastase, cathepsin G and proteinase 3 are serine proteinases that are produced during neutrophil development in the bone marrow and stored in azurophilic granules of mature neutrophils. Once released into the extracellular environment neutrophil serine proteases may markedly affect the extracellular matrix and cells. Neutrophil serine proteases have broad substrate specificity and degrade a variety of extracellular matrix proteins including elastin collagen 1-4 fibronectin and proteoglycans. Elastase mediated cleavage of immunoglobulins complement C3bi and the complement receptor C3B results in decreased phagocytosis of pathogens[32].

Both elastase and cathepsin G impair T-cell function through cleavage of CD2 CD4 and CD8 on the surface of T cells. The enzymatic activity of released elastase, cathepsin G and Proteinase 3 is tightly regulated by an antiproteinase screen that is composed of $\alpha_1$- proteinase inhibitor ,ALP, elafin and $\alpha_2$ macroglobulin

Exposure to specific mediators may induce matrix accumulation . Increased concentrations of growth factors or cytokines and production of local mediators lead to

alterations in the local matrix composition. Intracellular mediators such as interleukin 1, epidermal growth factor and interleukin 6, which promote mesangial proliferation, may also stimulate matrix synthesis[11].

Accumulation of ECM components can occur by increased synthesis. Increased matrix production may result from increased transcription of matrix genes, enhanced longevity of mRNA for these proteins or increased translation.In general an increase in glomerular cell products including collagen types 4, 5, 6,laminin, fibronectin,tenascin, and heparin sulphate proteglycan is noted, whereas the interstitial collagen types 1 and 3 are less often found and then more in crescents. Alternatively matrix degradation could be decreased in sclerosis. Lesser amounts of proteolytic enzymes may be synthesized that may be present but inhibited or not activated or the accumulating matrix proteins may have qualitative changes that make them resistant to degradation by proteases.

Defensins affect the balance between neutrophil serine proteinases and their inhibitors, since they bind to members of the serpin family of proteinase inhibitors that includes $\alpha_1$- proteinase inhibitor.

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## 1.6 Aim

The previously recognized association of atopy with nephrotic syndrome, the possible role of $\alpha_1$-proteinase inhibitor in the regulation of elastase and the suggested association of $\alpha_1$-proteinase inhibitor deficiency and membranoproliferative glomerulonephritis prompted the present study.

# Chapter 2

# Materials and methods

## 2.1 Study sample

Sixty patients (age range 3-18 years old) at present being followed up at the Paediatric Nephrology unit Johannesburg and Baragwanath hospitals with a diagnosis of nephrotic syndrome. (Defined as oedema, plasma albumin <25 grams per litre and a urine protein /creatinine ratio >20 milligrams of protein per mmol of creatinine) caused by FGS or minimal change nephrotic syndrome were included in the study. The group consisted of 20 black patients with FGS (8 girls) 8 black patients with MCNS (three girls) 7 White patients with FGS (three girls) and 15 white patients with MCNS (five girls). There were five coloured patients (mixed race) with FGS (1 girl) and 5 coloured patients with minimal change nephrotic syndrome (1 girl).

One hundred and forty two people were used as controls (Age range 5 to 45 years) These consisted of 90 Whites (38 woman) and 52 blacks (18 women). Blood donors from the South African blood transfusion Service and patients at the

Paediatric Nephrology Clinic who did not have any disease process associated with the immune system were used as controls ,that is patients with anatomical abnormalities. Each subject gave informed consent to participate in the study, which was passed by the Committee for research on human subjects of the University of Witwatersrand.

The diagnosis of FGS was histologically proven in all patients. Juxtamedullary glomeruli were evaluated in all cases. Children with a secondary cause of proteinuria such as immune complex mediated glomerulonephritis, congenital and hereditary causes of nephrotic syndrome were excluded. Hepatitis B, C and HIV were also excluded. None of the patients had membranoproliferative glomerulonephritis . None of the children whose kidneys had been biopsied had histological evidence of renal tuberculous infection.

### 2.1.1 Medication

19 of the 32 of all FGS patients were on steroids. Nine were on high doses (2 milligrams per kilogram) and ten were on lower doses (0.2 milligrams per kilogram of prednisone). Among patients with FGS very few responded to steroid therapy. The majority of patients with minimal change

nephrotic syndrome (80 per cent) were on lower doses (less than 0.2 milligrams per kilogram) of prednisone . Most of the patients with MCNS were in remission at the time of this study.

## 2.2 Phenotype study

$\alpha_1$-proteinase inhibitor can be detected easily by isoelectric focusing. In deficiency states arising from the Z allele a single base change in the gene alters the codon for amino acid residue 342 from the acidic residue glutamate to the basic residue lysine. This replacement results in retarded migration of the $\alpha_1$- proteinase protein in an electric field.

Plasma was separated on the day of collection and stored at minus 60 degrees Celcius until analysed. Phenotypes were identified by isoelectric focusing on polyacrilamide gel according to the method of Constant[34] modified as follows. A gel 0.5 millimetres thick was prepared by dissolving 0 .9% grams of acrylamide, 0.03 grams of bisacrylamide 2.67 grams of sucrose in 19.3 milliliters of water. One milliliter of ampholine mixture (Pharmacia,Uppsala,Sweden) (ph4.2 to 4.9) was added followed by 30 µl TEMED

(tetramethylethylenediamine) and one milliliter of ammomnium persulphate (20 mg in 2ml of distilled water) . Electrode paper strips were soaked with 0,1M NaOH for the cathode and 0.04 M $H_3PO_4$ for the anode. The gel was prefocussed for one hour at 1,800 volts 14mA, 6 W. The samples were applied on paper inserts to be cathode end of the gel and were focused for 45 minutes. The paper inserts were then removed and the gel focused for further two hours. After the migration of the gel was stained in the 0.1 per cent solution of Coomassie Brilliant Blue and the phenotypes read.

### 2.2.1 Control serum.

Dr Diane Cox (University of Toronto, Canada) kindly supplied control serum for the V variants.(ref code C2906-3)

### 2.3 Biochemical study:

In contrast to the phenotype study, each blood sample was collected in a heparinized tube. Plasma was separated and assayed within 24 hours of venesection. Plasma concentrations of $\alpha_1$-proteinase inhibitor were determined by the antibody precipitation laser nephelometric method. The

following reagents were obtained from Hoescht: (Calbiochem Corp, San Diego,CA) the antibody to $\alpha_1$-proteinase inhibitor, the substrate for elastase (ie sucinyltrialanyl-p-nitroanilide (SAPNA)) and N-protein standard plasma.(i.e. the standard for $\alpha_1$-protease inhibitor determination). Porcine pancreatic elastase(PPE) was obtained from Boerhinger Mannheim. All other reagents were of analytical grade. (Merck Darmstadt, Germany)

## 2.4 Genotypic study

In addition to the extensive variation found in the protein by electrophoretic methods further variation is found in the DNA sequence as recognised by restriction enzymes producing restriction fragment length polymorphisms. Using cloned genomic sequence as probes numerous DNA polymorphisms have been found with a variety of restriction enzymes.

In DNA polymorphic analysis using PCR, two oligonucleotide primers are required which are complimentary to the flanking sequences of the target segment of DNA which is to be amplified. The primers direct repeated rounds of localized DNA replication to produce an exponential increase in the number of copies of the target sequence.

After 20 -30 rounds the target DNA constitutes the bulk of the DNA present. The amplified DNA fragment can then be digested with an appropriate restriction enzyme and the resulting fragments separated by gel electrophoresis and directly visualized under ultra violet light when stained with ethidium bromide

Genotype study

Genomic DNA was extracted from EDTA containing to whole blood according to the method of Higushi(1989)[35]. The polymerase chain reaction was used to amplify exon III of $\alpha_1$-proteinase inhibitor gene containing a BstEII restriction site. The following 30-mer primers were used (Schwartz et al, 1989)

153 5' -CCCACCTTCCCCCTCTCTCCAGGCAAATGGG-3'

155 5 -GGGCCTCAGTCCCAACATGGCTAAGAGGTG-3'

The amplified sequence is 360bp long. In addition to the polymorphic BstE11 site this sequence has an obligatory BstE11 site. The fragment will either be cut into two(310bp -50bp) or three smaller fragments(227bp-83bp-50bp) following digestion with the enzyme BstE II. Amplification was performed in a total volume of 50 ml containing one µg

of DNA.0.2mM each dATP dGTP,dCTP and dTTP (Boehringer Mannheim): 100pmol each oligonucleotide primer 10mM Tris/HCL buffer pH 8.3. 50mM KCL AND 1.5mM $MgCl_2$ (0.01% gelatine) The samples were processed in a Hybaid DNA thermal cycler. The first cycle was carried out at 90 degrees Celsius for one minute followed by a cooling step bringing this sample temperature down to 52 degrees Celsius before adding 1.5 units of Taq polymerase.(Promega USA) The samples were then processed through 90 cycles consisting of denaturation at 93 degrees Celsius for one minute ,annealing at 52 degrees Celsius for one minute and extension at 72 degrees Celsius for one minute the last step was one of extension at 72 degrees Celsius for five minutes.

Restriction analysis of the amplified sequence was performed as follows: 10 µl of the reaction mixture was digested with two units of the BstEII (Promega USA) Electrophoresis was carried out in a 8% polyacrilamide gel with the restricted and uncut samples in the adjacent lanes.

## 2.5 Elastase inhibitory capacity

The elastase inhibitory activity was determined according to the subject's phenotype.

A volume of a hundred µl of a one in 50 dilution of plasma in 0,1M Tris HCL Buffer was incubated with 100µl PPE (50µl/ml Tris-HCL buffer ph=8.6) solution for two minutes at 37 degrees Celsius to allow binding, followed by addition of a 100µl SAPNA (succinyltrialanyl-p-nitroanilide)(10mg of the substrate was dissolved in a minimum of dimethyl sulpoxide and then diluted to 8ml with Tris HCL buffer ph=8.8). After further incubation for two minutes at 37 degrees Celsius the reaction was stopped by adding one milliliter of 0.5 M of citric acid. Absorbance was read at 405 nanometres. The unbound elastase activity ($D_0$) was determined by substituting buffer for the plasma. The difference between $D_0$ and the readings of the samples allowed calculation of inhibition due to $\alpha_1$-proteinase inhibitor as KU (international kilo units) of elastase bound to $\alpha_1$-proteinase inhibitor per liter of plasma. Correction for the effect of $\alpha_2$- macroglobulin was made, by measuring the residual activity of the plasma after

treatment with sufficient elastase to react with all the elastase binders and followed by an excess of the exogenous inhibitor. This inhibitor was a semi- purified preparation of $\alpha_1$-proteinase inhibitor which was prepared from human plasma using precipitation with the $(NH_4)_2SO_4$ at a final concentration of 2M thus ensuring that the $\alpha_2$-macroglobulin had been completely removed.

The assays were done randomly on the following groups of children ; eight children (5 white controls and one black control and two white patients with minimal change nephrotic syndrome) with the $M1Val^{213}M1Val^{213}$ phenotype, nine children (five black controls and four black patients with FGS) with the $M1Val^{213}$ $M1Ala^{213}$ phenotype, four black patients with FGS with the M 1 $Ala^{213}M1Ala^{213}$ phenotype, five black patients with FGS with the M 1 $Ala^{213}$ V phenotype, and three white patients with minimal change nephrotic syndrome with the M 1 $Ala^{213}$ S phenotype. Most of the patients were undergoing steroid therapy but this did not have any effect on the elastase inhibitory capacity.

The purified protein derivative (PPD) obtained from the Statens serum Institute was used for evidence of exposure to tuberculosis.

## 2.6 Statistics

The comparison of the distribution of the different variants in the various populations was done by means of the two-tailed Fisher exact test. The biochemical characteristic of the variants was compared by means of the student t test.

# CHAPTER 3

# Results

## 3.1 Distribution of PI variants

The M1 $Ala^{213}$ variant occurred more frequently in the black control group than the white control group (p= 0.0001). Phenotypic identification by isoelectric focusing in polyacrilamide gel is shown in figure 1.$M_1S$, $M_1M_1$, $M_1V$ are shown on this amplified section of the gel. An example of the Bst E 11 polymorphism in exon 3 is given in figure 2. In this way M1 $Val^{213}$ M1 $Val^{213}$ homozygotes M1 $Val^{213}$ M1 $ALA^{213}$ heterozygotes and M1 $ALA^{213}$ M1 $ALA^{213}$ homozygotes could easily be identified.

The distribution of the PIs of $\alpha_1$-proteinase inhibitor in all nephrotic patients and controls are shown in tables one, two and three respectively. A significant increase in the prevalence of the V variants was found in the black patients with a FGS (12%) as compared to the black controls (1 per cent) (p= 0.01). Similarly in five coloured patients with in FGS, 20 per cent had the V variants. The V variant was not present in the five coloured patients with minimal

change nephrotic syndrome. An increase in S variant was found in the white patients with a FGS and minimal change nephrotic syndrome (10 per cent) as compared to the white controls (2 per cent) but did not reach significance.

### 3.2 Biochemical characteristics

Table four shows the biochemical characteristics of the five different phenotype groups of $\alpha_1$ -proteinase inhibitor. There was no difference in plasma concentration between the two groups. However the specific elastase inhibitory capacity was found to be significantly decreased in plasma of patients and controls containing protease inhibitor $M1Ala^{213}M1Val^{213}$ , M1 $Ala^{213}M1Ala^{213}$ and $M1Ala^{213}V$ as compared to the plasma containing protease inhibitor $M1Val^{213}$ $M1Val^{213}$ (P = 0.048, p= 0.025, p = 0.004, p =0.006 respectively).

### 3.3 Elastase inhibitory capacity in FGS and MCNS

Furthermore as shown in table five, patients with FGS have significantly reduced elastase inhibitory capacity as compared to patients with minimal change nephrotic syndrome or controls (p= 0.02 and 0.005 respectively).

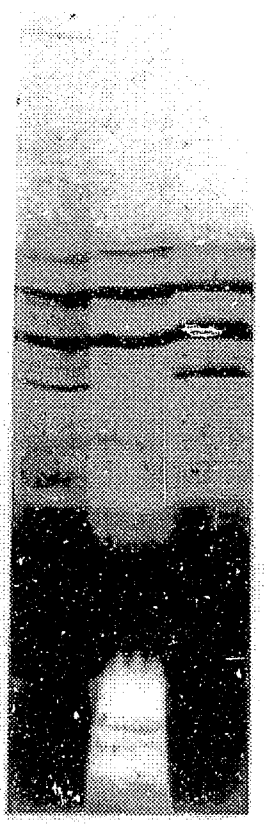


Fig1. Isoelectric focusing gel showing α1 AT variants including the V allele

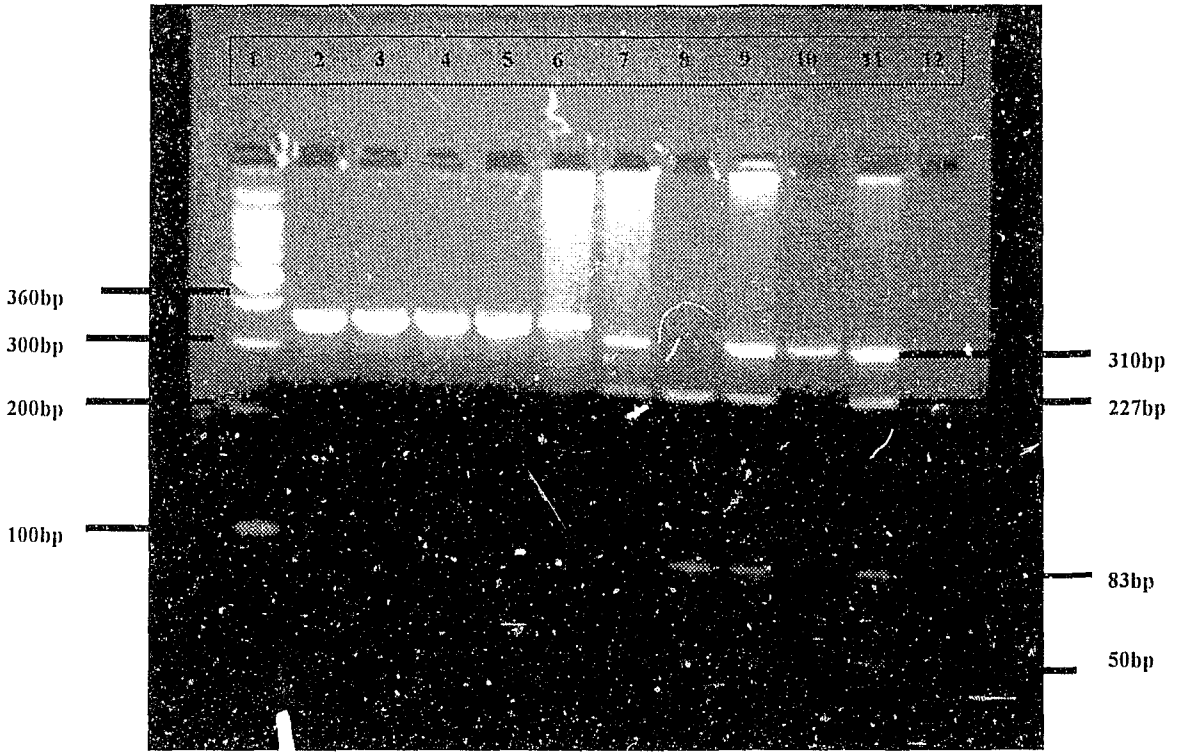


Figure2. DNA samples from different individuals haplotyped for the BstE11 polymorphism within exon 111 by digestion of the PCR product with Bst11 Lane 1 molecular weight markers lanes 7,9,11 restricted samples showing heterozygote ($ala^{213}$ $val^{213}$) lane 10 homozygote ($ala^{213}$ $ala^{213}$) and lane 8 homozygote ($val^{213}$ $val^{213}$). Lanes 2-5 show the corresponding undigested PCR products.

Of all the FGS patients, twelve out of the thirteen who required transplantation had previous exposure to tuberculosis (evidenced by PPD of greater than 15 millimeters on presentation of the nephrotic syndrome) all these were black or coloured patients. The phenotypes of these twelve patients consisted of six M 1 $Ala^{213}$ $M1Val^{213}$ four M 1 $Ala^{213}$ V and one M 1 $Ala^{213}$ F and one M 1 $Val^{213}$ $M1Val^{213}$ . The eight of the twelve black and coloured or white patients with FGS who had the M 1 $Ala^{213}$ V or S allele whose PPD was negative did not require a transplantation. In the patients who required transplantation and those who had not been followed over a similar period, the PPD positivity was not related to the duration of the nephrotic syndrome as it had been present at the onset of the disease.

Table 1. Frequency of $\alpha_1$PI variants in black, white and coloured patients with FGS.

| Variants | Blacks | Frequency | Whites | Frequency | Coloureds | Frequency |
|---|---|---|---|---|---|---|
| M1(Val$^{213}$) | 14 | 0.35 | 11 | 0.78 | 3 | 0.30 |
| M1(Ala$^{213}$) | 20 | 0.50 | 2 | 0.14 | 5 | 0.50 |
| V | 5 | 0.12 | | | 2 | 0.20 |
| F | 1 | 0.02 | | | | |
| S | | | 1 | 0.10 | | |
| TOTAL | 40 | | 14 | | 10 | |

1) Comparison of the frequency of the M1(Val$^{213}$) allele in black and coloured patients with FGS and black and coloured patients with MCNS by the 2-tailed Fisher's exact test shows a significant difference (p=0.04)

Table 2. Frequency of $\alpha_1$PI variants in black, white and coloured patients with MCNS.

| Variants | Blacks | Frequency | Whites | Frequency | Coloureds | Frequency |
|---|---|---|---|---|---|---|
| M1(Val$^{213}$) | 9 | 0.56 | 17 | 0.57 | 7 | 0.70 |
| M1(Ala$^{213}$) | 6 | 0.37 | 8 | 0.27 | 2 | 0.20 |
| M2 | | | 1 | 0.03 | 1 | 0.10 |
| M3 | | | 1 | 0.03 | | |
| V | 1 | 0.06 | | | | |
| S | | | 3 | 0.10 | | |
| TOTAL | 16 | | 30 | | 10 | |

2) Comparison of the frequency of the V allele in black and coloured patients with FGS and black and coloured patients with MCNS by the 2-tailed Fisher's exact test shows a significant difference (p=0.024)

Table 3. Frequency of $\alpha_1$PI variants in black and white controls.

| Variants | Blacks | Frequency | Whites | Frequency |
|---|---|---|---|---|
| M1(Val$^{213}$) | 46 | 0.44 | 128 | 0.70* |
| M1(Ala$^{213}$) | 55 | 0.52 | 34 | 0.20+ |
| M2 | 2 | 0.01 | 13 | 0.07 |
| V | 1 | 0.01 | | |
| S | | | 4 | 0.02 |
| Z | | | 1 | 0.01 |
| TOTAL | 104 | | 180 | |

* Comparison of M1(Val$^{213}$) in black controls vs white controls, p<0.0001

+ Comparison of M1(Ala$^{213}$) in black controls vs white controls, p<0.0001

Table 4. Biochemical characteristics of $\alpha_1$PI in the different phenotype groups.

| Phenotypes | n | Plasma level (g/L) | EIC* (KU/L) | Specific Activity (KU/g) | P value ** |
|---|---|---|---|---|---|
| M1(Val$^{213}$)M1(Val$^{213}$) | 8 | 1.44±0.015 | 47.99±4.05 | 33.76±2.93$^a$ | |
| M1(Val$^{213}$)M1(Ala$^{213}$) | 9 | 1.59±0.68 | 41.76±3.43 | 26.32±1.94$^b$ | 0.048 |
| M1(Ala$^{213}$)M1(Ala$^{213}$) | 4 | 1.41±0.197 | 30.77±7.73 | 21.19±3.21$^c$ | 0.025 |
| M1(Ala$^{213}$)V | 5 | 1.74±0.22 | 36.89±4.62 | 20.78±1.66$^d$ | 0.004 |
| M1(Ala$^{213}$)S | 3 | 1.30±0.14 | 34.82±5.95 | 20.63±2.07$^e$ | 0.006 |

mean values are mean±sem

*EIC=Elastase Inhibitory Capacity

**a versus b,c,d and e

Table 5 Comparisons of plasma elastase inhibitory capacity of $\alpha_1$PI in patients with FGS or MCNS and controls

| Group | no | Plasma elastase inhibitory capacity (KU/g) |
|---|---|---|
| Controls | 11 | 33.44(8.39) |
| Patients with FGS | 13 | 22.77(5.05) |
| Patients with MCNS | 5 | 32.51(7.75) |

Patients with FGS v controls p=0.005
Patients with FGS v patients with MCNS p=0.02

# CHAPTER 4

# DISCUSSION

FGS is found in 25-35% of black South African children with idiopathic nephrotic syndrome[36] [37]. FGS is the commonest cause of end stage renal disease (ESRD) in our population of patients The association between FGS in black South African children and tuberculosis has previously been reported [37].

Tuberculosis was found in 37.5% of children with FGS as compared to 6% in a group of children with MCNS, the latter being similar to the control population. In our previous study nine of the ten patients with FGS who required transplantation had evidence of infection by Mycobacterium tuberculosis. It has also been noted that black and hispanic children experience a disproportionate frequency of FGS with progression to ESRD suggesting that both genetic and environmental factors play a role[38].

$\alpha_1$-proteinase inhibitor is the major inhibitor of serine proteinases and plays a role in modulating interleukin-1 (IL-1) mediated inflammatory responses by inducing the

synthesis of IL-1 receptor antagonist[26]. It has been shown that $\alpha_1$-proteinase inhibitor can bind to extracellular matrix (ECM) in vitro and once bound retains 50% of its ability to inhibit elastase mediated ECM proteolysis[39].

The serine proteases elastase and plasminogen activators play an important role in remodeling of the kidney's ECM and may also play a role in the inflammatory response by augmenting the lymphocyte response.

In this study patients with the M1 Ala213 V and S alleles of the $\alpha_1$-proteinase inhibitor were found to have reduced EIC. Vaziri et al[40] reported a decreased $\alpha_1$-proteinase inhibitor activity in a group of nephrotic patients who also had markedly raised tissue type plasminogen activator levels. In this study we have found a significant increase in the prevalence of the V allele of $\alpha_1$-proteinase in black and coloured patients with FGS. 40% of the black patients who required transplantation were PI M1 $Ala^{213}$ V .The V allele was not found in any of the white groups but was found in two of the five coloured patients with FGS. Similarly an increase of the S allele was found in white patients with FGS and MCNS as compared to white controls.

This allele was not found in any of the other population groups.

It has recently been shown that the V allele results from Gly- Arg substitution at position 148[41]. The mutation is located at the lower pole of the protein in a poorly conserved region linking S1A with helix F in a region that is thought to be important in serpine proteinase complex formation. The replacement of a glycine residue with a large positively arginine in this negatively charged region may alter the structure of the molecule and could explain the reduced elastase inhibitory capacity of the V allele as shown in figure 3.

The V allele was first described on the M1 $Val^{213}$ base allele. It has now been described on the M1 $Ala^{213}$ allele and is associated with a silent mutation at position 158.

Its association with silent mutations and polymorphisms may be meaningful as the gene for $\alpha_1$-proteinase inhibitor lies in close proximity to the gene for cortisol binding globulin on chromosome 14 .The two genes lie so close to each other that variants of $\alpha_1$- proteinase inhibitor could

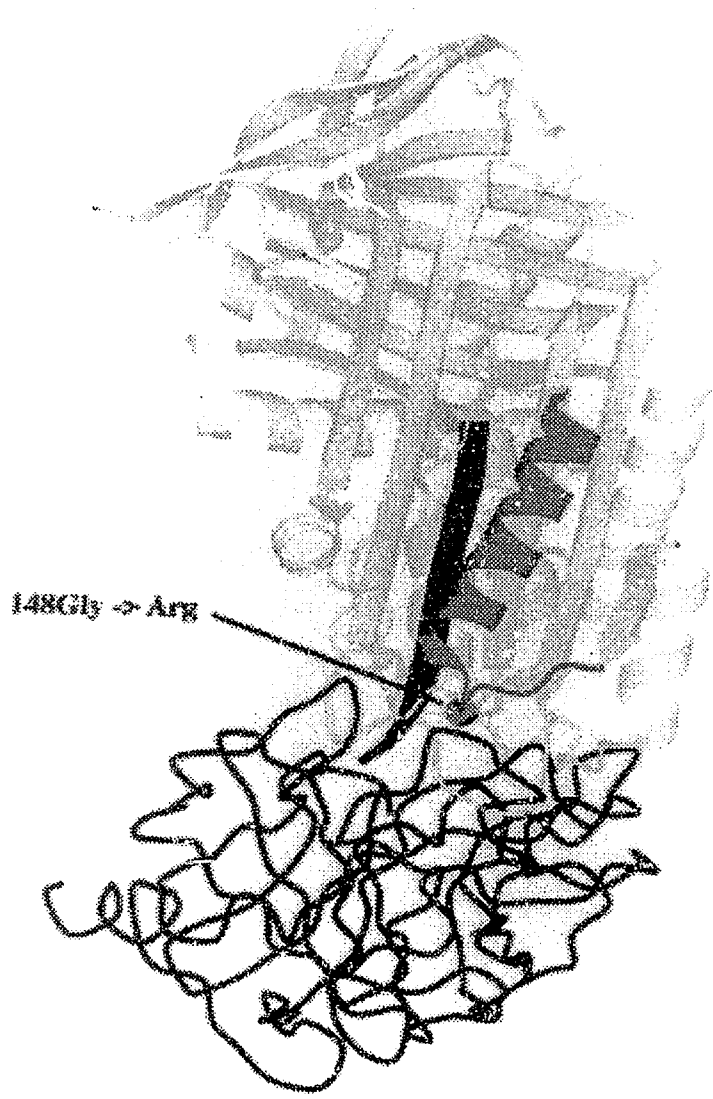


Fig3: The Gly-Arg substitution at position 148 on the quanternary structure of $\alpha_1$ PI V in a position between S1A and the F helix which may perturb the stability of the protease inhibitor complex

act as markers for variants of the CBG gene which plays an important role in the inflammatory response.

The PI MS has been associated with asthma[42] and a number of autoimmune diseases such as rheumatoid arthritis[9] systemic lupus erythymatosous and anterior uveitis[43]. These associations suggest that patients with these variants are subject to an enhanced immune response. An increased IL-2R expression in T lymphocytes in patients with MCNS has been observed suggesting an increased activation of T cells in these patients[44]. In vitro studies have shown a $\alpha_1$ proteinase inhibitor dose dependent inhibition of lymphocyte responsiveness to mitogens[45]. This could also play a role in our patients as shown by a decreased EIC which on the same basis should lead to an increased lymphocyte responsiveness.

The M1 (Ala $^{213}$) V phenotype was more prevalent in the black patients with FGS. It has previously been shown that the M1 Ala $^{213}$ allele is significantly increased in the black South African population and may contribute to the severity of asthma owing to a reduced EIC[4]. The black patients with FGS may therefore be predisposed to a more severe course of

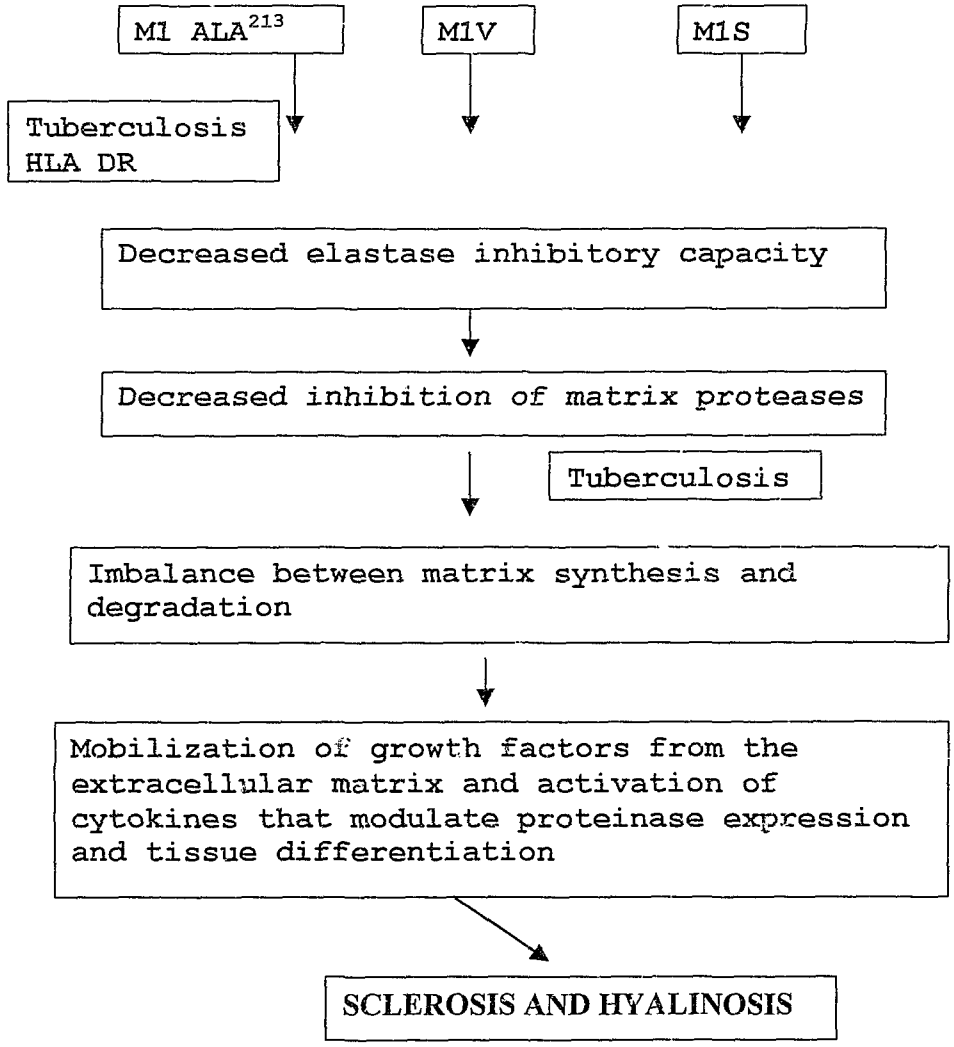


**Fig 4 .Proposed role of $\alpha_1$- proteinase inhibitor in the pathophysiology of focal glomerulosclerosis**

disease because of a decrease in the capacity to inhibit proteinases.

In our local experience FGS leading to end stage renal failure is far more prevalent in black patients often with previous exposure to tuberculosis (37%) than in white patients (6%)[46] Besides degrading ECM components extracellular proteinases may also contribute to mobilising growth factors and cytokines which further enhance the immune response. An increase in vivo elastase activity in subjects with $\alpha_1$- proteinase deficiency homozygous PIZ and heterozygous PIMS[47] has been noted.

In conclusion this study has shown that black and coloured nephrotic patients with FGS had an increased prevalence of the M1 Ala $^{213}$ V phenotype of $\alpha_1$-proteinase inhibitor and a corresponding decrease in EIC .It is possible that in the face of an inflammatory challenge such as infection with tuberculosis there may be diminished downregulation of proteinases and cytokines leading to dysregulation of the extracellular matrix resulting in scarring.

# CHAPTER 5

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