HOMOCYSTINURIA AND HYPERHOMOCYSTEINAEMIA IN THE WESTERN CAPE

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I declare that this thesis is my own work.

It is being submitted for the degree of

Doctor Technologiae (Biomedical Technology)

to the Cape Technikon, Cape Town.

It has not been submitted before for any degree or examination

at any other Technikon or tertiary institution.

The work was undertaken at the Division of Chemical Pathology,

University of Cape Town and Groote Schuur Hospital.

The opinions and the conclusions drawn are not necessarily

those of the Cape Technikon.

L Human ..... March 2002 .....

Lucille Human Date

I dedicate this thesis to my husband, Eben,

my two daughters, Christina and Karen

and my late brother, Gerhard.

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ABSTRACT

Research into the role of homocyst(e)ine in cellular functions was stimulated by homocystinuria, a severe autosomal recessive disorder caused by, in the classic case, deficiency of cystathionine β-synthase. Patients with homocystinuria have plasma homocyst(e)ine levels ten times that of reference values. This study was initiated with the presentation and investigation of a local family with clinical symptoms typical of that found in patients with homocystinuria. The free plasma homocystine level detected in the index case was 12 times higher and the plasma methionine level was a 1000 times higher than the respective normal reference ranges. The most common cause of homocystinuria worldwide is a defect in the cystathionine β-synthase enzyme. Methodology was developed to measure cystathionine β-synthase activity in fibroblast cultures obtained from skin biopsies from the extended family. A radioactive method followed by separation of the amino acids on an amino acid analyzer was used. Both the symptomatic siblings had cystathionine β-synthase enzyme activities <1% of the reference value, which was similar to activities found in known homozygotes for cystathionine β-synthase deficiency. Cystathionine β-synthase enzyme activity in the asymptomatic mother was in the lower half of the reference range while the father had cystathionine β-synthase enzyme activity well below the reference range at less than 10% of activity found in healthy individuals. On the basis of clinical symptoms and above parameters, homocystinuria due to cystathionine β-synthase deficiency was confirmed.

Molecular genetic investigations of the cystathionine β-synthase gene were performed on the symptomatic siblings and their parents, to detect the defect at the molecular level. Messenger RNA was extracted from the fibroblast cultures and reverse transcribed to complementary DNA. The cystathionine β-synthase gene was amplified using PCR. Each allele was then cloned and sequenced. The index case was confirmed to be a compound heterozygote for cystathionine β-synthase deficiency. The commonly found Celtic G to A transition at nucleotide 919 in exon 8 was detected on one allele and a C to a T point mutation at nucleotide 1058 in exon 10 was detected on the other allele. The C1058T (Thr353Met) mutation had not been reported before, but has subsequently been found in two other alleles. This amino acid change resulted in a change from a neutral polar threonine to a hydrophobic non-polar methionine. Both these mutations were also present in the symptomatic female sibling. The G919A (Gly307Ser) mutation was identified as originating from the father and the C1058T (Thr353Met) point mutation was identified as originating from the mother. Both parents were obligate heterozygotes for

cystathionine β-synthase deficiency with no clinical symptoms of homocystinuria. Initial detection of the G919A (Gly307Ser) point mutation was done by sequencing, but subsequent screening for this mutation was done by restriction enzyme methodology that was developed for this study in order to screen large numbers of patients rapidly. During this study two other unrelated families presented with clinical symptoms of homocystinuria. Amino acid analysis showed raised homocyst(e)ine and methionine values consistent with homocystinuria. Molecular investigations showed mutation T833C (Ile278Thr) on both cystathionine β-synthase alleles of the index case (F1086) from the one family and the index case (F1036) of the other family carried the T833C (Ile278Thr) and G1126T (Asp376Thr) mutations. The T833C (Ile278Thr) mutation is the second most commonly found mutation in the cystathionine β-synthase gene in populations residing in Europe. The nucleotide change at nucleotide 833 results in an amino acid change of a hydrophobic neutral isoleucine to a polar neutral threonine. The G1126T (Asp376Thr) mutation has not been reported before. This mutation results in a change of a negatively charged aspartate to a polar uncharged (neutral) threonine. This change is also in a highly conserved region of the cystathionine β-synthase gene. All four mutations were also in close proximity to the pyridoxyl 5'-phosphate binding site on the cystathionine β-synthase gene.

During this study there was a growing interest worldwide in marginally raised plasma homocyst(e)ine levels, a condition known as hyperhomocysteinaemia, as a risk factor for the development of several conditions with vascular implications. Accurate analytical procedures for the measurement of free plasma homocystine have been problematic due to redistribution between free and bound forms of homocyst(e)ine and the very low levels of circulating free homocystine. Total plasma homocysteine is approximately ten times higher than free homocystine. Existing methodology was adapted to establish a method for the measurement of total plasma homocysteine. Comparative results between free and total plasma homocyst(e)ine obtained in this study showed that total plasma homocysteine measurement reflected free homocystine measurement with no loss of additional information. Several investigators have used oral methionine to further emphasize small differences between reference ranges and hyperhomocysteinaemia. Homocyst(e)ine analysis on blood collected at 2 hourly intervals for 24 hours after oral methionine showed that homocyst(e)ine values reached peak levels at 6 hours after oral methionine. Through this study the use of oral methionine as part of measuring total plasma homocysteine was successfully introduced in the wards at Groote Schuur Hospital and various other institutions in the Western Cape.

The incidence of hyperhomocysteinaemia in the Western Cape was unknown and its relationship to vascular disease is uncertain. In this study the relationship between hyperhomocysteinaemia and the disease states; abruptio placentae, pre-eclampsia and peripheral vascular disease in the Western Cape was investigated. A significant increase in total plasma homocysteine values in patients from the abruptio placentae, pre-eclampsia and peripheral vascular disease groups was demonstrated for this study. Folate treatment for 6 weeks in patients with peripheral disease significantly lowered total plasma homocysteine values after oral methionine. No significant difference existed in total plasma homocysteine values between race groups or between men and females.

There are several factors that are known to affect homocyst(e)ine levels in the normal population namely age, sex, smoking, pregnancy, vitamin deficiency, and genetic problems such as heterozygosity for defects in the cystathionine β-synthase gene. Heterozygosity for defects in the cystathionine β-synthase gene has been well documented in Europe and an incidence of as high as 1:100, has been reported. The incidence of hyperhomocystinaemia in the Western Cape is unknown. Patients with hyperhomocysteinaemia were investigated for cystathionine β-synthase activity and then screened for possible "common" mutations in the cystathionine β-synthase gene found in the local homocystinuric familes. Cystathionine β-synthase activity can be measured in either fibroblast or lymphoblast cultures. Lymphobast cultures are easier to establish as it only requires a blood sample whilst establishment of fibroblast cultures require a skin biopsy. In addition lymphoblast cultures are more cost effective. The cystathionine β-synthase enzyme assay was adapted for measurement in cultured lymphoblasts. Cystathionine β-synthase activity in lymphoblast cultures of patients were found to be the same as the cystathionine β-synthase activity found in lymphoblast cultures of the control group, indicating the cause for the hyperhomocysteinaemia found in these patients was not heterozygosity for a defect in the cystathionine β-synthase enzyme. The cystathionine β-synthase gene was also screened for the G919A (Gly307Ser) mutation commonly found in patients with homocystinuria. The G919A (Gly307Ser) mutation was not detected in any of the individuals from the abruptio placentae study group. The other hyperhomocysteinaemia study groups were not investigated for this mutation.

As no association between a defect in the transsulphuration pathway of the methyl cycle and hyperhomocysteinaemia could be found, the re-methylation pathway was investigated in order to find a cause for the hyperhomocysteinaemia present in the vascular disease groups. A deficiency of the enzyme, methylenetetrahydrofolate reductase is one of the established causes for defective remethylation of homocyst(e)ine. This can cause an

increase in plasma homocyst(e)ine and can be due to insufficient dietary folate or a genetic defect in the enzyme. Previous investigators have linked the C677T mutation in the methylenetetrahydrofolate reductase gene with hyperhomocysteinaemia. Restriction enzyme analysis performed on PCR products from genomic DNA of individuals from the abruptio placentae, pre-eclampsia and peripheral vascular disease study groups showed that no difference in C677T genotype allele frequency existed between control and disease subjects of the abruptio placentae and pre-eclampsia study groups. Homozygosity for the C677T variant was however detected in patients with peripheral vascular disease. Total plasma homocysteine was higher than normal in patients homozygous for the C677T genotype. Ethnic diversity was demonstrated by the C677T genotype frequency of 34% in the Caucasian population with peripheral vascular disease, compared to 17.9% in the Coloured population and 4.4% in the African population.

The first part of the study concerning hyperhomocysteinaemia has confirmed a significant link between increased homocyst(e)ine and vascular disease, however no definite genetic link could be established as to the cause of hyperhomocysteinaemia. The second part of this study deals with the possible association between hypervariable markers in the fibrillin and elastin genes and hyperhomocysteinaemia and vascular disease. If hyperhomocysteinaemia is the cause of the vascular damage in these patients, then could there be a genetic variation in the genes coding for proteins found in vascular structures, that make these patients more susceptible to disease in the presence of hyperhomocysteinaemia? Previous investigators have implicated hyperhomocysteinaemia in hypertension. It is thought that abnormally increased homocyst(e)ine may contribute causality to atherosclerotic lesions independent from lipoproteins. In high blood pressure, the walls of the artery become stiff and inflexible, making the walls of the artery more susceptible to attack by sulphydryl compounds such as homocyst(e)ine. Fibrillin and elastin are an integral part of matrix structure and are found in cell walls. Genetic variations found in the fibrillin and elastin genes have been implicated in Marfan's syndrome, an autosomal dominant disorder of connective tissue with cardiovascular manifestations, supravascular aortic stenosis and Williams' syndrome respectively. Powell *et al.* (1996) reported that variations at the fibrillin-1 locus (Marfan gene) could have an effect on aortic elasticity, blood pressure and development of aneurysm. In an attempt to answer this question, genetic variations at two loci in the fibrillin gene and one locus in the elastin gene in individuals with hypertension and peripheral vascular disease, were investigated. Hypertension was classified as systolic blood pressure greater than 160mm Hg and pulse pressure greater than 70mm Hg.

Hypertensive, normotensive and peripheral vascular disease groups were genotyped for polymorphic markers (i.e., determining the number of repeats on each allele). Statistical analysis for chi^2 (distributions) of the different genotypes allowed us to determine whether specific variants associated with peripheral vascular disease and/or hypertension. Allele frequencies as well as genotype frequencies were also investigated, using R_{st} and G_{st} values as a measure of population differentiation. Statistical analysis for chi^2 distribution around median systolic blood pressures and pulse pressures enabled us to determine whether an association existed between a specific genotype and systolic blood pressure and/or pulse pressure. Data from the hypertensive patients was compared with data from Powell's study. A significant association between the fibrillin-1 genotype and systolic blood pressure and pulse pressure was detected (p=0.003 and 0.037 respectively). Mean systolic blood pressures were not significantly different between the different genotypes, but the 2-3 genotype however had the highest mean systolic blood pressure and pulse pressure. Our findings were similar to that of Powell's, if our assumption that Powell might have incorrectly scored the fibrillin-1 (penta repeat) alleles is correct and our fibrillin-1 (penta repeat) allele sizes were the same as his.

Inclusion of the rare alleles in the statistical analysis gave different results to when they were excluded. Powell did not include the rare alleles in his calculations. No significant association between a specific genotype and disease was found when statistical analysis was performed on the disease groups consisting of combined diverse ethnic origin. Statistical analysis on the individual race groups however showed an association between the elastin genotype and peripheral vascular disease in the Caucasian population (p=0.035), with a genotype frequency of 11.1% compared to 0% in the hypertensive group and 4.4% in the normotensive group. A significant association was found between the fibrillin-1 (penta repeat) genotype and patients with peripheral vascular disease from the Coloured population (p=0.044).

Statistical analysis on allele frequencies produced similar results than that of analysis on genotype frequencies. Different results were also obtained when race groups were separated. No association between allele frequencies and disease group was observed although allele frequency patterns showed differences between the disease groups and the normotensive group.

No convincing statistical evidence for an association between either genotype or allele frequency and peripheral vascular disease or hypertension was found. A trend towards an association however warrants further study, using a larger study population. Such an association could lead to an alternative method for pre-symptomatic screening and diagnosis of vascular disease.

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ABBREVIATIONS

AEC,	S-2-aminoethyl-L-cysteine hydrochloride
AMP,	adenosine 5'-monophosphate
AMV,	Avian Maloney virus
ANOVA,	analysis of variance
Asp,	aspartate
ATP,	adenosine 5'-triphosphate
bp,	base pair
BP,	blood pressure
cDNA	complementary DNA
cm,	centimeter
cpm,	counts per minute
dATP,	deoxyadenosine 5'-triphosphate
dCTP,	deoxycytosine 5'- triphosphate
del,	deletion
DEPC,	diethylpyrocarbomate
ddATP,	dideoxyadenosine 5'-triphosphate
ddCTP,	dideoxycytosine 5'-triphosphate
ddGTP,	dideoxyguanine 5'-triphosphate
ddTTP,	dideoxythymidine 5'-triphosphate
dGTP	deoxyguanine 5'-triphosphate
dH_2O,	distilled H_2O
DMEM,	Dulbecco's Modified Eagles medium
DMSO,	dimethylsulphoxide
dNTP,	deoxynucleotide triphosphates

DTT,	dithiothreitol
EBV,	Epstein Barr virus
EDTA,	ethylenediaminetetraacetic acid
Ex,	exon
FCS,	fetal calf serum
fig,	figure
g,	gravity
Gly,	glycine
GTE,	Glucose, EDTA, Tris-HCl
h,	hour
Hcy,	homocysteine
Hg,	mercury
Ile,	isoleucine
HPRT,	hypoxanthine-guanine phosphoribosyltransferase
HPT,	hypertension
ins,	insertion
IPTG,	Isopropyl-1-thio-β-galactoside
kb,	kilobase
kDa,	kilodalton
Met,	methionine
mg,	milligram
$MgCl_2$	magnesium chloride
M,	molar
mm,	milimeter
mM,	milimolar
ml,	millilitre
MOPS,	3-(N-morpholino)-propane sulphonic acid

MRNA,	messenger RNA
MTHFR,	methylenetetrahydrofolate reductase
mw,	molecular weight
nm,	nanometer
nmol,	nanomolar
NS,	not significant
nt,	nucleotide
OD,	optical density
PAGE,	poly acrylamide electrophoresis
PET,	pre-eclampsia
PEG,	poly ethylene glycol
PHA,	phytohemaglutinin
PLP,	phosphoribosyl phosphate
*p*mol,	picomolar
PNK,	phosphonucleotide kinase
pp,	pulse pressure
PSN,	penicillin/streptomycin/neomycin
PVD,	peripheral vascular disease
$RbCl_2$,	rubidium chloride
RE,	restriction enzyme
rpm,	revolutions per minute
rRNA,	ribosomal RNA
s,	second
SAM,	S-adenosylmethionine
SD,	standard deviation
SDS	Sodium dodecylsulphate
Ser,	serine
SSA,	salicylsulphonic acid

syst,	systolic pressure
TAE,	Tris Acetate EDTA
TBE,	Tris Borate EDTA
TE,	Tris EDTA
TEMED,	N,N,N',N'-tetramethylethylenediamine
tHcy,	total plasma homocysteine
Thr,	threonine
U,	unit
µg,	microgram
µl,	microlitre
UV,	ultra violet light
W,	watt
XGAL,	5-bromo-4-chloro-3-indolyl-β-galactoside

CHAPTER 1 INTRODUCTION

1.1 General Introduction

Homocystinuria is a condition, which is characterised by the accumulation of homocyst(e)ine in the blood and urine. It is known to be caused by defects in any one of the enzymes along the methyl cycle (methionine metabolism), namely cystathionine β-synthase, methylenetetrahydrofolate reductase and methionine synthase. The cystathionine β-synthase genes of patients who had been confirmed as homocystinuric, and immediate family members, were screened for defects. Other enzymes were also investigated in order to establish whether defects in them contributed to the homocystinuria in these patients.

Moderate elevation of homocyst(e)ine (hyperhomocysteinaemia) in plasma has been implicated as an independent risk factor for a variety of vascular disease states. Homocyst(e)ine is a thiol-containing amino acid derived from demethylation of methionine. Homocyst(e)ine is either irreversibly degraded by the vitamin B_6 dependent enzyme, cystathionine β-synthase, via the transsulphuration pathway or it is remethylated to methionine by cobalamin and folate dependent reactions. Homocyst(e)ine accumulates when either of these pathways is impaired, as a result of specific enzyme defects, or more commonly by dietary deficiencies, especially of folate. The total homocysteine concentration in serum/plasma reflects cellular homocyst(e)ine metabolism and is usually about 10 μmol/L. Patients with inborn errors in cystathionine β-synthase often have plasma values elevated above 100 to 200 μmol/L. Hyperhomocysteinaemia refers to total plasma homocysteine levels in the range 15-30 μmol/L (Ueland *et al.*, 1993) and can thus also sometimes be attributed to deficiencies in folate, vitamin B_6, or cobalamin metabolism (Brattström *et al.*, 1990).

No diagnostic laboratory in the Western Cape, South Africa offers a total plasma homocysteine assay as a routine screen for patients with disease states where hyperhomocysteinaemia is implicated. A reliable, accurate and sensitive assay for the measurement of total plasma homocysteine that is feasible in a routine diagnostic laboratory could assist clinicians in their management of patients with vascular disease and early detection of patients at risk. Challenging the methyl cycle (methionine metabolism) with oral methionine emphasizes the small differences found in total plasma homocysteine values in the healthy population and patients with suspected hyperhomocysteinaemia. Introduction of the oral methionine test could increase the

detection of defects in the methyl cycle (methionine metabolism) in patients with suspected hyperhomocysteinaemia.

The incidence, cause and relationship however between hyperhomocysteinaemia and vascular disease in the Western Cape are still unknown factors and it is important to assess the association between hyperhomocysteinaemia and vascular disease in order to establish whether hyperhomocysteinaemia is a problem and whether it can be controlled by vitamin treatment. Previous studies have shown that oral folic acid supplementation could correct hyperhomocysteinaemia in most of these patients. Thus folate status is an important factor in the management of hyperhomocysteinaemia and the recommended daily allowance may not necessarily be sufficient for those people with additional factors impairing homocyst(e)ine metabolism. The importance of folic acid in the diet is recognised in several countries where flour and bread is often supplemented with folic acid.

Several other factors have been suggested to cause an increase in plasma homocyst(e)ine namely genetic defects in enzymes associated with homocyst(e)ine metabolism such as heterozygosity for defects in the cystathionine β-synthase gene (frequency of between 1:100 and 1:200 in the healthy European population) (Mudd *et al.*, 1995) and the C677T thermolabile mutation in the methylenetetrahydrofolate reductase gene. The homozygous TT methylenetetrahydrofolate reductase genotype, where both alleles carry the C677T mutation, exists at a frequency of ±12% in the healthy European population (Brattström *et al.*, 1998). The incidence of heterozygous defects in the cystathionine β-synthase gene and the C677T mutation in the methylenetetrahydrofolate reductase gene is unknown in the Western Cape. Moderately elevated homocyst(e)ine concentrations found by previous investigators in patients with low plasma folate and homozygosity for the thermolabile methylenetetrahydrofolate reductase variant indicates that folate treatment may benefit patients with defective methylenetetrahydrofolate reductase. The attributability of the thermolabile variant to hyperhomocysteinaemia needs therefore to be assessed in the local population.

The sensitivity of current methodologies for the measurement of cystathionine β-synthase activity needs to be increased in order to determine heterozygous status. Molecular methodology needs to be established in order to screen for disease-carrying mutations in the cystathionine β-synthase gene. This will be useful if enzyme studies are not conclusive.

If cystathionine β-synthase and methylenetetrahydrofolate reductase enzyme and genetic studies fail to explain the cause for the raised homocyst(e)ine found in the disease groups

under investigation another genetic route will be investigated. Recent findings are suggestive of a significant linkage between the human fibrillin-1 gene and the incidence of aortic aneurysm, hypertension and Marfan's syndrome (Powell *et al.*, 1996). Cardiovascular manifestations are characteristic of Marfan's syndrome (Lee, 1991). A new polymorphism in the elastin gene has also successfully been used to isolate familial supravascular stenosis and Williams' syndrome, a congenital disorder, characterised by mental retardation and vascular abnormalities (Urban *et al.*, 1997). Fibrillin and elastin are an integral part of cell wall structures. An association between other vascular disease states and genetic variation in the fibrillin and elastin genes could be useful in pre-symptomatic diagnosis of vascular disease. The variety of mutations found in patients with cystathionine β-synthase deficiency makes it difficult to routinely screen for disease carrying mutations. Co-segregation of a specific copy of the fibrillin-1 or elastin genes with vascular disease groups under investigation in this study could result in a widely applicable method of molecular screening.

1.2 Principal aims of the study

The aims of the study were 1) to determine the defects in the cystathionine β-synthase gene in individuals with homocystinuria, 2) to assess the incidence and cause of hyperhomocysteinaemia in the Western Cape, and 3) to delineate the relationship between hyperhomocysteinaemia and vascular disease in the local population of the Western Cape.

The following points will be given special attention:

(1) The confirmation of homocysteinaemia in three patients and kindreds with homocystinuria and determination of the underlying mutation(s) in the cystathionine β-synthase gene.

(2) The establishment of methodologies (biochemical and molecular) for the measurement of cystathionine β-synthase deficiency and its relationship to patients with hyperhomocysteinaemia.

(3) The establishment of an inexpensive and reliable total plasma homocysteine assay and investigation of the relationship between free and total plasma homocysteine in certain groups of patients before and after oral methionine.

(4) The investigation of the relationship of homocyst(e)ine in patient groups with peripheral vascular disease, abruptio placentae and pre-eclampsia.

(5) The effect of folate treatment on patients with homocystinuria and peripheral vascular disease and hyperhomocysteinaemia.

(6) The establishment of the allele frequency of the thermolabile variant of the methylenetetrahydrofolate reductase gene in patient groups with peripheral vascular disease, abruptio placentae, pre-eclampsia and homocystinuric patients and their immediate family members.

(7) The establishment of the allele frequency of the thermolabile variant of the methylenetetrahydrofolate reductase gene in the different ethnic groups of the local population of the Western Cape.

(8) The establishment of an alternative and perhaps a more effective method for pre-symptomatic diagnosis of vascular disease. Individuals from the peripheral vascular and hypertensive disease study groups will be typed for disease carrying polymorphic markers (i.e. determining the number of repeats) found in the fibrillin and elastin genes. Each patient will be typed for specific polymorphic markers. Allele sizes and genotype frequencies will be compared across disease groups and at three different loci, two in the fibrillin-1 gene and one in the elastin gene.

CHAPTER 2 LITERATURE REVIEW

2.1 Homocystinuria

Homocysteine was first discovered in 1952 by De Vigneaud as a product of demethylation of methionine. Subsequently, various studies have been conducted to explain the role of homocysteine in the metabolism of methionine. Excessive accumulation of homocysteine and subsequent excretion into urine is caused by disturbances during methionine metabolism. This condition is known as homocystinuria and was first discovered in 1962, in mentally retarded patients by Carson and Neill and Gerritsen and Waisman. Fasting total plasma homocysteine usually exceeds 100 to 200μmol/L in these patients, compared to the reference range of 5 – 15μmol/L (Ueland *et al.*, 1993). Homocystinuria is a rare autosomal recessive condition usually resulting from deficiency of cystathionine β-synthase, an enzyme required in methionine metabolism for the conversion of homocysteine to cystathionine.

The clinical features of homocystinuria resemble that of vascular disease thrombo-embolism and premature arteriosclerosis (Kilmer, 1969), mental retardation (Skovby, 1985), skeletal abnormalities (Brenton, 1977), epilepsy (Walton and Treiman, 1988) and dislocated lenses. Worldwide incidence of homocystinuria is 1:200000 (Mudd *et al.*, 1985). Higher incidence (1:60000) of homocystinuria has been found in New South Wales and Ireland (Brenton, 1977).

2.1.1 Methionine metabolism

Homocysteine is formed from the essential amino acid, methionine (fig. 1). The enzyme, methionine S-adenosyl transferase, transfers the adenosyl moiety of ATP to methionine, forming S-adenosylmethionine. The tri-polyphosphate of the ATP molecule remains bound to the enzyme and is cleaved by a tri-polyphosphatase, in a second catalytic reaction, to form inorganic phosphate (Lombardini *et al.*, 1973). After removal of the tri-polyphosphate, the synthesis of S-adenosylmethionine becomes irreversible under physiological conditions (Mudd, 1973). A major function of the methyl cycle is the provision of methyl groups by S-adenosylmethionine (SAM) to various chemical processes in the body, e.g. DNA synthesis, creatine-creatinine formation in muscle and myelin formation in the brain. All these transmethylation reactions result in the formation of a common sulphur containing compound, S-adenosyl homocysteine.

Methyl transferase activity is regulated by the concentration of S-adenosyl homocysteine. When S-adenosyl homocysteine is present at a ratio of 1:4 with respect to S-adenosylmethionine, methyl transferase activity is inhibited (Poston and Stadtman, 1975). The enzyme S-adenosyl hydrolase, catalyses the cleavage of S-adenosyl homocysteine to adenosine and homocysteine. Rapid removal of homocysteine and adenosine, normally favours the forward motion of this reaction. At this point, homocysteine can either be remethylated to methionine or, transsulphurated to cysteine.

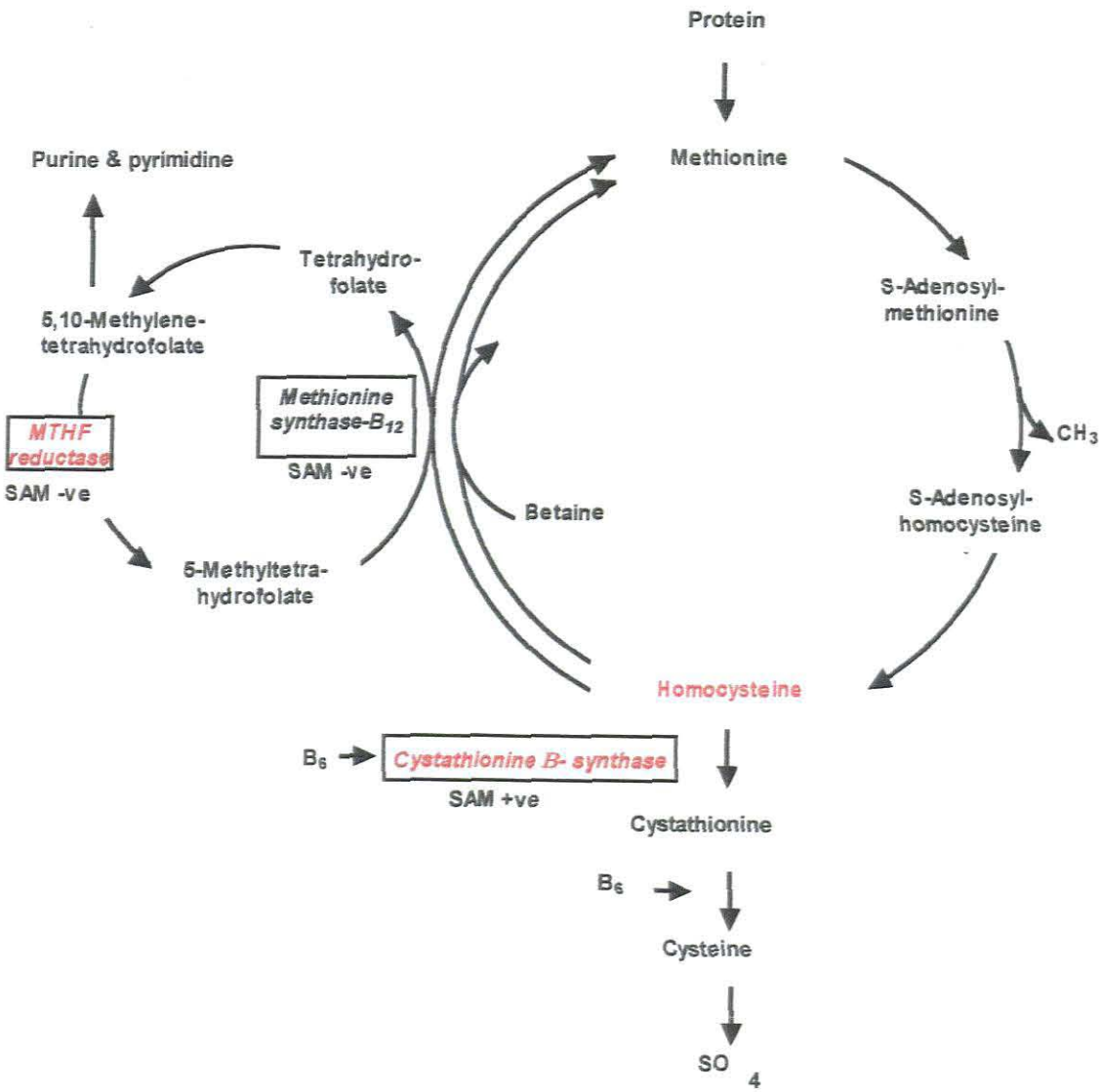


Figure 1. Methionine metabolism in the body (methyl cycle).

2.1.1.1 Remethylation of homocysteine to methionine

Remethylation of homocysteine to methionine can occur via two mechanisms. In the liver, remethylation is catalysed by the enzyme betaine methyl transferase. In the second reaction, 5'-methyltetrahydrofolate acts as the methyl donor for remethylation of homocysteine to methionine. 5'-Methyltetrahydrofolate is formed when 5'-10'-methylenetetrahydrofolate is reduced by the enzyme methylenetetrahydrofolate reductase. Remethylation of homocysteine is catalysed by the cobalamin-containing enzyme, 5' methyltetrahydrofolate homocysteine methyl transferase (Frasca *et al.*, 1988). An intermediate product, methyl cobalamin, is formed during this complex methyl transfer reaction.

2.1.1.2 Transsulphuration of homocysteine to cysteine

In the first reaction of transsulphuration, homocysteine condenses with serine to form cystathionine. Transsulphuration of homocysteine is catalysed by the vitamin B_6 dependant enzyme, cystathionine β-synthase (Mudd *et al.*, 1995). At physiological conditions, the equilibrium of the reaction is towards cystathionine formation, unless homocysteine is rapidly removed (Mudd *et al.*, 1995). Cystathionine is cleaved to cysteine and α-ketobutyrate by the vitamin B_6 dependant enzyme, cystathionase. Further cleavage of cysteine by cystathionase, results in the formation of sulphate and pyruvate (Mudd *et al.*, 1995).

2.1.1.3 Regulation of methionine and homocysteine homeostasis

To maintain intracellular methionine homeostasis and levels of S-adenosylmethionine that are sufficient for methyl transfer reactions, regulation occurs at various points in methionine metabolism. Low intracellular S-adenosylmethionine, resulting from decreased methionine, enhances remethylation, by increasing the activity of methylenetetrahydrofolate reductase. Low intracellular homocysteine and low vitamin B_{12} block tetrahydrofolate formation (Svardal *et al.*, 1986a). Folates are diverted from DNA synthesis to preserve them for methionine formation, thus decreasing the competition for methionine for protein synthesis. Increased methionine, homocysteine and S-adenosylmethionine activate the enzyme cystathionine β-synthase, resulting in increased transsulphuration (Svardal *et al.*, 1986b). The result is the removal of excess intracellular homocysteine and excretion into the urine.

2.1.2 Enzymes implicated in Homocystinuria

Homocysteine accumulates due to defects in any one of the enzymes involved in methionine metabolism. Defects in the three enzymes; cystathionine β-synthase, methylenetetrahydrofolate reductase and methionine synthase have been shown to cause an increase in homocysteine. The most common cause however is defective cystathionine β-synthase.

2.1.2.1 Cystathionine β-synthase

Homozygosity for molecular defects in cystathionine β-synthase is the most common cause of homocystinuria. Deficiency of cystathionine β-synthase activity leads to elevated levels of homocysteine as well as methionine in plasma and urine and decreased levels of compounds found distal to the block caused by defective cystathionine β-synthase, such as cystathionine and cysteine. Enzyme activity in most tissues, excluding red cells, of patients, homozygous for molecular defects of cystathionine β-synthase deficiency, ranges from 0 to 2% of that of reference values (Mudd *et al.*, 1995).

The cystathionine β-synthase enzyme is a tetramer with four identical 63kDa subunits (Skovby *et al.*, 1984). In its activated proteolised form, the enzyme is a dimer of 48kDa subunits (Kraus, and Rosenberg, 1983). Each of these subunits binds two substrates, serine and homocysteine, as well as three additional ligands, the coenzyme, vitamin B_6 (Kraus, J.P. 1978), an allosteric activator S-adenosylmethionine, which activates the enzyme two to four fold (Roper and Kraus, 1992), and a haem moiety (Kery *et al.*, 1998). The cystathionine β-synthase enzyme consists of a catalytic region, which stretches from amino acid 37 to 414 and a regulatory region, which stretches from amino acid 414 to 551. Kery *et al.* (1998) proposed a mechanism whereby S-adenosylmethionine binding to cystathionine β-synthase results in a conformational change of the enzyme. In non-activated cystathionine β-synthase, the catalytic site is partially hidden by the C-terminal domain. They propose that binding of S-adenosylmethionine displaces the C-terminal from the catalytic site, resulting in an increase in cystathionine β-synthase activity. This organization of cystathionine β-synthase in a catalytic region and inhibitory regulatory region is found in other enzymes, which have a common need for rapid regulation in response to cellular conditions. The crystal structure of cystathionine β-synthase has recently been described by Meier *et al.* (2001) as shown in fig. 2.

The crystal structure of cystathionine β-synthase

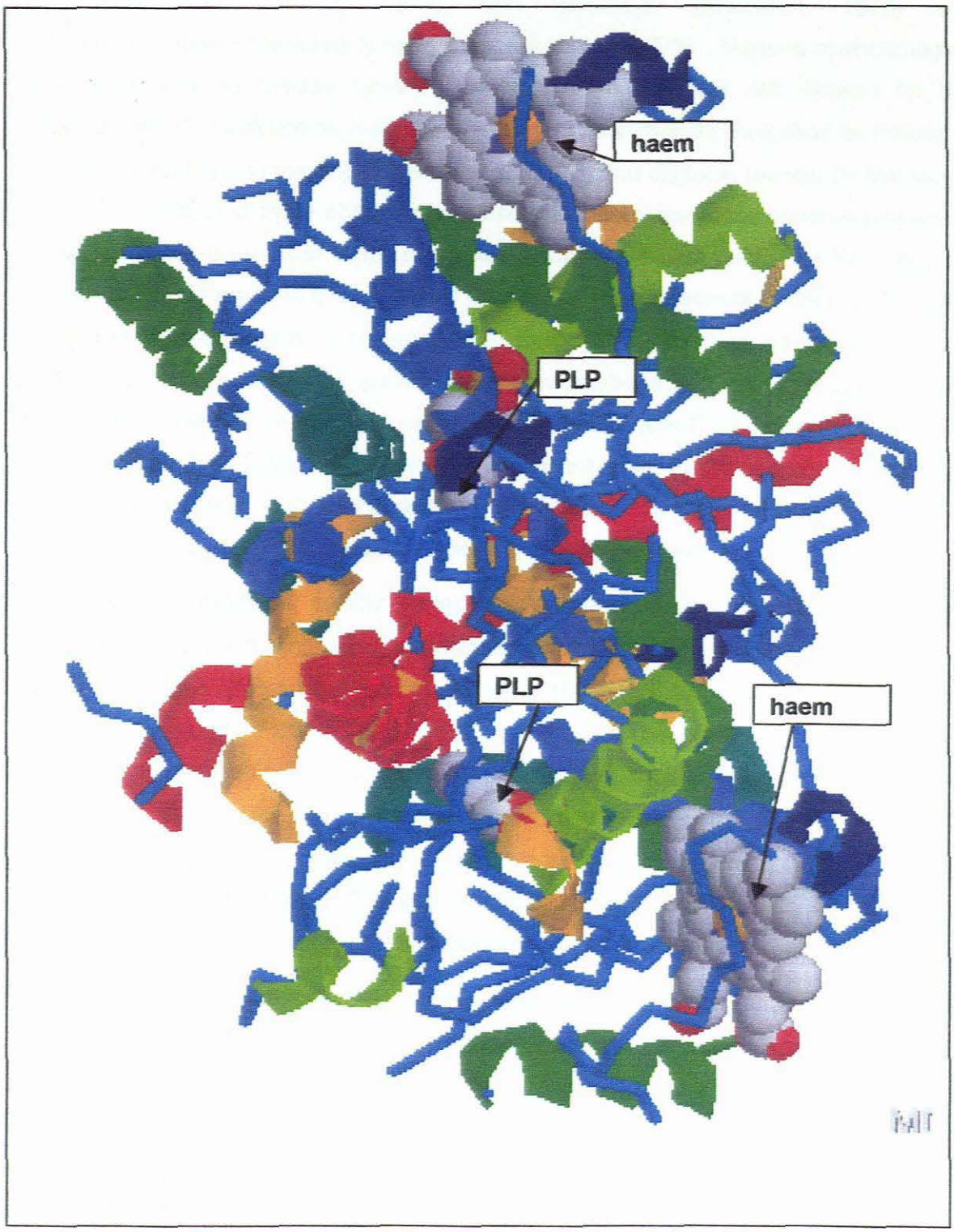


Figure 2. This is an X-ray crystal structure of a truncated form of the cystathionine β-synthase enzyme. A dimer formed by two subunits is displayed. Alpha helices are shown as helices and beta strands are shown as straight planks with arrowheads. The two ligands, haem and pyridoxal 5'-phosphate (PLP) are indicated on both subunits. Regions that are neither alpha helix nor beta strands are shown as smoothed backbone traces (Meier *et al.*, 2001).

Cystathionine β-synthase activity has been measured in many types of cells, namely, hepatocytes (Gaull *et al.*, 1974), skin fibroblasts (Uhlendorf, 1973) and phytohaemaglutinin stimulated lymphocytes (Goldstein, 1973). Various methodologies have been used to release cytosolic enzymes from prepared cell extracts for the measurement of cystathionine β-synthase activity. These include sonication by Fowler *et al.* (1978), freezing and thawing (Fleisher *et al.*, 1973) and digitonin release by Mackall *et al.* (1979). Yield of enzyme obtained by these methodologies varies considerably and it would be helpful to establish which one would result in the best yield. The Km value for serine was initially reported to be 1.15mM (Kraus, 1978); subsequent studies confirmed a Km of 4mM for both forms of the enzyme (Kraus, 1987). To measure cystathionine β-synthase enzyme activity, cell culture extract preparations are incubated with the two substrates, serine and homocyst(e)ine. Serine is titrated with [^{14}C] radio labelled serine. After incubation the cystathionine formed is measured either by paper chromatography or amino acid analysis and quantitated by scintillation counting. Enzyme activity is expressed in nmol cystathionine formed per mg protein per hour.

Cystathionine β-synthase activity varies significantly amongst the different tissues. Control cystathionine β-synthase values range from 9.85 ±9.5nmol/mg/hr in long-term skin fibroblast cultures (Nordstrom and Kjellstrom, 1992), to 1.73 to 3.10nmol/mg/hr in lymphoblast cultures (Fleisher *et al.*, 1972) and 90 to 888pmol/mg/4hr in short-term phytohaemaglutinin stimulated lymphocytes (Goldstein, 1973). The expression of the cystathionine β-synthase enzyme varies significantly amongst the different cell types. Enzyme measurements in cultured cells, is not perfect, as culture conditions may not reflect true *in vivo* conditions. The age of cultured cells also affect enzyme activity and activity decreases with an increase in cell culture age (Nordstrom and Kjellstrom, 1992).

The entire human cystathionine β-synthase gene has been cloned and sequenced fully. The human cystathionine β-synthase gene contains 23 exons, comprising 2554 nucleotides and 551 amino acids (Kraus, 1993). Exons 1 to 14 and exon 16 encodes the polypeptide. Exon 15 encodes 14 amino acids and is incorporated in relatively few mature human cystathionine β-synthase mRNA molecules. Most mRNAs have a 214 base pair insert (retained intron) at nucleotide 1674, at the 3' end, whereas a few transcripts lack this feature (Kraus, 1993). Several defects have been detected in the cystathionine β-synthase gene of patients with deficient cystathionine β-synthase activity.

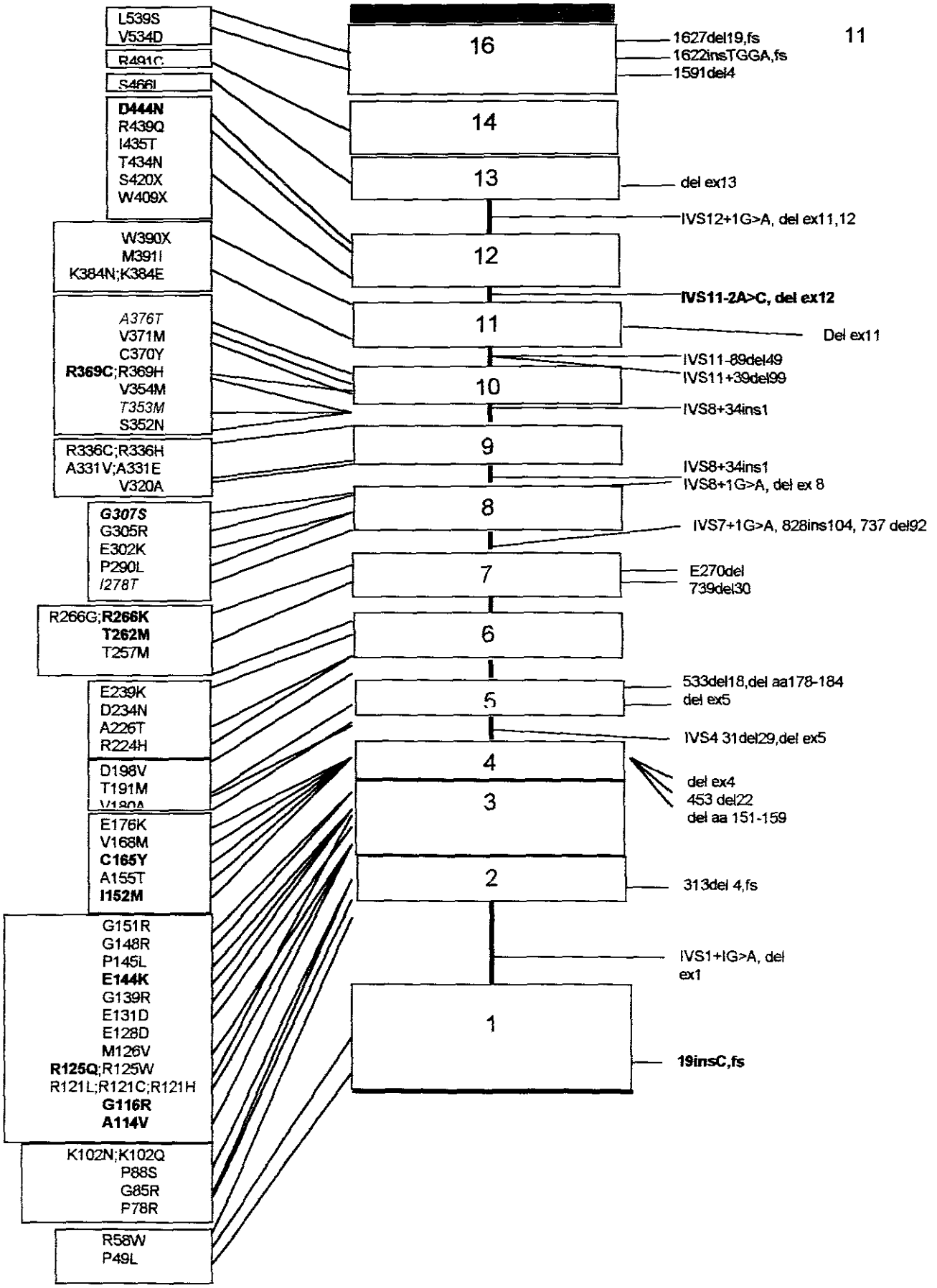


Figure 3. Location of human cystathionine β-synthase mutations. The exons in the coding region are drawn to scale; the introns are not. Shaded areas, denotes parts of 5'- and 3'-untranslated regions of cystathionine β-synthase mRNA found in exons 1 and 16, respectively. The mutations shown on the left are missense and nonsense; the mutations on the right are deletions, insertions, and splicing aberrations. The mutations shown in bold were detected in three or more alleles (Kraus *et al.*, 1999). The four missense mutations detected for this study are shown in blue italics.

In 310 examined homocystinuric alleles in several laboratories around the world, 100 different disease-associated mutations have been identified in the cystathionine β-synthase gene (fig. 3). Mutations have been detected from exon 1 to exon 16. Thus far, 66 missense mutations have been found in patients with defective cystathionine β-synthase activity. A relative common mutation found in mainly Celtic patients is the G919A (Gly307Ser) mutation, which alters a glycine to a serine at amino acid 307. This mutation has been detected in 71% of alleles. (Kraus, 1994; Gallagher *et al.*, 1995). Another commonly found mutation is T to C transition at nucleotide 833 and amino acid 278. Both these mutations are found in exon 8. The T833C mutation is present at a higher frequency in some countries, e.g. Kluijtmans *et al.* (1998) found that more than 50% of affected alleles in the Netherlands carried the T833C Ile278Thr mutation, whereas in other countries it is only present at a frequency of 25%. Although considerable progress has been made in the detection of specific mutations responsible for cystathionine β-synthase deficiency, most of the alleles studied, come from European origin and virtually nothing is known about the nature of homocystinuric mutations in African, Asian and South American populations.

2.1.2.2 Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase deficiency is the most common genetic abnormality found in the remethylation pathway. Methylenetetrahydrofolate reductase catalyses the conversion of 5'-10'-methylenetetrahydrofolate to 5'-methyltetrahydrofolate, which is the predominant circulating form of folate and the methyl donor for homocysteine remethylation. The methylenetetrahydrofolate reductase enzyme is a dimer and consists of two identical subunits of 74.5kDa each. The cDNA has been cloned and consists of 2200 base pairs, encoding a protein of 656 amino acids (Goyette, 1994). A point mutation at nucleotide 677, which result in a C to a T transition and an alanine to a valine substition, renders the enzyme thermolabile. Frosst *et al.* (1995) screened for the C677T polymorphism by amplifying a 198 base pair segment of genomic DNA. Restriction enzyme *Hinf* 1 digests this fragment into two fragments of 175 and 23 base pairs each. The digested DNA can be visualized by agarose gel electrophoresis.

Kang *et al.* (1991), suggested that thermolability in methylenetetrahydrofolate reductase is inherited as an autosomal recessive trait and showed that the thermolabile variant could be an inherited risk factor for coronary artherosclerotic disease. Frosst *et al.* (1995) detected a C to a T polymorphism at nucleotide 677, which caused thermolability and reduced catalytic activity of the enzyme and elevated homocysteine under impaired folate

status. This polymorphism was later confirmed to be a common determinant of plasma total homocysteine in the general population. Jacques *et al.* in 1996 reported that individuals, homozygous for the methylenetetrahydrofolate reductase thermolabile mutation, have moderately elevated fasting homocysteine concentrations when plasma folate concentration is in the lower range but not when folate is high, suggesting that folate treatment could benefit patients with defective methylenetetrahydrofolate reductase (Brattström *et al.*, 1998). Subsequently a large study by Brattström *et al.* (1998) concluded that there is no evidence for an association between increased cardiovascular risk and the thermolabile methylenetetrahydrofolate reductase genotype. Ueland *et al.* (2001) reported two aspects of this polymorphism to be associated with disease. Firstly the polymorphism might effect the increased total plasma homocysteine concentrations already caused by disease. Secondly the genotype might be associated with disease risk, mediated by altered folate and homocysteine metabolism

Botto and Yang (2000) reported that the C677T polymorphism was related to ethnicity. They reported homozygosity for the T allele (TT) to be ~10 % in the Caucasian population, ~20% in some Italian populations and less than 2% in Afro-Americans. If hyperhomocysteinaemia is caused by a defect in this enzyme, treatment with folate could lower plasma homocysteine.

2.2 Hyperhomocysteinaemia

The initial discovery that excessive homocyst(e)ine is associated with occlusive vascular disease in patients with homocystinuria (Wilcken and Wilcken, 1976; McCully, 1996) lead to numerous investigations into moderately elevated homocyst(e)ine (hyperhomocysteinaemia) in vascular disease. Results from these studies have now shown a definite correlation between this milder form of homocystinuria and vascular disease. Boushey *et al.* (1995) reported an incidence of hyperhomocysteinaemia of 20% to 30% in patients with cerebrovascular, peripheral vascular and coronary heart disease. Boers (1994) reported an incidence of 32% in patients with cerebrovascular, peripheral vascular and coronary heart disease compared to 2% in the normal population. In another study 23-47% of vascular disease patients had mild hyperhomocysteinaemia compared to 7% of 1400 healthy controls (Ueland *et al.*, 1992).

The common causes of hyperhomocysteinaemia are low serum or red cell folate concentrations, (Kang *et al.*, 1987; Stabler *et al.*, 1988.), vitamin B_{12} deficiency (Stabler *et al.*, 1988), the TT genotype for the C677T methylenetetrahydrofolate reductase polymorphism in combination with low folate status (Engbersen *et al.*, 1995; Brattström *et*

al., 1998) and heterozygosity for cystathionine β-synthase deficiency (Boers *et al.*, 1985; Clarke *et al.*, 1991). The prevalence of moderate hyperhomocysteinaemia in the general population initially was estimated to be 5-7% (Andersson *et al.*, 1992). The resistance to hyperhomocysteinaemia of South African "Blacks", compared with Caucasians, consuming the same diet, may also be genetic in origin (Ubbink *et al.*, 1995).

Phenotypic expression of some of the genetic defects along the methyl cycle can either be masked or augmented by other factors, like nutritional status, medication and endocrinological condition. Hyperhomocysteinaemia, caused by a defect in the methylenetetrahydrofolate reductase gene can be masked if folate intake was sufficient, or augmented if folate intake was insufficient, explaining the interaction between dietary and genetic factors (Sartorio, 1986). In most cases, the homocysteine status is thus the result of an interaction between genetic, physiological and environmental factors/ influences.

Hyperhomocysteinaemia has now been implicated as a risk factor for several clinical conditions, namely deep-vein thrombosis (Den Heijer *et al.*, 1996), peripheral vascular disease (Boers *et al.*, 1985), cerebral vascular disease (Brattström *et al.*, 1984), coronary heart disease (Ubbink, 1997) and spontaneous abortion or abruptio placentae (Steegers-Theunissen *et al.*, 1992). However it still has to be proved that hyperhomocysteinaemia is causative and not just a secondary event.

2.3 Factors influencing homocysteine homeostasis

2.3.1 Environmental factors

2.3.1.1 Dietary influence

Vitamin dependent enzymes along the methyl cycle necessitates sufficient dietary vitamin intake for maintaining normal levels of plasma homocysteine. Vitamin B_{12}, vitamin B_6 and folate are extremely sensitive to destruction by various food processes and the increased intake of processed foods low in vitamin content, in the Western world, could result in hyperhomocysteinaemia. Remethylation of homocysteine could be impaired by nutritional deficiency of vitamin B_{12} and folate. The formation of the intermediate product, methyl-cobalamin during remethylation reactions could be impaired by deficient vitamin B_{12}. The activity of folate dependant enzymes such as methylenetetrahydrofolate reductase is dependant on critical intracellular concentrations of folate. Deficient folate intake therefore could result in low methylenetetrahydrofolate reductase activity.

Several studies have indicated the association of hyperhomocysteinaemia with sub clinical deficiencies in vitamin B_{12} and folate. Lindenbaum *et al.* (1988) showed that patients with deficient vitamin B_{12}, but no evidence of macrocytosis or anaemia, had elevated total plasma homocysteine concentrations. Kang *et al.* (1987) demonstrated hyperhomocysteinaemia in 84% of patients with deficient serum folate. Brattström, (1996) reported that the average fasting total plasma homocysteine of 20 patients, deficient in vitamin B_{12}, was significantly higher (23.8 ±17μmol/L) than in healthy controls subjects (11.5 ±5.8μmol/L). The total plasma homocysteine was normalised 14 days after administration of hydroxy-cobalamin. Ubbink *et al.* (1993) also reported deficient vitamin B_{12}, vitamin B_6 and folate levels in South African men, with hyperhomocysteinaemia. A number of studies have shown that vitamin supplementation results in a correction of total plasma homocysteine concentrations to normal levels (Ubbink *et al.*, 1994; Franken, 1994; Rasmussen *et al.*, 1996). Folic acid supplementation is more efficient in lowering total plasma homocysteine than folate derived from food (Wei *et al.*, 1998). Vitamin B_6 supplementation lowers post oral methionine, total plasma homocysteine (Ubbink *et al.*, 1994). In the recent multi-centre European (COMAC) study (Robinson *et al.*, 1998), in which 750 patients and 800 controls were studied, low circulating folate and vitamin B_6 were demonstrated as risk factors for stroke, peripheral vascular disease and coronary arterial disease. Hyperhomocysteinaemia has been implicated as a risk factor in all these conditions.

2.3.1.2 Chemical agents (toxins)

The anti-cancer drug, methotrexate has been widely used in therapy of acute leukaemia, several solid tumours, psoriases, rheumatoid arthritis and nonmalignant tumours. Methotrexate reduces the amount of reduced folates and thereby inhibits several pathways dependant on folate (Refsum *et al.*, 1989). The anaesthetic, nitrous oxide oxidises cob(I)alamin to cob(II)alamin, which can no longer function as a methyl carrier. 6-Azauridine triacetate inactivates vitamin B_6 and increases urinary excretion and plasma level of homocysteine (Slavik *et al.*, 1982). Anti- convulsants cause folate deficiency (Lambie and Johnson, 1985).

2.3.2 Physical factors

2.3.2.1 Hormonal influence

Premenopausal women have lower plasma homocysteine values than men of the same age and postmenopausal women (Wouters *et al.*, 1995). This suggests that homocysteine metabolism may be influenced by the female sex hormones. The fact that decreased (as much as 50% lower) total plasma homocysteine is found during pregnancy (Walker *et al.*, 1999), a state characterised by high levels of estrogen, supports this hypothesis. It has also been shown that some contraceptives increase plasma homocysteine, whereas some lower plasma homocysteine (Wong and Kang, 1988). An increase in the demand for folate (5'-10-methylenetetrahydrofolate) for DNA synthesis during cell division could be the cause for lower homocysteine found during pregnancy.

2.3.2.2 Age

A decrease in absorption or decreased intake of vitamins could be the cause for higher plasma homocysteine levels found in older men and women (Selhub *et al.*, 1993). The strong negative correlation for age and cystathionine β-synthase activity reported by Nordstrom and Kjellstrom in 1992, could also be the cause of higher plasma homocysteine levels found in the elderly.

2.4 Conditions associated with hyperhomocysteinaemia

2.4.1 Abruptio placentae

In abruptio placentae the placental attachment to the uterus is disrupted by haemorrhage. This haemorrhage, which could be caused by arteriolar rupture into the deciduas, may cause vascular damage (Barron, 1988). Bleeding then results in spontaneous abortion. Recurrent early abortion has been linked to hyperhomocysteinaemia (Steegers-Theunissen *et al.*, 1992; Wouters *et al.*, 1993). It has been shown that low levels of folate and premature abortion occur frequently in patients with high parity and poor social status. Steegers-Theunnissen *et al.* (1992) showed that 25% of a group of women with recurrent spontaneous abortion and abruptio placentae had total homocysteine levels higher than 2 SD of that of total plasma homocysteine found in healthy pregnant subjects. Owen *et al.* (1997), has also demonstrated an association between abruptio placentae and hyperhomocysteinaemia. Steegers-Theunissen *et al.* (1992) could however show no correlation between mild hyperhomocysteinaemia and cystathionine β-synthase deficiency in subjects with recurrent abortion, suggesting that the cause for mild hyperhomocysteinaemia in this group of patients could be dietary or an enzyme defect in the remethylation pathway.

During pregnancy the demand for folate increases due to rapid cell division and purine and pyrimidine synthesis. This increase in demand for folate requires folate supplementation during pregnancy. Folate treatment during pregnancy has failed however to reverse damage already caused (Wouters *et al.*, 1993) and it has been suggested that folate treatment should start well before pregnancy.

The cause of hyperhomocysteinaemia in abruptio placentae patients could be due to deficient dietary folate and or defective methylenetetrahydrofolate reductase, because deficient folate has been shown to augment the effect of the thermolabile variant of methylenetetrahydrofolate reductase. The poor dietary habits of the impoverished communities of the Western Cape could be a contributing factor towards abruptio placentae amongst these communities. Jacques *et al.* (1996), found that individuals who are homozygous for the methylenetetrahydrofolate reductase thermolabile variant have elevated fasting homocysteine concentrations when plasma folate concentration is in the lower range but not when folate is high. This suggests that folate treatment could benefit patients with the thermolabile methylenetetrahydrofolate reductase defect.

Vollset *et al.* (2000), recently suggested that impaired vitamin B status could be the cause of a moderately elevated total plasma homocysteine in patients with abruptio placentae. The impaired vitamin B status could affect homocysteine remethylation, biological

methylation and DNA synthesis, and thereby normal faetal growth. The higher total plasma homocysteine found in abruptio placentae in turn, could be the cause of the vascular damage found in this condition.

2.4.2 Pre-eclampsia

The condition, pre-eclampsia is characterised by a group of conditions namely proteinuria, hypertension and oedema during gestation or within 7 days of delivery. Pre-eclampsia usually begins after the 32nd week of pregnancy but may begin earlier. Homocysteine levels decrease during normal pregnancy (Walker *et al.*, 1999). Recent studies however have shown women with pre-eclampsia and non-pregnant women with a history of pre-eclampsia to have raised homocysteine (Dekker *et al.*, 1995; De Vries *et al.*, 1997; Leeda *et al.*, 1998).

Hyperhomocysteinaemia may result in damage to the vascular endothelium (Celermajar *et al.*, 1993; Kanani *et al.*, 1999). The underlying mechanism has not yet been elucidated, but several possibilities exist. These include the generation of a pro-thrombotic endothelial surface, formation of free radicals, impaired endothelium dependant vasodilation, and proliferation of vascular smooth muscle cells and enhanced collagen production. Hyperhomocysteinaemia has now become the focus of research into the etiology and prevention of pre-eclampsia because of the association of endothelial disruption with pre-eclampsia (Dekker *et al.*, 1998).

In South Africa, hypertensive disorders of pregnancy are the most common causes of maternal mortality, but previous studies of hyperhomocysteinaemia and pre-eclampsia have largely involved only white North American and European women (Guba *et al.*, 1999). Studies investigating homocysteine levels in antenatal women with pre-eclampsia are also few, and none has as yet looked at the effects of oral methionine on total plasma homocysteine levels. Oral methionine is considered to be essential for the diagnosis of hyperhomocysteinaemia in atherosclerotic and thrombotic vascular disorders (Urban *et al.*, 1997).

2.4.3 Peripheral vascular disease

Peripheral vascular disease is a condition that occurs due to atherosclerosis of large and medium sized arteries and mainly affects the legs. These patients usually experience pain on exertion, which disappears with rest. Ulceration and gangrene eventually develops due to impaired blood supply, which subsequently may result in amputation. A

multi-centre European study has demonstrated low circulating folate and vitamin B_6 as risk factors for peripheral vascular disease (Robinson, 1998). Loncar *et al.* (2001), and Dilley *et al.* (2001) have associated hyperhomocysteinaemia with peripheral vascular disease. The incidence of peripheral vascular disease in the local population is high. Studies of hyperhomocysteinaemia and vascular disease have mainly involved Europeans and North Americans and prevalence of hyperhomocysteinaemia in the local peripheral vascular disease population has not yet been established.

2.5 Homocyst(e)ine measurement and blood collection methods

Total homocysteine is the term used for all forms of homocysteine derivatives which release homocysteine by reduction. Measuring free homocystine to identify moderately elevated levels of homocyst(e)ine is difficult, because free homocystine is rapidly converted to the protein-bound form at any temperature. Unbound forms of homocyst(e)ine represent only 15% of total homocysteine, making it very difficult to measure small increases as is found in hyperhomocysteinaemia accurately.

Homocyst(e)ine was first measured in the laboratory in by Carson *et al.* (1962) when they first investigated patients with the inherited metabolic disorder, homocystinuria. In early clinical studies, homocyst(e)ine was measured in the acid-soluble fraction of plasma, as homocysteine-cysteine mixed disulfide. Increased levels of homocyst(e)ine in the plasma or serum is used as a cellular marker for human disease. However in freshly prepared plasma, homocysteine exists as a combination of the mixed disulphide, homocystine, some free thiol and as Kang *et al.* (1979) has demonstrated, approximately 70% of plasma homocysteine is bound to albumin in plasma. The sum of all these homocyst(e)ine species in plasma is referred to as total plasma homocysteine.

After plasma separation, a marked redistribution takes place between free and bound homocysteine, even at -20°C most of the homocysteine become protein bound (Refsum *et al.*, 1985). Therefore without immediate acid treatment and centrifugation falsely low values are obtained when the free form is measured. Reliable measurement of free plasma homocysteine therefore requires immediate acid treatment and centrifugation of plasma. Care must be taken when sampling for total plasma or serum homocysteine. Total plasma homocysteine increases when whole blood is left standing at room temperature, as a result of continuous production and release of homocyst(e)ine from red blood cells. To prevent false elevated results the blood sample is placed on ice and the plasma separated from the red cells by centrifugation within 1 hour of the specimen being collected.

Patients with homocystinuria excrete large amounts of homocysteine in the urine, and blood concentrations can be as high as ten times that of reference values. These high values are easily detected with any of the various methods available. However patients with hyperhomocysteinaemia have homocysteine values that are only slightly raised and sometimes even fall within the normal range. The narrow differences and considerable overlap between homocysteine values of patients with hyperhomocysteinaemia and reference groups is a major limitation in analytical procedures, especially when only free homocystine is measured. Free homocystine measurement has therefore been abandoned due to the impracticality of handling these samples and total plasma homocysteine, which is the sum of the free and the bound forms, is now the preferred measure of plasma homocysteine.

Whether total plasma homocysteine will be used as a marker for cardiovascular risk, nutritional assessment or neonatal homocystinuria, depends on the test's availability for the routine laboratory. For the routine laboratory to provide such a service, the assay should be sensitive, especially for the levels encountered during hyperhomocysteinaemia. The throughput should be relatively high and the methodology should be simple and preferably fully automated.

2.6 Oral methionine test

To emphasize the small difference between homocysteine levels found in normal control subjects and patients with hyperhomocysteinaemia, the cycle can be challenged with oral methionine. Fasting homocysteine and homocysteine at intervals after oral methionine is determined. After oral methionine, plasma homocyst(e)ine concentration increases to such an extent that transsulphuration catalysed by cystathionine β-synthase is favoured. Defective cystathionine β-synthase activity should then result in an abnormal increase of plasma homocysteine in plasma and in some instances in urine. Andersson *et al.* (1990) showed that homocysteine values reached peak values at 6 hours after oral methionine. An increase in total plasma homocysteine after oral methionine, of ±26μmol/L is indicative of defective methionine metabolism (Robinson, 1998).

Controversy whether oral methionine is necessary for the diagnosis of hyperhomocysteinaemia, has lead to various studies with methionine challenge, especially in patients heterozygous for enzyme deficiencies involved in methionine metabolism.

2.7 Hyperhomocysteinaemia and vascular damage

The question whether mildly elevated homocysteine contributes to the pathogenesis of atherosclerotic vascular disease or whether it is only a marker for increased risk, has not yet been answered. In a recent review, Brattström and Wilcken (2000) hypothesise that mild hyperhomocysteinaemia in association with atherosclerotic cardiovascular disease is a consequence rather than a cause of disease (fig. 4). They speculate that impaired renal function, caused by hypertension and atherosclerosis, could be an important cause of elevated plasma homocysteine in vascular disease patients, because of impaired clearance of homocysteine as shown by Guttormsen *et al.* (1997).

Interaction between certain conditions, vascular disease and elevated plasma homocysteine

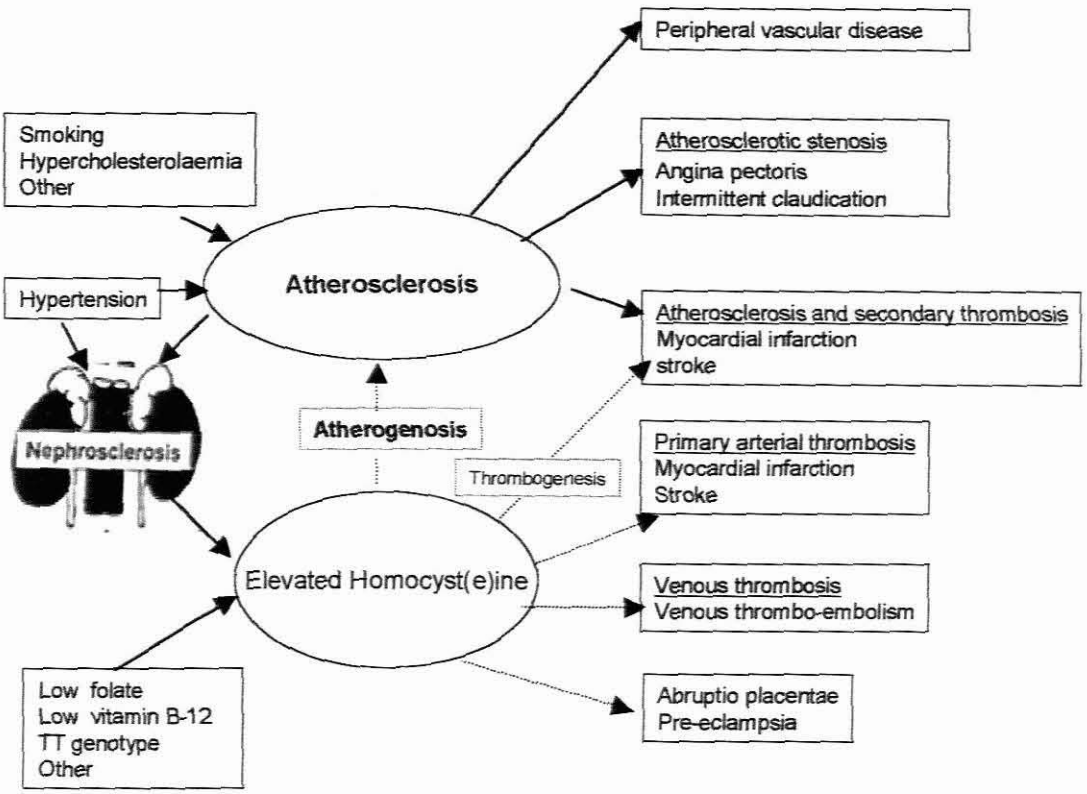


Figure 4. A schematic description of the proposed interactions between impaired renal function, poor folate or vitamin B_{12} status, the TT genotype of the C677T methylenetetrahydrofolate reductase polymorphism, smoking, hypercholesterolaemia and atherosclerosis and elevated homocyst(e)ine. Hypertension and atherosclerosis lead to nephrosclerosis and decline in renal function, which in turn leads to elevated plasma homocysteine. This mechanism may explain much of the association between plasma homocysteine and atherothrombotic vascular disease (modified from Brattström and Wilcken, 2000).

Several studies so far, have also shown that the extent of vascular damage depends on the homocyst(e)ine concentration (Boushey *et al.*, 1995; Refsum and Ueland, 1998). Extensive research has been done to elucidate the mechanism whereby homocysteine may cause vascular damage. According to McCully (1990) many patients with coronary heart disease, stroke or peripheral vascular disease have no significant abnormalities of serum lipoproteins or cholesterol. Previous investigators have observed atherosclerotic lesions in children with high homocysteine, but a normal lipid profile. The atherosclerotic lesions in children with homocystinuria are mainly fibrous and fibro-calcific, unlike the complex atherosclerotic plaques that contain cholesterol crystals and lipid deposits. It has been shown that highly purified cholesterol (protected from oxidation) is not atherogenic in animals, while cholesterol oxides are highly atherogenic in animals. Pathasarathy S. (1987) showed that sulfhydryl compounds such as homocysteine, modify low-density lipoprotein in the presence of cupric or ferric ions to oxycholesterols and other oxidized lipids and proteins. These factors all suggest that homocysteine may play a role in the pathogenesis of vascular disease. The excess homocysteine, caused by defective remethylation or transsulphuration, aggregates lipoprotein and these aggregates are taken up from the blood by the vascular macrophages of the arterial intima resulting in foam cell formation. Foam cells release lipids and cholesterol into fibro-lipid plaques. Foam cells also release homocysteine thiolactone, which in turn act on thioretinaco ozonide of the mitochondria to form thioco. This process results in smooth muscle cell hyperplasia and fibrosis. The resulting overproduction of oxygen radicals causes intimal damage, activates elastase, incites thrombogenesis and increases calcium deposits. These processes may result in the pathological features of atherosclerotic plaques (McCully, 1996). Several investigators have also implicated an increase in homocysteine to cause a decrease in endothelial-dependant brachial artery dilatation (Bellamy *et al.*, 1999).

Genetic links to raised total plasma homocysteine and various disease states, has to date not been established. Heterozygosity for defects in the cystathionine β-synthase gene has not been linked to vascular disease. Some investigators have established poor association with the methylenetetrahydrofolate reductase gene and others have found no link. Another genetic route might be to look for a link between vascular disease and other genes associated with vascular structures.

2.8 Fibrillin and elastin

2.8.1 Fibrillin

Fibrillin forms an integral part of matrix structures as found in arterial walls. Mutations in the fibrillin gene have now been found in patients with Marfan's syndrome (Lee, 1991). Diagnosis of Marfan's syndrome has in the past relied solely on clinical criteria and the relevance of this information is that Marfan's syndrome may provide a precedent for involvement of mutations or polymorphisms of the fibrillin gene in cardiovascular disorders. Marfan's syndrome is a genetic disorder of the connective tissue and is characterised by skeletal, cardiovascular and ocular abnormalities. The incidence of Marfan's syndrome is approximately 1:20000, with life expectancy severely reduced because of cardiovascular complications. Marfan's syndrome is the leading cause of death from aortic aneurysm in adults under the age of 40.

Fibrillin also functions to link elastin to other matrix structures, and serves as a scaffold for elastin deposition (e.g. in the aorta). Both the elastin and fibrillin-1 (*FBN1*) genes have recently been cloned and characterised (Indik 1987; Maslen, 1991). Lee (1991) used a $(TAAAA)_n$ repeat in the fibrillin-1 gene on chromosome 15 in analysis of linkage of this gene to Marfan's syndrome.

The human fibrillin gene encodes a protein in the extra-cellular matrix. The gene for fibrillin-1 is 110kb and it contains 65 exons (Reinhardt *et al.*, 1995). Several of the introns contain informative microsatellites (base pair repeats). Powell *et al.* (1996) reported that variations at the fibrillin-1 locus (Marfan gene) could have an important effect on aortic elasticity, blood pressure and development of aneurysm.

2.8.2 Elastin

Excess elastin accumulation by skin fibroblasts is characteristic of genetic diseases such as Buschke-Ollendorff syndrome, Hutchinson-Gilford progeria and keloid (Davidson, 1995). Deletions of all or large parts of the elastin gene have however been reported in patients with Williams syndrome, a congenital disorder characterised by mental retardation, vascular abnormalities including supravulvular aortic stenosis and a loquacious personality (Urban *et al.*, 1996). Foster *et al.* (1993), described a highly informative marker of $(AC)_n$ repeats in intron 17 of the elastin gene for the analysis of inheritable disorders of connective for which elastin is a candidate gene. Elastin is an insoluble fibrous protein that contributes to the elastic properties of the extra-cellular matrix. The elastin gene consists of 34 exons and is approximately 47kb of genomic DNA

(Tassabehji *et al.*, 1997). Polymorphic markers (microsatellites) are located in introns 17 and 18. A dinucleotide repeat in intron 17 consists of 8 alleles, with sizes between 161 and 175 base pairs. Surprisingly, few authenticated descriptions are known of defects in the elastin gene itself, which might cause disorders of elastic tissue. The product of the elastin gene is intimately associated with fibrillin in the construction of the microfibrillar structure of elastic tissue and could itself be a candidate gene for association with hypertension (Li *et al.*, 1997). Genetic variation in the fibrillin and elastin genes may be associated with other vascular disease states, namely peripheral vascular disease and hypertension in the local population.

2.8.3 Microsatellite analysis

Microsatellites or short tandem repeats are simple base sequences that consist of repeated units in tandem of one to five base pairs (bp) *e.g.* $(CA)_n$, $(AAT)_n$ or $(GATC)_n$ where n is the number of times the unit is repeated. Microsatellites are classified as mono-, di-, tri-, tetra- or pentanucleotide repeats depending on the number of base pairs within each repeat unit. Microsatellites are randomly and widely dispersed throughout eukaryotic genomes, occurring in organisms as evolutionarily diverse as yeast, *Drosophila* and vertebrates (Tautz and Renz, 1984). Mononucleotide, dinucleotide and tetranucleotide repeats are found only in non-coding regions of the genome. These types of microsatellites are generally highly polymorphic, and mutations in them would disrupt the triplet reading frame of a coding region, thus affecting the resultant gene product. These so-called pathogenic microsatellites are stably inherited in normal individuals, from one generation to the next. Consequently, they are frequently studied in connection with human disease, and are used as genetic markers for tracing inheritance patterns in human pedigrees.

The theory behind the use of microsatellites as genetic markers in linkage studies is as follows. Assume a mutation occurs in the fibrillin-1 gene, which then causes a form of hypertension. If this gene was linked to a microsatellite of a certain size say size 173 base pairs, then if all the hypertension in a population are caused by this one mutation, then, when subsequent mutations in the gene have occurred, all patients with hypertension will carry at least one copy of the 173 allele.

Powell *et al.* (1996), studied the $(TAAAA)_n$ penta repeat microsatellite at locus 21946 in the fibrillin-1 gene in patients with hypertension. They concluded that a significant association appears to exist between fibrillin-1 genotype and arterial pulse pressure in these patients with hypertension. Another microsatellite of $(CA)_n$ repeats at locus 21945 in

the fibrillin-1 gene has been used by Pereira *et al.* (1994) in familial studies of Marfan's syndrome. Foster *et al.* (1993), described a dinucleotide $(AC)_n$ repeat in the elastin gene.

Statistical analysis of microsatellite data is a rapidly evolving field. For earlier forms of molecular data, such as that produced by allozymes, Wright's F-statistics (Wright, 1965), e.g. F_{st} or the closely related G_{st} have most frequently been used as standard measures of population differentiation. However, the very rapid mutation rate in microsatellites, together with a stepwise and reversible mutation model have necessitated the development of more appropriate statistics that take these factors into account. R_{st} (Slatkin, 1995) is one such measure, and the Division of Chemical Pathology at Groote Schuur Hospital have been involved in developing appropriate methods for its use in better defining accurate population differentiation (O'Ryan *et al.*, 1998).

2.8.4 Hypertension

Hypertension due to atherosclerosis is one of the commonest chronic diseases affecting the general population and has been implicated as a common and important risk factor for vascular disorders including peripheral vascular disease (Makin *et al.*, 2001). Arteriosclerosis, a generic term for thickening and hardening of the arterial wall, and the cause of hypertension, is responsible for the majority of deaths in the Western world. One type of arteriosclerosis is atherosclerosis, the disorder of the larger arteries that underlies most coronary artery disease, aortic aneurysm and arterial disease of the lower extremities. It is likely that both genetic and environmental factors contribute to the incidence and severity of the disease. As fibrillin-1 and elastin form integral components of arterial walls it might be informative to establish if an association exists between polymorphic markers in either of these genes and hypertension.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

Reagent	Supplier
Dulbecco's modification of Eagle's minimal medium (DMEM)	Gibco Life Technologies (Paisley, Scotland) and BioWhitaker (Walkersville, Maryland 21793, USA)
Foetal calf serum (FCS)	Highveld Biological Supplies (Kelvin, South Africa)
Penicillin, streptomycin and neomycin	Boehringer Mannheim (Germany)
Ficoll Hipaque	Sigma (USA)
Bisbenzamide fluorochrome stain	Hoechst (Germany)
Hanks barium salt	Highveld Biological Supplies (Kelvin, South Africa)
γ^{32}PATP and [3-^{14}C]-serine	Amersham Life Science (Buckinghamshire, England)
Thermus aquaticus polymerase (*Taq*)	Gibco Life Technologies (Paisley, Scotland)
Avian Maloney virus (AMV) reverse transcriptase	Boehringer Mannheim (Germany)
Dithiothreitol	Boehringer Mannheim (Germany)
NADH	Boehringer Mannheim (Germany)
Fast-Link DNA Ligation kit	Epicentre Technologies (Madison, WI 53713, USA)
RNase Inhibitor, and was obtained from	Promega (Madison, WI 53711-5399, USA).
Wizard Plus SV Minipreps	Promega (Madison, WI 53711-5399, USA).
pGEM-T Vector	Promega (Madison, WI 53711-5399, USA).
Qiaex II gel extraction kit	Qiagen (Santa Clarita, CA 91355, USA)

3.1.1 Reagents

Except where otherwise mentioned, all reagents were of Analar grade. For preparation of reagents see Appendix 1.

3.1.2 Primers

Incorrect primers for the cystathionine β-synthase gene cDNA amplification

PRIMER	POSITION	T_m	SEQUENCE
CβS 01	F -71	57°C	5'-GAAAGCTGTGGGTGCAGAGG-3' outer reverse cDNA amplification primer. (no position on correct sequence)
CβS 02	R 1393	55°C	5'-AGGGCTCAGGAAAGCGAAGG-3' outer forward cDNA amplification primer. (no position on correct sequence)
CβS 03	F 349	57°C	5'-AGTACCGCAACGCCAGCAAC-3' forward nested cDNA primer for the amplification of the 3' end of CβS mRNA. (F 208 in correct cDNA sequence)
CβS 04	R 896	57°C	5'-GCCACCACCAGGGCTTCTTC-3' outer nested cDNA amplification primer. (R 498 in correct sequence)

Table 1. Forward primers are designated F and reverse primers R. The 3'- terminal base of each primer used for cDNA amplification is numbered according to the CβS sequence Genbank 3233.D. This sequence was subsequently found to be incorrect according to the published sequence of Kraus *et al.* (1993).

Primers for the cystathionine β-synthase gene cDNA amplification and sequencing

PRIMER	POSITION	T_m	SEQUENCE
CβS 1	F -107	59°C	5'-AGTAAAACAGCATCGGAACACCAGG-3' outer forward cDNA amplification primer.
CβS 2	F -57	60°C	5'-GTCTCCTTACAGAGTTTGAGCGGTGC-3' nested forward cDNA amplification primer.
CβS 6	F 665	58°C	5'-AGTACCGCAACGCCAGCAAC-3' forward nested cDNA primer for the amplification of the 3' end of CβS mRNA.
CβS A	R 1955	58°C	5'- TCTCTTTTGCCTTTAATCCACTCTGC-3' outer reverse cDNA sequencing primer.
CβS B	R 1928	60°C	5'-CAGTCATTGCCTGTGTTCATCCTACC-3' outer reverse nested cDNA amplification primer.
CβS 3	F 186	57°C	5'-GTCCCCACATCACCACACTGC-3' forward cDNA sequencing primer.
CβS 4	F 382	59°C	5'-GAGGATGCTGAGCGCGACG-3' forward cDNA sequencing primer.
CβS 5	F 570	61°C	5'-GACGCCCACCAATGCCAGG-3' forward cDNA sequencing primer.
CβS C	R 1212	58°C	5'-GCCACCACCAGGGCTTCTTC-3' inside reverse cDNA primer for the amplification and sequencing of the 5' end of the CβS mRNA.
CβS 7	F 769	62°C	5'-ACGGGCGGCACCATCACG-3' forward cDNA sequencing primer.
CβS 8	F 1106	64°C	5'-GCTGCGTGGTCATTCTGCCCG-3' forward cDNA sequencing primer.
CβS 9	F 1140	55°C.	5'-CTACATGACCAAGTTCCTGAGCG-3' forward cDNA sequencing primer.
CβS 10	F 1295	58°C	5'-GGCACACCATCGAGATCCTCC-3' forward cDNA sequencing primer.
CβS 11	F 1359	60°C	5'-GGTAATCCTGGGAATGGTGACGC-3' forward cDNA sequencing primer.
CβS 12	F 1487	64°C	5'-TGGGCAGGCTCTCGCACATCC-3' forward cDNA sequencing primer.
CβS 13	F 1559	60°C	5'-GCACCGGGAAGTCCAGTCAGC-3' forward cDNA sequencing primer.

Table 2. Forward primers are designated F and reverse primers R. The 3'- terminal base of each primer used for cDNA amplification or and sequencing is numbered according to the published CβS sequence (Kraus *et al.*, 1993).

PCR and sequencing primers for genomic cystathionine β-synthase (exon 8) DNA

PRIMER	POSITION	T_m	SEQUENCE
CβEX8 1	Intron 7	59°C	5'-ctgccttgagccctgaagcc-3' forward intronic genomic primer for the amplification and sequencing of exon 8
CβEX8 A	Intron/exon 8	55°C	5'-ctggactcgacctacCGTCCT-3' reverse intronic/exonic genomic primer for the amplification and sequencing of exon 8
CβMIS B	Intron/exon 8	64°C	5'-ACCGTGGGGATGAAGTCGCAGC-3' reverse genomic mismatch primer for the amplification of exon 8 with a *Pvu* II restriction site

Table 3. CβSEX1 and CβSEX1 are primers used by Kozich *et al.* (1993) for the amplification of exon 8 in the cystathionine. β-synthase gene. CβMIS B is the primer designed for this study to create a *Pvu* II restriction enzyme site.

3.1.3 Study groups and patient information

All the studies for this thesis were supported by the Medical research Council and Ethics Committee of the University of Cape Town (UCT). Informed consent for both clinical and genetic investigations was also obtained from each participant (see examples of consent forms in appendix 2 and 3).

3.1.3.1 Families with homocystinuria

Family 1: The index case was a young boy (F489) with lens dislocation and homocystinuria. He had learning problems at school and died at the age of 13 years from a massive cerebrovascular accident. His sister (F488) was diagnosed with dislocated lenses and homocystinuria at the age of 2 years and she is mentally retarded. Both grand parents have a history of ischaemic heart disease, but the parents (F487 and F486) are both clinically well although they are obligate heterozygotes for cystathionine β-synthase deficiency. Two brothers from father side and three brothers and three sisters from the mother's side were also screened for defects in the cystathionine β-synthase gene. No clinical history of the aunts and uncles was available.

Family 2: The index case, a boy (F1036), was diagnosed with homocystinuria at the age of two. He has visual impairment and dislocated lenses. He performs well at school and has a normal lipid profile. The father is hypercholesterolaemic and has premature ischaemic heart disease with a total plasma homocysteine value of 30μmol/L. The mother's total plasma homocysteine is normal at 7.5μmol/L.

Family 3: The index case was a 35 year old man (F1086) with visual problems since childhood, bilateral lens dislocation and mild retardation. No family history was available but both sisters had normal total plasma homocysteine values and were not investigated further.

Homocysteine was measured in EDTA preserved blood collected from these patients. Molecular testing was performed on genomic DNA or cDNA from cultured fibroblasts obtained from skin biopsies or lymphoblasts cultured from blood.

3.1.3.2 Hyperhomocysteinaemia study groups

Abruptio placentae

A case control study was undertaken where total plasma homocysteine in 21 patients, whose pregnancies were complicated by abruptio placentae, were compared with total plasma homocysteine in 19 control patients with uncomplicated pregnancies and carefully matched for age, gravida, parity and ethnic group. Both control and patient groups were from the local Coloured community.

Total plasma homocysteine was measured 24 to 48 hours post partum in both the control and affected groups. Patients were recruited into the study group, if the index pregnancy was complicated by abruptio placentae, at a gestation of ~32 weeks or when the birth weight was less than 1000g, if the gestation was unknown. Abruptio placentae was clinically diagnosed before delivery and confirmed after delivery when the retro-placental clot covered more than 15% of the placental surface.

In the affected group of 21 patients, 10 babies were delivered by Caesarean section and 11 were delivered vaginally. Of the 19 controls, 10 were delivered by Caesarean section and 9 were delivered vaginally. All women from both groups were healthy with no evidence of renal, liver or vascular disease.

Pre-eclampsia

44 Women admitted to Groote Schuur Hospital with a diagnosis of pre-eclampsia and over 20 weeks gestational age, but who did not require immediate delivery, were recruited as subjects. Women with other medical complications of pregnancy e.g. diabetes, connective tissue disorders and cardiac disease, were excluded. Pre-eclampsia was defined according to the criteria defined by the International Society for the Study of Hypertension. These requirements were; a documented normal blood pressure prior to 20 weeks gestation with the subsequent development of 90mm Hg or more on two

occasions six hours apart, plus proteinuria of at least 0.3g in a 24 hour urine collection. Control women with uncomplicated pregnancies were recruited within one week of trial entry for each subject, from community based Midwife Obstetric Units in the Western Cape. Controls were matched for gestational age (within 1-2 weeks), ethnic group (African or Coloured) and residential area. Control women (53) were subsequently seen for antenatal care by a sister at Groote Schuur Hospital where they delivered. Controls were excluded from analysis if they developed pre-eclampsia or non-proteinuric hypertension during pregnancy.

Total plasma homocysteine was measured before and post oral methionine in women from both the control group and pre-eclampsia group at 20 weeks gestation. Total plasma homocysteine between controls and women with pre-eclampsia was initially compared in the combined ethnic groups (African and Coloured). Subsequent comparisons were done between total plasma homocysteine between controls and women with pre-eclampsia within their ethnic groups. Total plasma homocysteine was also compared between the two ethnic groups.

Peripheral vascular disease study group

Patients with early onset peripheral vascular disease, recruited from the Surgical Department at Groote Schuur Hospital, were investigated for hyperhomocysteinaemia. The group comprised of 39 men (5 Caucasians, 26 Coloureds and 8 Africans) and 31 females (4 Caucasians, 24 Coloureds and 3 Africans) between the ages of 35 to 50 years. Total plasma homocysteine has not been measured in the local general population for this study. European normal ranges (Andersson *et al.*, 1992) were used to classify patients as hyperhomocysteinaemic.

Hypertension

156 Patients with hypertension and who regularly visited the hypertension clinic at Groote Schuur Hospital were investigated. Hypertension was classified as systolic blood pressures > 160mm Hg and pulse pressures > 70mm Hg (Hypertension clinic, Groote Schuur Hospital). The control subjects (n=79) with systolic blood pressures <160mm Hg and pulse pressures < 70mm Hg, were sampled from appropriate local population and matched for ethnicity. They mainly consisted of patients visiting the hospital for minor ailments and had no evidence of vascular disease. Matching for age and sex was not required in this form of autosomal gene analysis. Patients with high pulse pressure (large

difference between systolic and diastolic pressures) were of particular importance for this study, since this picture would be consistent with pathology of the arterial elastic tissue.

3.2 Methods

3.2.1 Tissue culture techniques

Standard tissue culture procedures were used (Adams, 1980).

3.2.1.1 Fibroblasts

Primary cultures

Human fibroblasts were obtained from skin biopsies, 3 x 3mm in size, taken from the forearm. The skin biopsies were transported in a container with 3ml Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (Delta), this will be referred to as complete medium, to which penicillin (30mg/L), streptomycin (50mg/L) and neomycin (25mg/L) (PSN), was added. The tissue was finely sliced into ±1 x 1mm pieces, the tissue fragments then dispensed into several 35 x 10mm petri dishes and covered with a sterile coverslip. The coverslip was placed on top of the skin fragments to keep them in place, thus enabling the fibroblasts to attach to the surface of the tissue culture dish. 2.5ml of complete medium containing PSN was added to each petri dish and the dishes were incubated in a Hotpack humidified incubator in an atmosphere of 10% CO_2 at 37°C. The tissue culture medium was changed twice weekly until a confluent growth of cells was obtained out of the explant. The coverslips were inverted, cells side up, and transferred into a new 35mm petri dish. When cell growth in both the original dishes and the dishes containing the coverslips were confluent, the cells were trypsinized with 0.125% trypsin / 0.5 mM EDTA, (Adams, 1980) and seeded into a 50ml tissue culture flask (Greiner). The first trypsinization was designated as passage 1. Subsequent subcultures were performed in the ratio of one flask into two flasks and were recorded as the next passage number.

Maintenance of fibroblast cultures

Once the fibroblast cultures were established, antibiotic (PSN) use was discontinued. Antibiotics will mask low-level bacterial and fungal infections. Antibiotics will also mask the introduction of mycoplasma due to unsatisfactorily sterile technique. The fibroblasts were maintained with complete medium, which was replaced twice weekly. Subculturing was performed when the fibroblasts were confluent and was done by decanting the growth medium, washing the flask out twice with trypsin/EDTA (Adams, 1980) to remove any foetal calf serum as this inhibits the action of trypsin. The cells were incubated at 37°C, containing 2ml of trypsin, with intermittent shaking until they were detached from the substratum. The cell suspension was diluted with the required amount of complete medium and divided into two flasks. Before freezing and during experimentation the cultures were regularly screened for mycoplasma (see paragraph 3.2.1.3, page 34).

Storage of fibroblasts

The fibroblasts were frozen at an early passage number in complete medium plus 10% glycerol at 1°C per minute and stored in liquid nitrogen.

3.2.1.2 Lymphocytes

Transformation of lymphocytes to lymphoblasts

A volume of 10ml heparinised blood was diluted with 10ml complete medium (DMEM + 10% foetal calf serum + PSN). Diluted blood (10ml) was layered onto 5 ml Ficoll Hypaque in a 15 ml sterile centrifuge tube. This mixture was centrifuged at 2500rpm for 20 minutes. The lymphocytes (layer between Ficoll and plasma) were carefully removed without aspirating too much Ficoll. The lymphocytes were washed first in 15ml and then in 5ml complete medium at 1000rpm for 10 minutes. The medium was discarded after which 2,5ml complete medium, 2.5ml concentrated Epstein Barr virus (EBV) and 250µl phytohaemaglutinin (PHA) was added to the cells. This mixture was transferred to a 50ml flask and incubated standing upright, at 37°C for ± 3 days. After three days, as much medium as possible was removed and 2.5ml fresh complete medium was added.

Maintenance of lymphoblast cultures

The medium was changed twice weekly. If the cell growth was vigorous, cells were removed before fresh medium was added and when cell growth was scanty, care was taken not to add too much medium. Once there were sufficient cells, the culture was transferred to a 250ml flask.

Storage of lymphoblasts

A 50ml cell suspension was centrifuged at 1000rpm for 10 minutes. The cell pellet was suspended in 7ml 10% glycerol made up in DMEM + 20% foetal calf serum. The cell suspension was then frozen in 1ml aliquots in liquid nitrogen.

3.2.1.3 Mycoplasma screening

A Bisbenzamide fluorochrome stain, (Hoechst No. 33258) was used to screen for mycoplasma, using a modification of the method described by Chen (1977). Fibroblasts were grown in 35x 10mm petri dishes on a coverslip in antibiotic free complete medium for four to five days, fixed with methanol:acetic acid (3:1) , dried and stained with fluorescent stain, (0.5ug/ml Hoechst 33258 in Hanks Barium salt Solution (Highveld)), for 5 minutes and examined for mycoplasma using a fluorescent microscope (Nikon). Smears of the lymphoblast cultures were checked similarly. It is essential to keep the cultures mycoplasma-free, since these organisms may contribute to overall metabolic fluxes observed. The fibroblast and lymphoblast cultures used in this study, were found to be free of mycoplasma contamination at all times

3.2.2 DNA Extraction

3.2.2.1 Small scale DNA extraction from whole EDTA blood

A rapid method for small scale DNA extraction from whole blood was often used throughout this study. The extraction was performed in 1.5ml microfuge tubes. Whole blood was stored at –4°C to lyse the red blood cells. To 100µl lysed whole blood and 100µl sterile water for test and blank respectively, 400µl NH_4Cl_3 was added. The tubes were mixed well by vortexing, incubated at room temperature for 20 minutes and centrifuged for 30 seconds in a microfuge. The pellets were washed three times in cold isotonic saline and resuspended in 200µl 0.05M NaOH. The mixtures were boiled for 10 minutes, after which the NaOH was neutralised with the addition of 25µl 1M Tris-HCl, pH

8.0. A volume of 3µl of the above preparation was used in subsequent PCR reactions. A commercially available kit method (Qiagen) was also used for small scale DNA extraction from whole blood.

3.2.2.2 Large scale DNA isolation/extraction from whole EDTA blood

Frozen, anticoagulated (EDTA), whole blood was used for this procedure. To 10ml thawed blood in a 50ml sterile tube, 20ml lysis buffer was added and the tubes were mixed well. The mixture was then centrifuged for 10 minutes at 3000rpm and the supernatant discarded. The pellet containing white blood cells, was washed 3 to 4 times, with lysis buffer, until the supernatant was clear. To the washed pellet the following was added, 2.25ml saline/EDTA, 250µl 10% SDS and 100µl freshly prepared Proteinase K. The pellet was suspended after the addition of the saline/EDTA, and then incubated at 56°C overnight. After the addition of 225µl 6M NaCl, the tubes were mixed well and centrifuged in a microfuge at 10000rpm for 10 minutes. The supernatant was then transferred into 12ml polypropylene tubes. DNA was precipitated by the addition of 2 volumes 100% ice-cold ethanol and mixed by inversion. Strands of high molecular weight DNA was clearly visible at this stage. The DNA was pelleted by centrifugation at 3000rpm for 10 minutes. Care was taken not to dislodge the pellet when the supernatant was discarded. The DNA pellet was washed twice with 70% ethanol, and allowed to air dry. The pellet was dissolved in 200µl TE (Tris-HCl pH 8.0, 1mM EDTA) at 4°C with occasional mixing.

3.2.2.3 DNA extraction from cell cultures

Both Lymphoblasts and fibroblasts are first digested before DNA is extracted.

Fibroblasts

Cultured fibroblasts from a confluent 250ml flask, were washed once with 10ml isotonic saline. To the adhered cells, 2 ml digestion buffer and 5mg Proteinase K was added. The closed flasks were incubated overnight at 37°C. The next day, the lysate was transferred into sterile 10ml polypropylene tubes.

Lymphoblasts

The lymphoblast suspension was transferred to a sterile 50ml centrifuge tube. The cells were centrifuged at 1000rpm for 10 minutes at room temperature. The culture medium was discarded and the lymphoblasts were washed once with 10ml isotonic saline. Two ml digestion buffer and 5mg Proteinase K was added before the tubes were incubated overnight in a 56°C waterbath.

DNA extraction

To digested lymphoblasts and/or fibroblasts, an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) reagent was added. The mixture was centrifuged at 10000rpm for 10 minutes at room temperature. The upper aqueous layer was transferred to new sterile 10ml polypropylene tubes, using pipette tips of which the tips have been widened to prevent shearing of the DNA. The DNA was again extracted with 2ml phenol:chloroform:isoamylalcohol reagent. The mixture was finally centrifuged at 10000 rpm and room temperature for 10 minutes. The aqueous upper layer was transferred to a new tube without disturbing the denatured protein at the interphase, and 2 volumes of 100% ethanol and 0.2 volumes of 10M ammonium acetate was added. The tubes were mixed by inversion and incubated at room temperature for 15 minutes, then centrifuged at 10000rpm and room temperature and for 15 minutes. The supernatant fluid was discarded. The pellet was washed in 300µl 70% ethanol and centrifuged at at 10000rpm and room temperature for 15 minutes. The DNA was dried under vacuum and the pellet was reconstituted in 200µl Tris-EDTA (TE).

3.2.2.4 Quantitation of DNA

Concentration of nucleic acids and contamination from protein were assessed as follows: The absorbance of a 10µl aliquot in 490µl water was measured at 260nm and 280nm in a quartz cuvette. The 260nm/280nm ratio was used to detect the presence of contaminating proteins. The ratio of 260/280nm should be greater than 1.8. A ratio of less than 1.8 indicates a shift towards 280nm as a result of contaminating protein. A conversion factor of 1 O.D. unit = 50µg DNA/ml was used to quantify the amount of DNA present.

3.2.3 Extraction of mRNA

3.2.3.1 Single step RNA extraction from fibroblasts and lymphoblasts

Extreme care must be taken to prevent contamination with RNAses during this procedure. Gloves were worn throughout the procedure and RNAse free aerosol pipette tips were used. The total RNA was extracted from cultured fibroblasts or lymphoblasts using the single step, acid guanidinium thiocyanate-phenol:chloroform method, modified from Chomzynski, and Sacchi, (1987). All aqueous reagents, which did not contain guanidinium thiocyanate were treated with diethylpyrocarbonate (DEPC) to destroy ribonuclease activity. This was achieved by the addition of 200µl DEPC per 100ml of reagent and mixing well before autoclaving for 30 minutes.

Fibroblasts

The culture medium was removed from the mono-layer of fibroblasts and the cells were washed with 10ml isotonic saline. The washed cells were homogenised in 1ml denaturing solution per 10^7 cells. The lysate was passed several times through a 21 gauge needle to shear high molecular weight DNA, thus enabling efficient separation of DNA from RNA in subsequent steps of the protocol. The homogenate was transferred to sterile 10ml polypropylene tubes and mixed sequentially with 0.1ml 2M sodium acetate, pH 4.0; 1.0ml H_2O saturated phenol; 0.2ml chloroform:isoamylalcohol, 49:1. Tubes were mixed thoroughly by inversion after the addition of the sodium acetate and the phenol. The suspension was incubated on ice for 15 minutes. After incubation the mixture was centrifuged at 10000g at 4°C for 20 minutes, yielding an upper aqueous phase containing the RNA separated from the proteins and DNA, which remain in the interphase and organic phase.

The aqueous phase was transferred to a clean tube. RNA was recovered from the aqueous phase by the addition of 2 volumes of 100% ethanol, followed by precipitation at –80°C for 15 minutes. The precipitate was pelleted by centrifugation at 10000g and 4°C for 20 minutes. The resulting RNA pellet was dissolved in 300µl denaturing solution, precipitated with 2 volumes 100% ethanol at –80°C for 30 minutes and washed once in 75% DEPC treated ethanol, and left at room temperature for 10 minutes to dissolve residual guanidinium, that could contaminate the pellet and later affect enzyme assays. The RNA pellet was dried under vacuum and dissolved in 200µl DEPC treated water, 400µl 100% ethanol was added and the RNA stored at –80°C. The RNA was recovered for cDNA synthesis by centrifugation after the addition of 10% of the original volume (20µl)

DEPC treated 3M sodium acetate, pH 5.2. The pellet was washed in 1ml 75% ethanol, dried under vacuum and suspended in 200µl DEPC treated water.

Lymphoblasts

Lymphoblast cultures were centrifuged at 1000rpm for 10 minutes in a Sigma 3E-1 centrifuge. The culture medium was discarded. The cells were washed once in 10ml sterile isotonic saline. The cell pellet was transferred to a 10ml polypropylene tube and 1ml denaturing solution was added. After this step the exact procedure as for fibroblasts were followed.

3.2.3.2 Quantitation of total RNA

The absorbance of a 10µl aliquot in 490µl water was measured at 260nm and 280nm. The 260nm/280nm ratio was used to detect the presence of contaminating proteins. A conversion factor of 1 OD_{260} unit = 40µg RNA/ml was used to quantify the amount of RNA present (fig. 5). Total RNA extraction from confluent fibroblast monolayers, and 10^6 Lymphoblasts, cultured in 250ml tissue culture flasks yielded between 15 - 25µg of RNA, with a 260nm/280nm ratio >1.8.

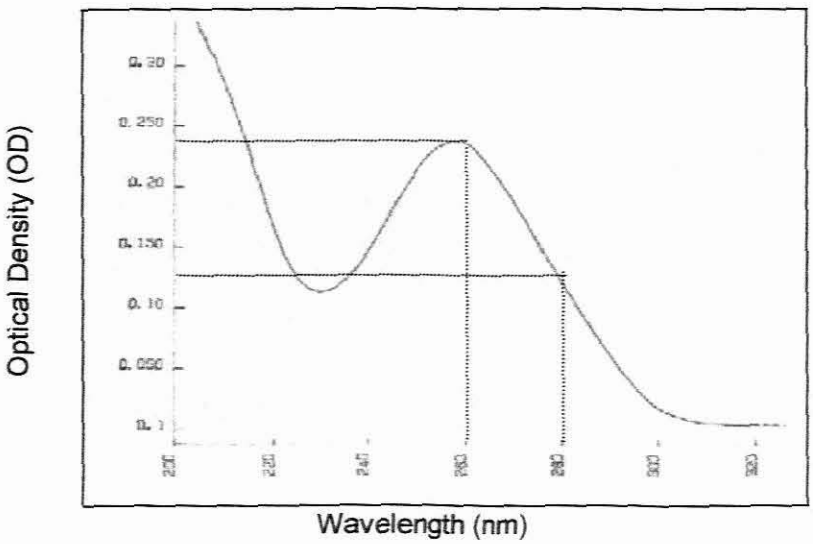


Figure 5. Scan of RNA at 260 and 280nm. OD reading at 280 and 260nm was 0.12 and 0.23 respectively. The 260nm/280nm ratio was 1.92.

3.2.3.3 Visualisation of RNA

To determine the quality of RNA extracted, 5.0μl extracted RNA was heated at 60°C for 5 minutes and then treated with 1.0μl of 10ng/ml ethidium bromide; 10μl formamide; 2.0μl formaldehyde; 2.0μl 10x loading buffer; 2.0μl 10x MOPS. This mixture was applied to a 1.2% formaldehyde/agarose gel, which was then run at 150V for 1 hour. Visualisation of the isolated RNA on formaldehyde agarose gels showed that the RNA was not degraded, with sharp 28s and 18s rRNA bands (fig. 6).

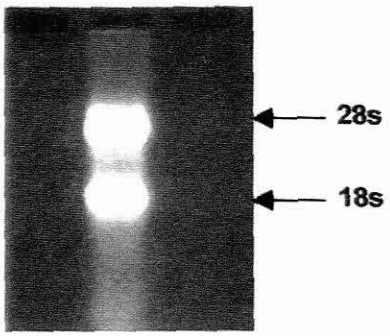


Figure 6. Isolated RNA on a formaldehyde agarose gel, showing the 18s and 28s bands. RNA, stored as an ethanol precipitate, was stable for at least a year when stored at –80°C.

3.2.3.4 cDNA synthesis from mRNA

cDNA synthesis was primed by an oligo dT primer, a 15 base primer of oligo deoxythymine. Avian Maloney virus (AMV), reverse transcriptase (Boehringer) was used for primer extension. All aqueous reagents were DEPC treated and previously mentioned precautions were taken to prevent RNAase degradation of the RNA. To 10μg (μl) RNA, 5μg oligo dT primer and 44μl DEPC treated water were added. The RNA was denatured at 65°C for 5 minutes and the primer was annealed by snap cooling the mixture on ice. The following reagents were added sequentially; 20μl 5x reaction buffer, 10μl deoxynucleotide phosphate stock (5mM each), 5 units RNAsin, a commercial RNAase inhibitor (Promega) and 80 units AMV reverse transcriptase. DEPC water was added to a final volume of 100μl, before incubation at 42°C for 1 hour.

A 100μl cDNA synthesis reaction volume proved useful for optimising PCR, but was subsequently reduced to 25μl, with a proportional reduction in the amount of template, reverse transcriptase and other components of the reverse transcription reaction. First strand cDNA preparations were stable for at least a year at –20°C.

3.2.4 The Polymerase Chain Reaction (PCR)

3.2.4.1 PCR components

Buffers and $MgCl_2$ concentration

Several buffers are available for PCR. Two buffers at a 10x concentration were prepared from autoclaved stock solutions; a 10x Tris/KCl/gelatine buffer containing 100mM Tris-HCl pH 8.3, 500mM KCl, 0.1% gelatine and a variable amount of $MgCl_2$ in the range 15 to 50mM. A second 10x buffer contained 1% Triton X-100 in 700mM Tris-HCl pH 8.8 with 15 to 50mM $MgCl_2$. The buffers were filter sterilised, divided into aliquots and stored at –20°C. Ten times detergent based buffers containing 15 mM $MgCl_2$, or no $MgCl_2$, supplied by some polymerase manufacturers, were also used.

$MgCl_2$ stimulates the polymerase and increases the T_m of double stranded DNA. $MgCl_2$ concentration also has an effect on the specificity of the PCR reaction. $MgCl_2$ was optimised for each primer set. Low $MgCl_2$ concentrations lead to low yields of DNA, whereas high $MgCl_2$ concentrations lead to the accumulation of non-specific products. The optimum $MgCl_2$ concentration can be determined by performing a titration over a range of 1mM to 5mM in 1mM steps. Additives such as bovine serum albumin or gelatin are sometimes added to bind protein inhibitors, which may be present in the template preparation. Dimethylsulphoxide (DMSO) and formamide have been reported to improve the specificity of PCR by destabilising non-specific amplification.

DNA polymerase

A number of thermostable DNA polymerases are available, for example, *Vent*, *Pyrococcus* and *Taq*. *Taq* polymerase is the least stable of the three mentioned. *Taq* polymerase does not have a 3' to 5' proof reading ability, which means it has the highest frequency of replication error. In addition, a terminal transferase actively modifies the 3' end of the PCR product by adding an additional adenine nucleotide. *Vent* and *Pyrococcus* have much greater stability than *Taq*, a higher fidelity due to 3' to 5' exonuclease activities and do not modify the 3' end of the PCR product. They are however more expensive. An advantage of *Taq* is its high processivity, compaired to the other two. DNA polymerase from a variety of manufacturers was used at 2.5U per standard 100µl reaction volume.

Template

A DNA extraction blank was always prepared along with the template to ensure that the DNA extraction reagents had not been contaminated by DNA or PCR products. The DNA blank should was amplified along with the DNA template.

Deoxynucleotide triphosphates (dNTPs)

To prevent contamination, to each of the nucleotides, dATP, dTTP, dGTP and dCTP, sterile water was added directly to the manufacturers container containing 50mg so as to make up a stock solution of 20mM of each of the four nucleotides. The pH was adjusted to 7.0, to prevent acid hydrolysis during long term storage. Equal volumes of the four 20mM dNTP solutions were combined to yield a final concentration of 5mM for each dNTP. The combined dNTPs were then divided into aliquots and stored at –80°C.

Mineral oil

A light molecular biology grade mineral oil was used when thermocyclers requiring an overlay was used, to prevent evaporation during thermocycling. In this study mineral oil from Sigma was used.

Primers

A primer analysis software program, OLIGO version 3.4 from National Biosciences Inc., MN, USA was used to design the primers used in this study. DNA oligonucleotide primers were prepared on an automated DNA synthesiser by the department of Biochemistry, University of Cape Town, South Africa and numbered according to the base pairs in the gene sequence, with the A of the ATG start codon numbered as base 1. Primers were diluted to a concentration of 50pmol/µl in TE buffer, divided into aliquots and stored at –20°C.

Thermocyclers

Techne PHC-2 and Techne Gene-E (Techne, Cambridge, UK) thermocyclers were used.

3.2.4.2 PCR conditions

The standard PCR reaction used during this study contained the following: 50*p*mole of each PCR primer, 200µM of each of dATP, dGTP, dCTP and dTTP, in a buffer containing 10mM Tris-HCl pH 8.3, 50mM KCl, 0.01% gelatine and an appropriate amount of $MgCl_2$ in the range 1.5mM to 5.0mM, as established for each primer pair by a $MgCl_2$ titration. The equivalent of 1µg of total reverse transcribed RNA or 0.1 to 1µg of genomic DNA was used for each reaction. cDNA synthesis or DNA extraction blanks were amplified for each patient to control for contamination during template preparation. A PCR blank containing all the components of the reaction, except the template, was used as a control for contamination of the amplification reaction components. The reaction was overlaid with 70µl mineral oil to prevent evaporation during 30 to 40 cycles of amplification if the thermocycler required it.

Temperature profiles

A typical temperature profile consisted of denaturing at 94°C for two minutes, annealing of primer at between 50°C and 60°C for 45 to 60s and extension at 72°C for 3 to 4 minutes. Extension was sometimes performed in a stepwise manner. Specific PCR profiles for the amplification of genes used in this study are discussed in results.

3.2.5 Agarose Gel Electrophoresis and Visualisation of DNA fragments

PCR products were separated by agarose gel electrophoresis in 1x TAE buffer and visualised on a long wavelength ultra violet (UV) light box after ethidium bromide staining. The concentration of agarose varied between 1.5% and 4%, depending on the size of the PCR product to be separated. Low melting point agarose or MetaPhor agarose (FMC BioProducts, Rocklands, ME, USA) was used at concentrations of 2% to 4%, as appropriate for low molecular weight fragments amplified.

3.2.5.1 Molecular Weight Markers for Gel Electrophoresis

Lambda DNA was digested to completion (overnight at 37°C) with *Hind* III or *Dra* I, in a buffer supplied by the restriction endonuclease manufacturer. *Hind* III digestion yielded fragment sizes of 23 130, 9 415, 6 557, 4 361, 2 322, 2 027, 564 and 125 base pairs. Digestion with *Dra* I produced fragment sizes of 8 596, 8 370, 7 834, 6 816, 6 038, 3 599, 2 303, 2 152, 1 075, 696, 533, 228, 174 and 90 base pairs.

A standard reaction mixture for enzyme digestion of Lambda DNA was:

Lambda DNA	10µl
10X Buffer	1µl
Enzyme	1µl

This mixture was incubated overnight at 37°C.

A commercial DNA marker, Bio Marker Low, with DNA fragment sizes 1 000, 700, 500, 400, 300, 200, 100 and 50 base pairs, was purchased from Bio Ventures Incorporated (Murfreesboro, Tennessee, USA).

3.2.6 Purification of PCR products for either restriction enzyme digestion, sequencing or cloning

3.2.6.1 Phenol/chloroform extraction and filtration

Millipore Ultrafree-MC 30 000 and 100 000 NMWL filter units were used to separate high molecular weight PCR products from low molecular weight primers and primer dimers. Five or one 100µl PCR reactions, depending whether product was used for restriction enzyme analysis or sequencing, were extracted with an equal volume of TE buffered phenol:chloroform:isoamyl alcohol (25:24:1). DNA in the aqueous phase was precipitated by the addition of 0.2 volumes 10M ammonium acetate and 2 volumes of 100% ethanol. After incubation for 20 minutes at –80°C, the precipitate was recovered by centrifugation at 10000g, washed with 70% ethanol and then dried under vacuum. The pellet was suspended in 300µl TE buffer and loaded into the cartridge of the filter unit and centrifuged at for 2 minutes at 7500g in a variable speed fixed angle microfuge. The filtrate was discarded and the high molecular weight DNA retained by the membrane, was then washed twice with 300µl of TE. The DNA was recovered from the membrane by rinsing with 20µl TE buffer.

3.2.6.2 Gel Purification

Five PCR reactions of a 100µl each were combined, the mineral oil overlay removed by extraction with an equal volume of chloroform and the DNA in the aqueous phase was ethanol precipitated. The DNA pellet was suspended in 20µl TE buffer and loaded into a single lane of an agarose gel. After electrophoresis, the gel was stained with ethidium bromide and the position of the DNA band to be isolated, visualised under UV light. Care

was taken to avoid damaging DNA during UV visualisation, by using a hand held, long wavelength, low intensity UV lamp.

3.2.6.3 Agarose gel extraction using commercial kits

Two commercially available kits, Promega Magic or Wizard Prep (Promega, Madison, Wisconsin, USA) and Qiaex gel extraction kit (Qiagen, Hilden, Germany), both based on the binding of DNA in solubilised agarose gel slices to a proprietary DNA binding matrix, were also used.

3.2.6.4 Micro dialysis

0,025μm VS Millipore (cat no VSWPO2500) micro dialysis filters were floated on distilled water. An appropriate volume of PCR reaction, usually about 22μl if enzyme digest is performed subsequently, was pipetted onto filter. After 5 minutes, 18μl was pipetted off the filter and placed in a 1.5ml reaction vial. The dialysed, amplified DNA was then used in the digestion reaction.

3.2.7 Restriction enzyme digestion

Restriction enzymes (RE) are enzymes that will recognize short sequences of double stranded DNA as targets for cleavage. See fig. 7.

The Pvu II enzyme recognition site

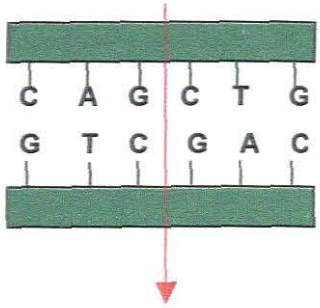


Figure 7. Recognition site of the restriction enzyme *Pvu* II. ↓ Indicates where the enzyme cuts.

This ability of the enzyme can be used to determine whether a point mutation is present in a gene. When the DNA fragment is digested with a suitable restriction enzyme (RE), it is cleaved into distinct fragments. The reaction mixture for the enzyme digestion with restriction endonucleases usually contained in the final volume of 15μl, was the following:

10X buffer M	1.5μl
RE (1 unit)	0.5μl
template	13.0μl

Certain enzymes required the DNA fragment to be purified before restriction enzyme digestion. In order to confirm that the enzyme is working, a lambda DNA control or a known positive PCR was processed identically. For this reaction the 13.0μl template was replaced with 1.0μl lambda DNA in 12.0μl sterile H_2O. Both mixtures were incubated at 37°C for 2 hours. The resulting fragments can then be visualised by agarose gel electrophoresis (see fig. 8).

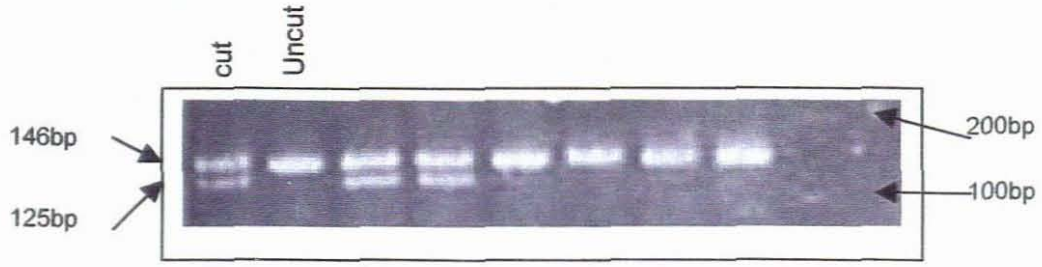


Figure 8. A *Pvu* II digest of a 146 base pair (bp) genomic DNA PCR product of the CβS gene. The PCR product is digested into two fragments, 21 and 125 base pairs in the mutant sequence. The right hand lane, show a 100 base pair marker.

3.2.8 Cloning PCR products for sequencing of an allele

PCR products were cloned to obtain a copy of a single allele of the cystathionine β-synthase gene. The cystathionine β-synthase gene is fairly large at ± 2000 base pairs. Cloning of PCR fragments yield sufficient amounts of a single allele in order to sequence the allele. The PCR fragment is inserted into with a bacterial plasmid. The PCR fragment replicates along with the bacterial plasmid resulting in large amounts of the original PCR fragment. This fragment is retrieved from the plasmid by a restriction enzyme digestion.

3.2.8.1 Preparation of competent cells

Competent DH5α cells were obtained from the Inherited Metabolic Disease Unit at the University of Cape Town. They were prepared to a competency of 10^6 to 10^7 by a rubidium chloride/calcium chloride method as described in Ausubel *et al.* (1994). PCR products were gel purified to remove unincorporated primers, primer dimers and non-specific amplification products prior to cloning into the plasmid pGEM-T. Generally, 50ng of plasmid was ligated to a 10 fold molar excess of PCR product. The single 3'-A added by *Taq* polymerase (Clark, 1988) is complementary to a single 3'-T present at each end of the linearised vector, pGEM-T. This enables cloning of *Taq* polymerase generated PCR products without having to blunt-end the PCR products before cloning. PCR products were cloned into a pGEM-T vector (Promega, Madison, Wisconsin, USA) following the manufacturers recommended protocol.

3.2.8.2 Ligation of PCR product to plasmid

A ligation reaction was set up in a 1.5ml reaction vial as described below.

pGEM-T Vector (50ng)	1μl
PCR product (1μg DNA, gel purified)	6μl
T_4 DNA ligase 10X buffer	1μl
10 mM ATP	1μl
T4 DNA Ligase (1 Weiss unit/μl)	1μl

This mixture was incubated at room temperature overnight

3.2.8.3 Transformation

Two agar plates, containing 100μg ampicillin were prepared beforehand, and coated with 4μl of 100mM (in water) isopropyl-1-thio-β-galactoside (IPTG) and 40μl 20mg/ml dimethyl formamide, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal). The tube containing the ligation mix was centrifuged to collect the contents at the bottom of the tube. Into a microfuge tube on ice, 3μl ligation mix was pipetted. An aliquote of competent DH5α cells were removed from the –70°C storage and placed on ice until just thawed. The cells were then mixed by gently flicking the tube. 3μl of ligation mix was careful added to 50μl of thawed cells. This mixture was left on ice for 45 minutes. The cells were heat shocked by heating at 37°C for exactly 30s and 950μl 2x TY broth was added to the tubes after which the tubes were shaken for 1 hour at 37°C, to allow expression of the ampicillin gene. After the incubation the tubes were centrifuged for 30s in a microfuge. Excess medium was discarded. The pellet was suspended and 50μl was plated out onto XGAL/IPTG/AMP plates with a glass rod. The plates were incubated at 37°C overnight.

The next day the recombinants (clones unable to metabolise XGAL due to insertional inactivation of the α-peptide coding region of the enzyme β-galactosidase after induction with IPTG) were selected and grown to saturation in 2x TY medium overnight at 37°C. The recombinants are white and the plasmids without the insert are blue, making it easy to distinguish between the colonies.

Plasmid DNA was prepared by a modified alkaline lysis miniprep method as described by Kraft *et al.* (1988) as follows. The mini cultures were centrifuged in a 1.5ml microfuge tube, 1.5ml at a time. The pellet was suspended in 100μl GTE (50mM glucose, 10mM EDTA and 25mM Tris-HCL, pH 8.0) and incubated at room temperature for 5 minutes. Freshly prepared, 0.2N NaOH/1% SDS (200μl) and 150μl ice cold potassium acetate, pH 4.8 (7ml glacial acetic acid and 3ml distilled H_2O and 15ml 5M potassium acetate), were added sequentially and after each addition the mixture was mixed and incubated on ice for 5 minutes. The microfuge reaction tubes were then centrifuged in a microfuge for 3 minutes. The DNA in the supernatant was precipitated with 1ml cold 100% ethanol and incubated at room temperature for two minutes. The pellet was washed with 70% ethanol and suspended in 10μl TE (Tris-EDTA). The presence of an insert was confirmed on a 0.8% agarose gel, by comparing the mobility of the intact recombinant plasmid to that of a plasmid containing no insert. The plasmids that contained an insert were cultured overnight at 37°C in 20μl 2x TY medium and ampicillin, in 50ml sterile screw cap tubes.

3.2.8.4 Miniprep method for the purification of cloned products

A volume of 1.5ml of the cell culture was transferred to 1.5ml microfuge reaction tube and centrifuged for 2 minutes at 10000rpm in a microfuge. The supernatant was discarded. An additional 1.5ml culture was added and the tubes were centrifuged again for 2 minutes. The last supernatant was removed completely. The cells were suspended in 100µl ice cold TE, by vortexing. After 5 minutes incubation at room temperature, 200µl freshly prepared solution, containing 0.2N NaOH and SDS1% was added. The viscous samples were mixed by inversion and incubated for 5 minutes on ice. 150µl of ice cold potassium acetate (7ml glacial acetic acid and 3ml distilled H_2O and 15ml 5M potassium acetate), pH 4.8 was added, mixed briefly by vortexing and incubated on ice for 5 minutes. Following centrifugation in a microfuge at 4°C for 5 minutes, the supernatants were transferred to new microfuge tubes after which centrifugation was repeated. The approximate 400µl supernatant was transferred to new tubes, avoiding the carry over of any precipitate. The supernatants were incubated with 1µl (50µg/ml) RNAse A at 37°C for 30 minutes. Proteins were extracted with an equal volume of phenol/chloroform (1:1, saturated with 10mM Tris-HCl, 1mM EDTA pH 8.0 (TE)) by vortexing and centrifuging in a microfuge for 2 minutes at 10000rpm. The aqueous phase was transferred to a clean 1.5ml reaction tube and DNA was precipitated with 2.5 volumes of ice cold 100% ethanol at –70°C for 1 hour. The DNA pellet was washed once with cold 70% ethanol. The supernatant was discarded and the pellets were dried under vacuum. DNA pellets were dissolved in 16.8µl distilled H_2O. The following solutions were then added with mixing in between; 3.2µl 5M NaCl and 20µl 13% polyethylene glycol 8000 (PEG). The mixture was incubated on ice at 4°C for a minimum of 30 minutes. The DNA was precipitated by centrifugation at 10000rpm and 4°C for 10 minutes. The pellets were rinsed in 70% ethanol, dried under vacuum and reconstituted in 20µl distilled H_2O.

3.2.9 DNA sequencing

Initially all sequencing was performed using the Sanger dideoxy method (Sanger *et al.*, 1977) with the Sequenase Version 2.0 kit purchased from United States Biochemical, Cleveland, Ohio, USA. Later, automated sequencing became available. Automated sequencing was performed by the Core Facility at The University of Cape Town using a ThermoSequenase dye terminator cycle sequencing kit (Amersham Life Science) with an ABI 373 DNA sequencer (Perkin Elmer) and at Stellenbosch University using the Big Dye method using a ABI Prism Big Dye terminator cycle sequencing ready reaction kit, version 2.0 by Applied Biosystems, CA, USA.

3.2.9.1 Alkaline denaturation of cloned PCR products

3 to 5µg of purified plasmid was alkaline denatured, before sequencing using the basic Sequenase protocol (United States Biochemical Corporation, 1987) without detergents. For the alkaline denaturation, to 20µl DNA, 2µl freshly prepared solution of 2N NaOH/2mM EDTA was added, this was mixed well and incubated for 5 minutes on ice at room temperature. Then 8µl 1M Tris-HCl, pH 4.5, and 3µl 3M sodium acetate were added sequentially and mixed again. DNA was precipitated with 75µl ice cold 100% ethanol for 20 minutes and centrifugation at 4°C for 5 minutes and then rinsed with 200µl ice cold 70% ethanol, centrifuged for 2 minutes at 10000rpm and 4°C, supernatant fluid removed and the pellet dried under vacuum.

3.2.9.2 Manual sequencing of PCR products from either cloned material or cleaned PCR products

Four 100µl PCR reaction volumes where combined and gel purified by one of the methods described. The basic Sequenase protocol (United States Biochemical Corporation, 1987) as suggested by the manufacturer, was modified according to the methods of Casanova *et al.* (1990) and Bachman *et al.* (1990). The protocol differed from the standard Sequenase protocol by the method of annealing primer to template and the inclusion of detergents in all the sequencing steps. The detergents, Tween 20 and MP 40 at 0.5% were included in all steps of the Sequenase protocol (Bachman *et al.*, 1990). Primers were annealed to heat denatured template by snap cooling on ethanol/dry ice (Casanova *et al.*, 1990). The 10µl annealing reaction contained 3 to 5µg of purified PCR product, 5 pmole of the sequencing primer, 2 µl sequencing buffer (USB) and 0.5% Tween 20 and MP40 1:1 detergent. After annealing, the following were added on ice: Tween 20 and MP40 1:1 to 0.5%, 2µl dGTP labelling mix diluted 1:4 (USB), 1µl 0.1M dithiothreitol (USB) and 0.5 to 1µl [^{35}S] dATP. The manganese, containing buffer (USB) was included when sequencing close to the primer. The labelling reaction was started with 2µl diluted Sequenase enzyme (USB) and incubated for 3 minutes at 18°C. 3.5µl of the labelling mix was transferred to tubes containing 2.5µl of each of dideoxynucleotides, ddA, ddG, ddC, ddT termination mixes (USB) at 37°C. The extension/termination reaction was terminated after 5 minutes by the addition of 4µl stop solution (USB).

3.2.9.3 Denaturing polyacrylamide gel electrophoresis

Sequencing reactions were separated on 6% polyacrylamide gels (19:1 acrylamide to bis-acrylamide) containing 8M urea as a denaturant, using 1x TBE as the electrophoresis buffer. Samples were heat denatured at 95°C for 1.5 minutes before application to the gel. Dried gels were auto-radiographed against ß-Max film (Radiochemical Centre, Amersham, UK) for periods of 24 hours to 2 weeks.

3.2.9.4 Automated sequencing of PCR products from either genomic DNA or cDNA

The Core Facility at The University of Cape Town performed automated DNA sequencing using a ThermoSequenase dye terminator cycle sequencing kit with an ABI 373 DNA sequencer. The following is a brief description of the manufacturer's protocol used. To 1µg of DNA and 5pmol primer in a volume of 12µl, 8µl of Sequencing Reagent Pre-mix was added. The reagent pre-mix contained 125mM Tris, pH9.5, 5mM MgCl2, T7 DNA polymerase, *Thermoplasma acidophilum* thermostable inorganic pyrophosphatase, Nonidet P40, Tween 20, 6.25% glycerol, 1.25mM dITP, 0.25mM each of dATP, dCTP, dTTP and dGTP, and uniquely dye-labeled dideoxy ATP, dideoxy CTP, dideoxy TTP and dideoxy GTP. The sequencing reactions were allowed to proceed using 30 cycles of the following temperature profile: 30s at 96°C, 15s at 45°C and 4 minutes at 60°C. The completed sequencing reactions were precipitated with 70µl 5mM $MgCl_2$ in 70% ethanol, re-suspended in 4µl formamide loading buffer and separated on a 4.75% PAGE gel and each uniquely labeled dideoxy terminator detected by its fluorescence. Sequencing reactions with Big Dye was performed according to manufacturers recommendations, with automated sequencing performed by The University of Stellenbosch.

3.2.10 Homocyst(e)ine measurement

3.2.10.1 Free plasma homocystine measurement

The method for measuring free homocystine was essentially an in-house method for measuring plasma and urine amino acids on a Beckman amino acid analyzer (Beckman System 6300 series, Beckman Instruments Palo Alto Calif. USA), currently in use in the Inherited Metabolic Disease Unit of the routine diagnostic laboratory at Groote Schuur Hospital.

Five ml blood was collected by venepuncture into vacutainer tubes, containing ethylene diamine tetra acetic acid (EDTA), as anticoagulant. The plasma was separated from the red blood cells within 30 minutes. 50µl S-2-aminoethyl-L-cysteine hydrochloride (AEC) (internal standard) standard was added to 1ml plasma and the plasma was then deproteinised with 100µl 30% salicylsulphonic acid (SSA). In the laboratory, the pH of the deproteinised fluid was checked and if not < 2.0, an additional 50µl of a 50% SSA was added. After 15 minutes, the protein precipitate was removed by centrifugation in a microfuge at 10000rpm for 5 minutes. The pH of the supernatant fluid was adjusted to 2.0 with 5N NaOH, and then filtered through a 0.2µm filter (Millipore).

The protein free filtrates were analysed with post-column ninhydrin derivitization on the amino acid analyser. The column, stainless steel, 0.4 x 10cm, packed with a spherical cation-exchange resin, was bought, pre-packed, from Beckman Instruments. Separation of amino acids was achieved by elution with four Beckman buffers, Li-A, D, E and F and conditions according to the program sheet in fig. 9. Absorption at wavelengths 440 and 570nm was recorded continuously on a Pantos Unicorder U-225M recorder. Each chromatographic run was 132 minutes long after which the column was rejuvenated with a regeneration buffer containing 1% lithium hydroxide. Homocystine eluted at 86 minutes and the AEC standard eluted at 102 minutes as shown in fig. 10.

SYSTEM 6300 PROGRAM SHEET

Date:

Program number:

METHOD: Free homocystine

Cation:

FLOW RATE: 20 mL/hour

Select		Function	Value	
B 1	Li A	*b1*	131	minutes
B 2	Li D	*b2*	30	minutes
B 3	Li E	*b3*	73	minutes
B 4	Li F	*b4*	80	minutes
B 5		*b5*		minutes
B 6		*b6*	130	minutes
T1		*t1*	130	minutes
°C		*t1*	33	°C
T2		*t2*	15	minutes
°C		*t2*	65.5	°C
T3		*t3*	75	minutes
°C		*t3*	70	°C
REAG		*r0*	132	minutes
SOLV		*So*	129	minutes
READ DATA		*rd*	132	minutes
RECY		*rt*	142	minutes
LINK		*tE*		
FLOW		*FL*	20	mL/hour
FILE		*IF*		
SMPL		*SA*		

INSTRUMENT PARAMETERS

PAR 1 *tF*	1 minutes	PAR 4 *PL*	3000 psig
PAR 2 *FC*	344.6	PAR 5 *FS*	60
PAR 3 *PC*	36.5	PAR 6 *FL*	20 minutes
		PAR 7 *TO*	2.5 °C/minute

Figure 9. Program sheet showing the conditions under which homocysteine was separated on the Beckman amino acid analyser

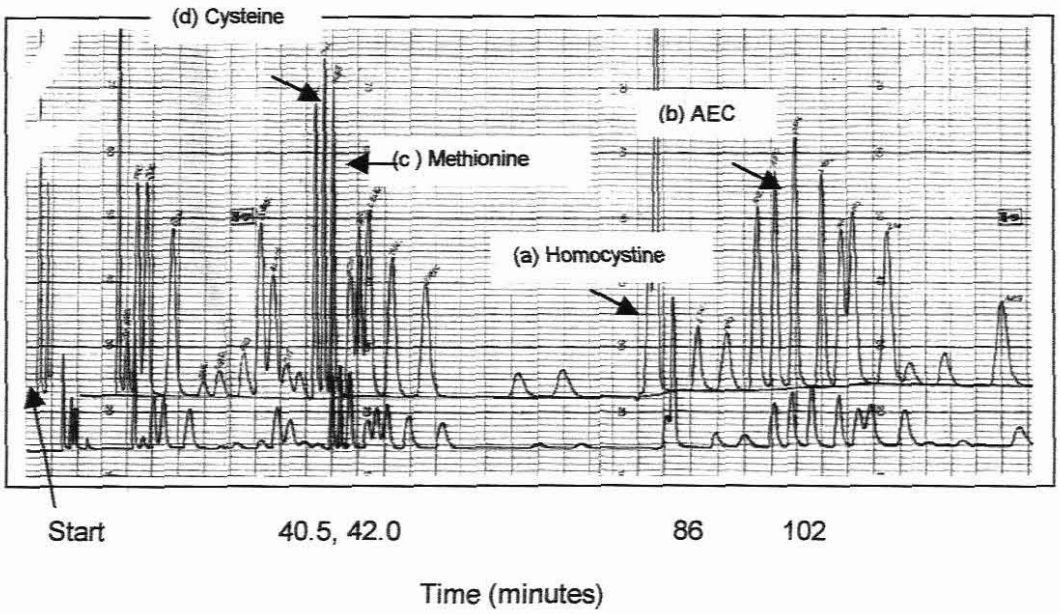


Figure 10. A chromatogram (top trace) showing the absorbance at both 440nm and 570nm of a plasma sample for the measurement of free homocystine. Peak (a) homocystine (elution time 86 minutes). Peak (b) AEC (elution time, 102 minutes), (c) methionine , elution time 42 minutes and (d) cysteine, elution time 40.5 minutes. The bottom trace is a duplicate trace at a less sensitive scale.

3.2.10.2 Total plasma homocysteine measurement

Measurement of total homocysteine consists of two stages;

- reduction of the different forms to homocysteine and
- separation and quantification of the amino acid.

Blood was collected by venepuncture into vacutainer tubes containing ethylene diamine-tetra-acetic-acid (EDTA) as anticoagulant. The plasma was separated from the red blood cells within 30 minutes and stored at - 80°C until analysis. Plasma total homocysteine is stable for several months when stored at - 80°C. Storage of plasma samples at - 80°C for 5 months during this study, lowered plasma total homocysteine levels by ± 3%.

Reduction was adapted from Brattström *et al.* (1984) and achieved by incubating 500µl plasma with 10µl 2.5mM norleucine internal standard (final concentration of 50µmol/L) and 25µl 1M dithiothreitol (DTT) solution at 37°C for 1 hour. The protein was precipitated by the addition of 100µl 50% Salicyl sulphonic acid (SSA), incubated at room temperature for 15 minutes and centrifugated in a microfuge at 10000rpm for 5 minutes. Separation and quantification of homocysteine was achieved by adapting the method (Beckman) for

measuring plasma amino acids, currently in use in the routine diagnostic laboratory. The supernatant fluid was filtered through a 0.2μm Millipore filter and the pH was adjusted to 2.0 with 5N NaOH. The supernatant was again centrifuged before an aliquot was analysed for total plasma homocysteine on an amino acid analyser (Beckman System 6300 series, Beckman Instruments Palo Alto Calif. USA) with a post-column ninhydrin derivitization.

The column, stainless steel, 0.4 x 10cm, packed with a spherical cation-exchange resin, was obtained pre-packed from Beckman Instruments. Separation of homocysteine, methionine and norleucine was achieved by elution with Beckman buffer, Li-D and conditions according to the program sheet as shown in fig. 11. Absorption at wavelengths 440 and 570nm was recorded continuously on a Pantos Unicorder U-225M recorder. Each run lasted 39 minutes.

Homocysteine eluted at 16 minutes, methionine at 16.8 minutes and norleucine at 27 minutes, as shown on the chromatographic run in fig. 10. After every run, the column was regenerated for 2 minutes with a buffer containing 1% lithium hydroxide. Ninhydrin reagent was replaced with water to prevent crystallisation of the ninhydrin at high pH in the reaction chamber during the regeneration procedure.

In the reduced form, homocysteine (16 minutes) elutes much earlier than that of homocystine (86 minutes), an internal standard (norleucine) that elutes earlier than AEC (102 minutes) was therefore used (fig. 12). This substitution shortened the chromatographic run, thus increasing the capacity of this assay. The internal norleucine standard was used as a direct standardisation of the size of the homocysteine peak and corrected for injection errors.

SYSTEM 6300 PROGRAM SHEET

Date:

Program number:

METHOD: Total homocysteine

Cation:

FLOW RATE: 20 mL/hour

Select		Function	Value	
B 1		*b1*		minutes
B 2	Li D	*b2*	42	minutes
B 3		*b3*		minutes
B 4		*b4*		minutes
B 5		*b5*		minutes
B 6		*b6*	40	minutes
T1		*t1*	40	minutes
°C		*t1*	50	°C
T2		*t2*		minutes
°C		*t2*		°C
T3		*t3*		minutes
°C		*t3*		minutes
REAG		*r0*	43	minutes
SOLV		*So*	39	minutes
READ DATA		*rd*	48	minutes
RECY		*rt*	50	mL/hour
LINK		*tE*		
FLOW		*FL*		
FILE		*IF*		
SMPL		*SA*		

INSTRUMENT PARAMETERS

PAR 1 *tF*	1 minute	PAR 4 *PL*	3000
PAR 2 *FC*	344.5	PAR 5 *FS*	60
PAR 3 *PC*	36-37	PAR 6 *FL*	20 minutes
		PAR 7 *TO*	2 °C/minute

Figure 11. Program sheet showing the conditions under which homocysteine was separated on the Beckman amino acid analyser.

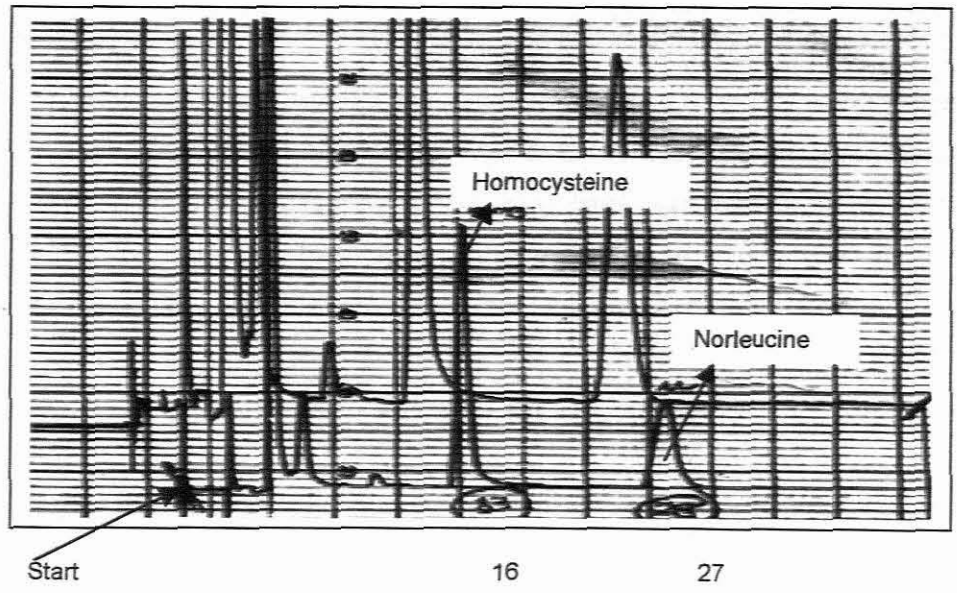


Figure 12. Chromatogram of a homocyst(e)ine standard (20μmol/L) and norleucine standard (50μmol/L). Peak (a) homocyst(e)ine (elution time, 16 minutes) Peak (b) methionine (elution time, 16.8 minutes. Peak (c) norleucine (elution time, 27 minutes).

The homocysteine concentration was calculated using an external homocysteine standard and an internal norleucine standard.

$$\frac{\text{patient peak height for Hcy}}{\text{standard peak height for Hcy}} \times \frac{\text{Standard peak height of norleucine}}{\text{patient peak height for norleucine}} \times \text{standard Hcy concentration}$$

= Homocysteine (Hcy) in μmol/L

3.2.10.3 Oral methionine

EDTA blood samples for homocyst(e)ine (free and total) determination were taken after an overnight fast and treated as above. Methionine (100mg/kg body weight) in 200ml orange juice was then administered orally to the patient. Another EDTA blood sample was taken 6 hours after methionine load. During this period of 0 to 6 hours, patients were put on a standard methionine reduced breakfast and lunch, which contained less than 14mg of methionine.

3.2.11 Cystathionine β-synthase measurement

3.2.11.1 Cell lysis for the release of cystathionine β-synthase enzyme

Washed fibroblasts and lymphoblast suspensions were lysed using three methods: sonication using a Branson sonicator (Fowler *et al.*, 1978), freezing and thawing (Fleisher *et al.*, 1973), in a dry ice ethanol mixture and treatment with digitonin (Mackall *et al.*, 1979). The protein concentrations were determined using the Bio-Rad method (paragraph 3.2.11.2, page 58) with bovine serum albumin as standards.

Sonication

Lymphoblasts were collected by centrifugation at 1000rpm at 4°C for 10 minutes, and then washed twice with cold phosphate buffered saline, pH 7.4. The final pellet was suspended in 100µl sonication buffer. A cell extract was then prepared from this suspension by sonication on ice with a Branson sonifier 250 sonicator (Branson Sonic Power Company, USA) at 10% of the duty cycle, 10% maximum output and 10 x 10 second bursts. The cell debris was sedimented by centrifugation at 10000g at 4°C in a Sigma 302K bench top centrifuge (Sigma, West Germany). The supernatant fraction was transferred to a clean microfuge tube and stored at 4°C for enzyme assay and protein determination.

Digitonin

Lymphoblasts were collected by centrifugation at 1000rpm at 4°C for 10 minutes, and washed twice with cold phosphate buffered saline, pH 7.4. The washed pellet was suspended in a solution containing digitonin 3mg/ml; sucrose 250mM; and EDTA 3mM and treated for 1 minute at –4°C. Cell debris was sedimented at 10000g in a Sigma 302K bench top centrifuge (Sigma, West Germany) at 4°C for 5 minutes. The supernatant fluid was transferred to a clean microfuge tube and stored at 4°C for enzyme assay and protein determination.

Freezing and thawing

The cells from each lymphoblast culture were collected by centrifugation at 1000rpm and 4°C for 10 minutes and washed twice with cold phosphate buffered saline pH 7.4. The washed pellet was suspended in buffer containing Tris-HCl (pH 8.6), 0.05M; and pyridoxal phosphate, 1mM. Lymphoblast extract was prepared by freezing and thawing the cell

suspension 6 times on dry ice, followed by centrifugation in a Sigma 302K bench top centrifuge (Sigma, West Germany) at 10000g and 4°C for 5 minutes. The supernatant fluid was transferred to a clean microfuge tube and stored at 4°C for enzyme assay and protein determination.

3.2.11.2 Protein determination

Protein concentration of the lymphoblast extract was determined by the Bio-Rad method (Bradford, M.M. 1976). Bovine serum albumin was used as a standard.

A volume of 5µl cell extract was diluted 1:20 with distilled water. A 100µl volume of diluted extract was added to 2ml distilled water and 0.4ml Bio-Rad protein assay dye reagent concentrate, (Bio-rad laboratories, Richmond Califomia) in a standard 10 x 10mm cuvette. The contents of the reaction cuvette was immediately mixed and the absorbance at 595nm, was read within 30 minutes in a Beckman spectrophotometer (DU-62) against a blank reaction mixture containing water instead of cell extract. Bovine serum albumin dilutions, at concentrations of between 50 - 200µg/ml were processed simultaneously.

3.2.11.3 Cystathionine β-synthase assay

The incubation reaction mixture was modified as described by Fowler *et al.* (1978) and contained in a final volume of 100µl: 50µl cell extract, +/- 200µg protein; Tris/HCl (pH 8.6), 100mM; Pyridoxal 5'-phosphate, 1mM; serine-3-[^{14}C], 30nmoles containing 1.5µCi; L- serine, 500nmoles; L-homocysteine, 15mM prepared freshly from its thiolactone. The reaction was initiated by the addition of the homocysteine solution after a 5 minute incubation at 37°C, after which each reaction was incubated at 37°C for 4 hours. After the 4 hour incubation period, the protein was precipitated with 50µl 50% SSA and centrifugation at 10000rpm for 5 minutes. The pH of a 100µl supernatant fluid was adjusted to 2.0 with 8µl 5N NaOH before separation using an amino acid analyser.

3.2.11.4 Chromatography for the separation of cystathionine and serine

In order to separate the radioactive cystathionine formed in the reaction, from the radioactive serine, the protein free supernatant was centrifuged again and analysed using an amino acid analyser (Beckman System 6300 series, Beckman Instruments, Palo Alto Calif. USA) with conditions according to program sheet in fig. 15. Ninhydrin was replaced

with distilled water to prevent possible quenching of the radioactivity during scintillation counting.

Separation of cystathionine was achieved by eluting for 30 minutes with an elution buffer containing lithium citrate. Initial temperature was 33°C. At 15 minutes, a temperature gradient of 2.5 °C/minute started, which finally resulted in a temperature of 65.5 °C at 51 minutes. At 30 minutes, a buffer, containing 96% water, 2% lithium citrate and 1% lithium chloride, pH 3.0, was introduced. After 52 minutes the column was regenerated with a regeneration buffer containing 99% water and 1% lithium hydroxide. Initially samples were collected at one minute intervals from between 10 to 50 minutes. The cystathionine peak eluted at 42 minutes. The elution time of cystathionine was confirmed by adding excess cold cystathionine and using ninhydrin to visualise the exact position of cystathionine. The cold cystathionine peak eluted at the same time as the [^{14}C] cystathionine.

One minute fractions, from 39 to 50 minutes (cystathionine peak) were collected during subsequent experiments as shown in figure 13. The fractions containing cystathionine were collected in 10ml Packard Hionic Fluor scintillation fluid and counted in a Beckman LS6000IC liquid scintillation counter using an open window. To determine the specific activity, 10µl of the original protein free supernatant was also counted. The amount of cystathionine formed was quantitated by combining the radioactivity in the fractions under the cystathionine peak and subtracting from this a blank value (the sum of the counts in the same fractions, collected from a reaction mixture containing no cell extract).

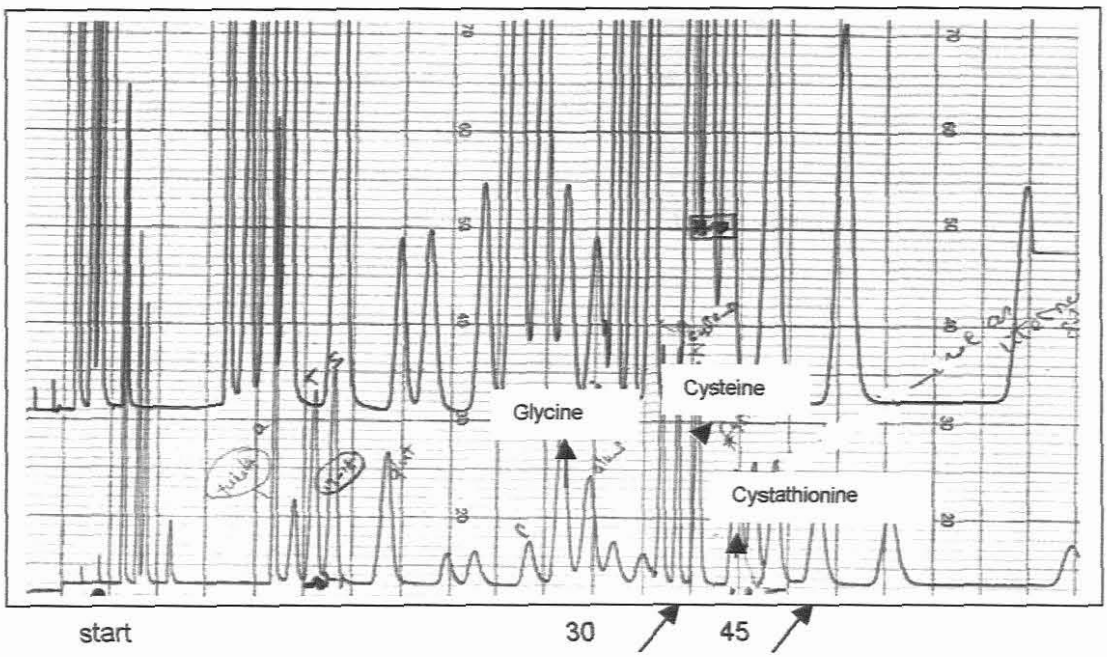


Figure 13. Chromatogram, showing radio-labelled peaks and sampling points of glycine, cysteine and cystathionine. Arrows indicate start of collection at 39 minutes and end of collection at 50 minutes.

Calculation for cystathionine β-synthase (CβS) determination

CBS enzyme activity was calculated as follows. One unit of CβS enzyme activity is defined as that which produces 1 nmol of L-cystathionine/hour. Enzyme specific activity, is expressed in units per milligram cell protein

$$\frac{^{a}\text{cpm in cystathionine and cysteine peak}}{^{b}\text{total cpm}} \times \frac{^{c}\text{serine}}{1} \times \frac{1}{^{d}\text{protein conc.}} \times \frac{^{e}\text{dilution}}{1} \times \frac{1}{^{f}4}$$

Figure 14. Calculation for cystathionine β-synthase determination. [a]Radioactivity in cystathionine and cysteine peak of patient minus radioactivity in cystathionine and cysteine peak of blank. [b]Radioactivity in reaction mixture. [c]Serine concentration in reaction mixture, in nmoles. [d]Protein concentration of cell extract in reaction mixture, in mg. [e]Dilution factor after protein precipitation (130/100). [f]Duration of incubation in hours.

SYSTEM 6300 PROGRAM SHEET

Date:

Program number:

METHOD: Cystathionine

Cation:

FLOW RATE: 20 mL/hour

Select		Function	Value	
B 1	Li A	*b1*	52	minutes
B 2	Li D	*b2*	30	minutes
B 3		*b3*		minutes
B 4		*b4*		minutes
B 5		*b5*		minutes
B 6		*b6*	51	minutes
T1		*t1*	51	minutes
°C		*t1*	33	°C
T2		*t2*	15	minutes
°C		*t2*	65.5	°C
T3		*t3*		minutes
°C		*t3*		°C
REAG		*r0*	53	minutes
SOLV		*So*	50	minutes
READ DATA		*rd*	63	minutes
RECY		*rt*	65	minutes
LINK		*tE*		
FLOW		*FL*	20	mL/hour
FILE		*IF*		
SMPL		*SA*		

INSTRUMENT PARAMETERS

PAR 1 *tF*	1 minutes	PAR 4 *PL*	3000
PAR 2 *FC*	344.6	PAR 5 *FS*	60
PAR 3 *PC*	36.5	PAR 6 *FL*	20 minutes
		PAR 7 *TO*	2.5 °C/minute

Figure 15. Program sheet showing the conditions under which cystathionine was separated on the Beckman amino acid analyzer.

3.2.11.5 Microsatellite analysis

Microsatellites are repeat sequences of a short block of nucleotides at specific sites in normal DNA. They are usually found in introns and are not involved in the coding of genes. The most common repeats are $(CA)_n$ or $(CT)_n$ repeats. Fragments of genomic DNA at two loci in the fibrillin-1 gene and one locus in the elastin gene were amplified and sequenced in order to determine the number of these repeats in each allele for every individual of the hypertension and peripheral vascular disease study groups.

Candidate genes

The fibrillin-1 and the elastin genes were investigated for polymorphisms in intron 5 at locus 21495 and intron 28 at locus 21496 in the fibrillin-1 gene and in intron 17 in the elastin gene.

Samples

Genomic DNA was extracted from EDTA blood obtained from individuals of the peripheral vascular disease and hypertensive disease groups and normotensive controls by standard methodology (paragraph 3.2.2, page 34). Hypertension was defined as a systolic blood pressure pressures > 160mm Hg (Hypertension Clinic, Groote Schuur Hospital, Cape Town, South Africa). Some hypertensive patients were already on medication, which was a factor that needed to be taken into consideration when results obtained for this study were evaluated. The normotensive group consisted of patients that attended Groote Schuur Hospital for minor ailments, had no history of vascular disease and whose systolic blood pressures were below 160mm Hg.

Primers

The primers for PCR were designed using OLIGO software version 3.4 with the aim of producing primers with similar and relatively high optimal temperature (T_m). The primers were prepared by the Biochemistry Department of the University of Cape Town. The concentrated primer stocks were diluted to a final concentration of 50*p*mol through the use of their optical density (OD) readings and stored at –20°C until use. Primers for the amplification of the fibrillin-1 microsatellite polymorphisms were designed from the flanking sequences of loci 21945 and 21946 (table 4) and primers for the amplification of the

elastin microsatellite polymorphism were made according to primers used by Foster *et al.* (1994).

Primers for microsatellite amplification

PRIMER	T_m	SEQUENCE
F1	47°C	5' TCTCCAATTAATCATGGCAC 3'
F2	47°C	3' CTTTATTAGAACCCCTCGAA 5'
F5	51°C	5' AAA GTA GCG ATG AAA ACA AAA GTC 3'
F6	51°C	3' TA GCC CTC AAC TTA CCA TCG 5'
E1	63°C	5'-GGG ATC CCA GGT GCT GCG GTT 3'
E2	50°C	5'-ATG AGA CGT GGT CAA GGG TAT 3'

Table 4. Primers used for the amplification of microsatellites at loci in the fibrillin and elastin genes. F1 and F2 were the forward and reverse primers respectively used for the amplification of microsatellite $(CA)_n$ at locus 21945 in the fibrillin-1 gene. F5 and F6 were the forward and reverse primers respectively used for the amplification of microsatellite $(TAAAA)_n$ at locus 21946 in the fibrillin-1 gene. E1 and E2 were the forward and reverse primers respectively used for the amplification of the microsatellite $(AC)_n$ in intron 17 of the elastin gene.

End-labelling of primer for gene amplification

A 20µl cocktail containing 10x PNK buffer, 2.0µl; 50µM primer, 2.0µl; dH_2O, 12µl; [γ^{32}] ATP, 2µl (4 *p*mol); T4 PNK 5U/µl, 2µl, was incubated at 37°C for 30 minutes, then 90°C for 3 minutes and centrifuged in a microfuge and stored at –20°C.

PCR

A cocktail was prepared for ten samples, containing 10x *Taq* buffer, 10µl; 50mM $MgCl_2$, 3µl; 5mM dNTP, 4.0µl; unlabelled primer (50*p*moles/µl), 1µl; dH_2O, 66.35µl; *Taq* polymerase 0.65µl; primer (labelled), 10.0µl. This mixture was kept on ice while 0.5ml reaction tubes containing 0.5µl DNA was prepared. Labelled primer was added to the cocktail after which 9.5µl of the cocktail was added to 0.5µl of DNA. The tubes were centrifuged briefly in a microfuge and 20µl mineral oil was added to each tube.

The temperature profile was annealing at 94°C for 2 minutes and 35 cycles of denaturing at 94°C for 45s, annealing at 55°C for 45s and extension at 72°C for 45s. This was followed by a long extension at 72°C for 5-10 minutes. 10µl stop solution was added to each tube and samples were stored at –20°C until polyacrylamide gel electrophoresis.

The products of each PCR varied in size according to the number of microsatellite repeats between the two primers with fixed positions. These products (marker alleles) were fractionated according to size by electrophoresis (60 watt) through a denaturing 6% polyacrylamide gel and visualized by autoradiography. The size of the marker alleles was determined by observing their co-migration with the extension products of a sequencing AT ladder made from DNA derived from the M13 polycloning site.

End-labelling of primer for preparation of A/T ladder for microsatellite gels

A 20µl cocktail containing 10x PNK buffer, 2.0µl; -40 primer (5µM), 1.6µl; dH_2O, 8.4µl; [γ^{32}] ATP, 6µl (4 *p*mol); T4 PNK 5U/µl, 2µl, was incubated at 37°C for 30 minutes, then 90°C for 3 minutes and spun in a microfuge and stored at –20°C.

Preparation of A/T ladder

A cocktail containing dH_2O, 5µl; M13 single stranded DNA (0.2 µg/µl), 10µl; reaction buffer, 4.0µl; [$\gamma^{32}P$] –40 primer (0.5-2.0 *p*mol), 5.0µl, was incubated at 65°C for 2 minutes and allowed to cool slowly to room temperature for 15 to 30 minutes after which the reaction mixture was centrifuged in a microfuge and kept cool on ice. The ratio of primer (*p*mol) to DNA (*p*mol) should be 1:1 to 5:1. To this mixture the following was added, dH_2O, 7.0µl; DTT (0.1M), 2.0µl; sequenase (dilute 0.5µl sequenase with 0.5µl pyrophosphate and 3µl buffer), 1.0µl. This was mixed gently and centrifuged briefly in a microfuge. Termination tubes (ddATP and ddTTP) were prepared as follows. 4µl each of the ddATP and ddTTp mix was added to two ddxTP red tubes from sequenase kit. 16µl of the "M13" cocktail was added to each tube. These were mixed gently and incubate at 37°C for 5 minutes and 70°C for 7 minutes (this timing is critical). 14µl stop solution was added to each tube. The two reactions were then combined into one tube, mixed and centrifuged in a microfuge. This mixture was stored at –20°C. An aliquote of 2.5µl was heated at 90°C for 2 minutes before being loaded onto gel.

Polyacrylamide gel electrophoresis

Pouring of gel

Two glass plates sizes, one, 20 x 20cm and one, 20 x 22cm were cleaned with detergent and rinsed well with water, after which it was cleaned with dH_2O and ethanol until plates were spotless. The shorter plate was gel-slicked. 4 to 5 drops gel-slick was added to the

plate, spread evenly and left to dry. The plate was then polished with dry paper. The spacers were dampened with H_2O and placed on the sides of the long plate. The gel mix was prepared by adding 80µl TEMED to 100µl 6% polyacrylamide solution. This solution was mixed quickly and poured onto the long plate. The gel was layered from the bottom up. The top spacer was placed into position and both the sides and the top were clamped. The gel was left to polymerase for 1 hour.

Running the gel

The polyacrylamide was cleaned off the plates. The top spacer was removed and the backing plate and side arms were added. The gel was placed in the rig and 1x TBE buffer was added to the bottom and top of the tanks. The top well was cleaned by squirting buffer through the wells with a syringe. The gel was pre-run for 30 minutes at 60 to 75W after which the wells were cleaned again and the comb added. The level of the buffer in the top tank was marked to check for leaks. Wells were marked with sample identification. The PCR mixtures were heated at 94°C for 2 minutes before loading. 4µl sample as well as AT ladder at regular intervals was loaded as quickly as possible. The gel was run for the required time. At 65W it takes the 2nd blue dye (xylene cyanol) 3 hours to reach the bottom of the gel. The temperature of the gel was monitored, while running. A temperature of 65°C was found to be the best. Higher temperatures lead to cracking of glass plates and lower temperatures lead to incomplete denaturing of the sequencing products.

Autoradiograph exposure and developing

Plates were disassembled. Filter paper (Whatmans 3mm) was cut to the size of the gel. Filter paper was pressed onto the gel and carefully pealed from one side. The gel should stick to the paper. The exposed side of the gel was wrapped with cling wrap and placed in a gel drier at 80°C for 1 hour to dry, after which the dried gel was placed into a developing cassette and autorad film added in the darkroom with red light only, on. Exposure was for 12 hours to 3 days depending on the radioactivity.

In the darkroom with red light only on, the gel was placed into developer for 90 seconds, stop solution for 30s and the fixer for 5 minutes. The gel was then washed with running water for 30 minutes and dried. The microsatellites were then scored by sizing them according to the AT ladder. An example of a gel is shown in figure 16. The pitfalls in reading these gels are explained in chapter 4, paragraph 4.3.2, page 123.

Autoradiograph of a typical polyacrylamide gel with fractionated marker alleles

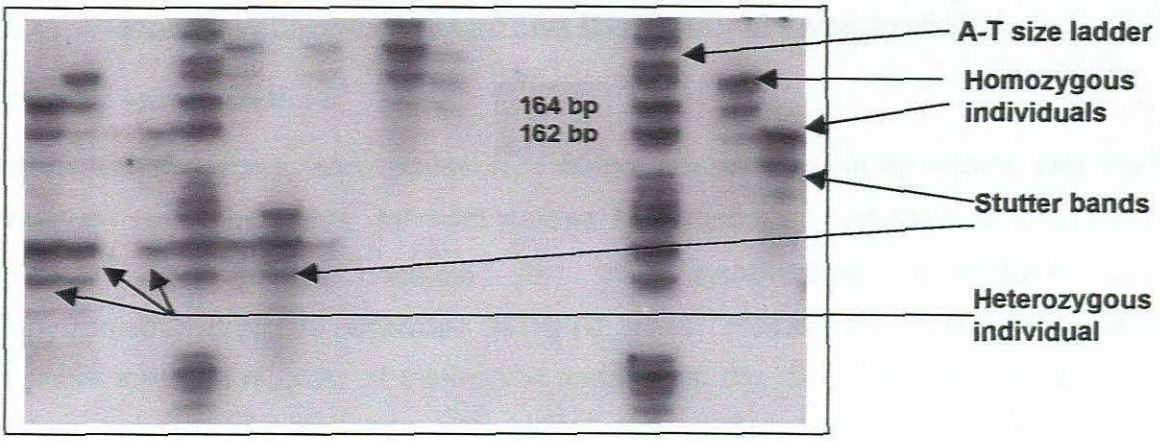


Figure 16. Example of a polyacrylamide gel of the fibrillin-1 f1 microsatellite locus, showing homozygous and heterozygous individuals and slippage products ("stutter bands").

CHAPTER 4 RESULTS

4.1 Homocystinuria: Genetic and Biochemical Investigation

4.1.1 Introduction

We investigated three index cases with homocystinuric clinical symptoms and their immediate family members, for total plasma homocysteine, cystathionine β-synthase activity, and genetic defects in the cystathionine β-synthase and methylenetetrahydrofolate reductase (MTHFR) genes. Homocystinuria is an autosomal recessive inherited disorder of methionine metabolism (fig. 17). Genetic deficiencies in two enzymes involved in methionine metabolism, namely cystathionine β-synthase and methylenetetrahydrofolate reductase, are the major causes of homocystinuria. The index case of family 1 was diagnosed as homocystinuric by a massive increase in free plasma homocystine. Cystathionine β-synthase is an enzyme that catalyses the removal of homocysteine during methionine metabolism. It is the first step of the transsulphuration pathway. (fig. 17). The method of Fowler *et al.* (1978) for the measurement of cystathionine β-synthase activity, was modified for this study. Cystathionine β-synthase activity was measured in cultured fibroblasts and/or transformed lymphoblasts. Standard tissue culture procedures were used.

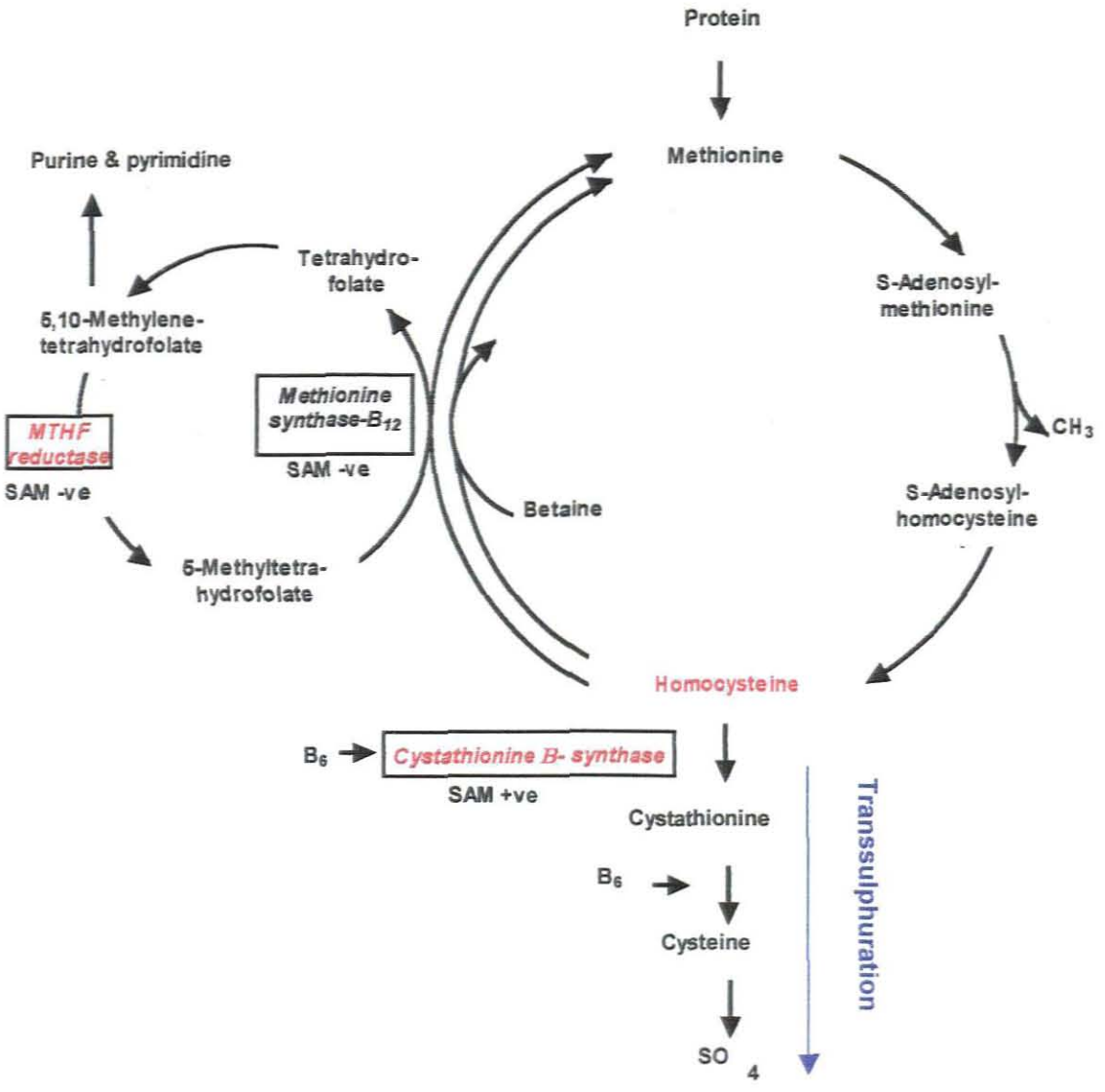


Figure 17. The methionine metabolic pathway. Cystathionine β-synthase catalyses the first step during transsulphuration, where homocysteine is transsulphurated to cystathionine. Methylenetetrahydrofolate reductase (MTHFR) catalyses the formation of 5'-methyltetrahydrofolate which is needed for the remethylation of homocysteine.

4.1.2 Cystathionine β-synthase method development

4.1.2.1 Procedure for the release of the cystathionine β-synthase enzyme from cell cultures

Three methods for the release of cytosolic enzymes from prepared cell extracts for the measurement of cystathionine β-synthase activity were investigated. These were (1) sonication, (Fowler *et al.*, 1978), (2) freezing and thawing, (Fleisher *et al.*, 1973), and (3) digitonin release, (Mackall *et al.*, 1979). These methodologies were compared to establish which one would give the best consistent yield of enzyme. The sonication procedure initially gave the best yield, measured as enzyme activity, 18.1 (±0.60)

nmoles/mg protein/hour, compared to 14.3 (±0.60) and 11.8 (±0.35)nmoles/mg protein/hour by the digitonin and freezing and thawing procedures respectively. Subsequent analysis however showed that the sonication procedure gave inconsistent yields. Repeated comparisons between digitonin release of the enzyme and release by sonication, showed digitonin release to be the most reproducible procedure. Digitonin release was therefore used for the preparation of cell extracts for cystathionine β-synthase enzyme determinations.

Protein determination

The protein concentration of the lymphoblast extract was determined by the Bio-Rad method, (Bradford, 1976). Bovine serum albumin dilutions, at concentrations of between 50 - 200 μg/ml were processed simultaneously. Protein concentrations were read off a graph shown in fig. 18.

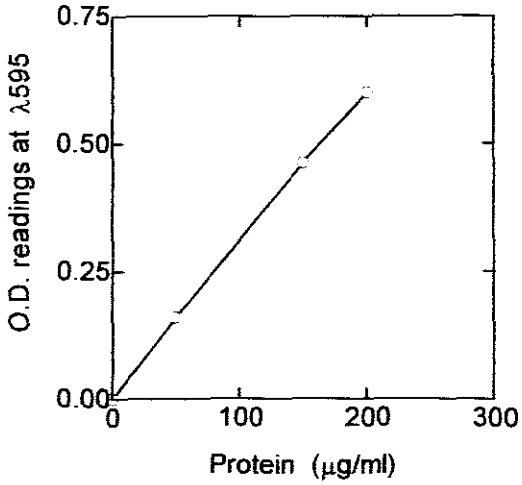


Figure 18. Protein standard curve based on duplicate determinations of bovine albumin dilutions. O.D. readings were plotted against protein concentration in μg/ml. Error bars denote mean ± SEM. Standard protein values were 0 (0 ±0.0), 50 (0.161 ±0.0005), 150 (0.462 ±0.003) and 200 (0.601±0.01). Figures before brackets denote protein concentration in μg/ml and figures inside brackets denote O.D. readings at 595nm ±SEM.

4.1.2.2 Determination of cystathionine β-synthase activity

Cystathionine β-synthase irreversibly condenses homocysteine and serine to form cystathionine. The assay to measure enzyme activity is based on the conversion of L-[^{14}C]-3-serine to [^{14}C]-cystathionine by lysates of fibroblast or lymphoblast cultures. Reaction products as shown in fig. 19, are separated by chromatographic methods (fig. 20) and the amount of radioactive cystathionine formed, determined as the amount of enzyme activity. One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 nano mole (nmole) of cystathionine per hour.

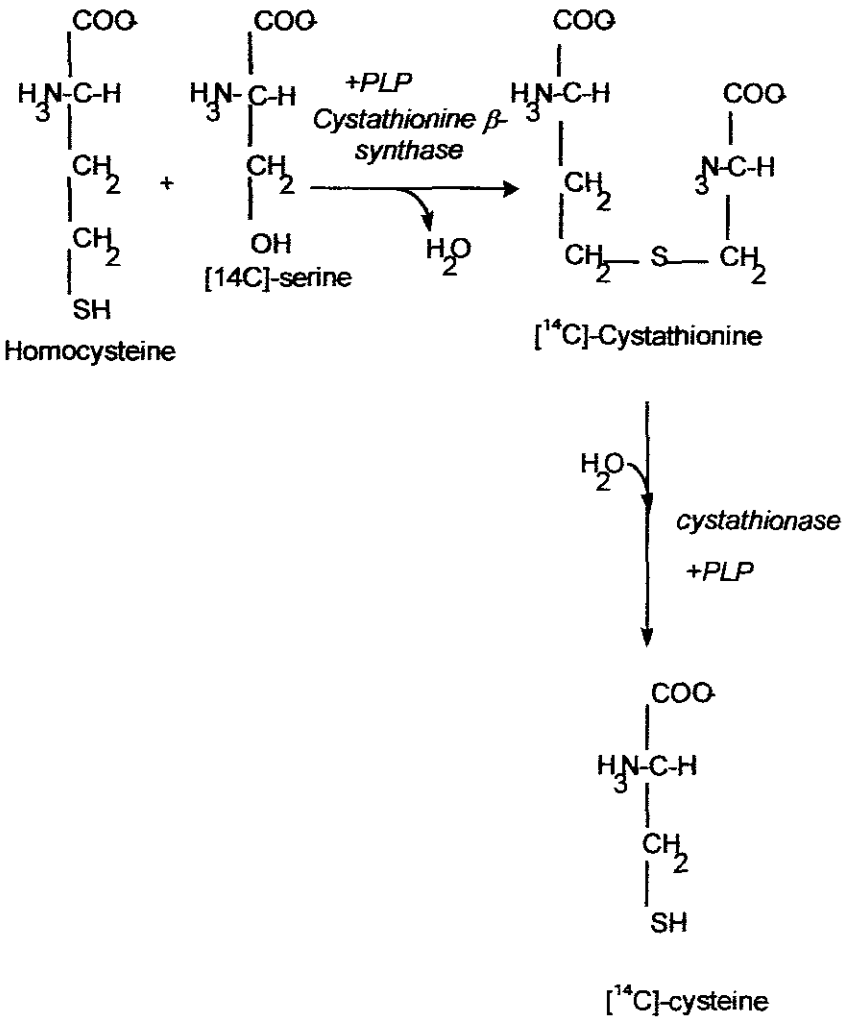


Figure 19. Reaction between radio-labelled serine and homocyst(e)ine in lymphoblast extract.

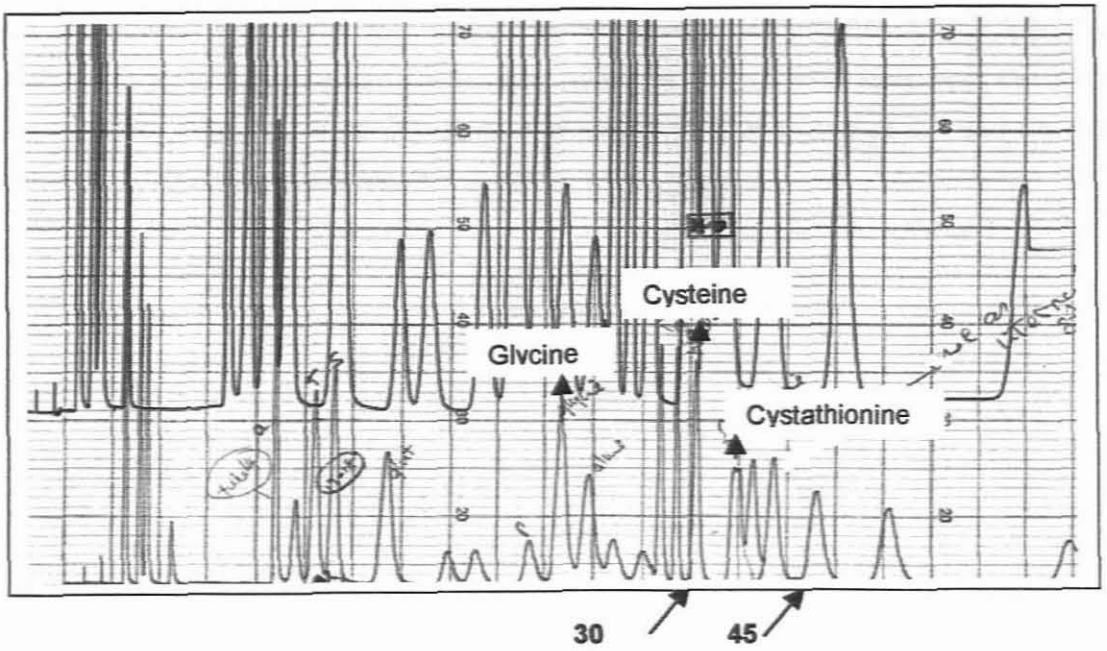


Figure 20. Chromatogram, showing radio-labelled peaks and sampling points of glycine, cysteine and cystathionine. Arrows indicate start of collection at 39 minutes and end at 50 minutes.

When [^{14}C] serine is incubated with a lymphoblast or/and fibroblast cell lysate, possible products that may form by alternative metabolic pathways are glycine, pyruvate, cystathionine and cysteine as shown in fig. 21.

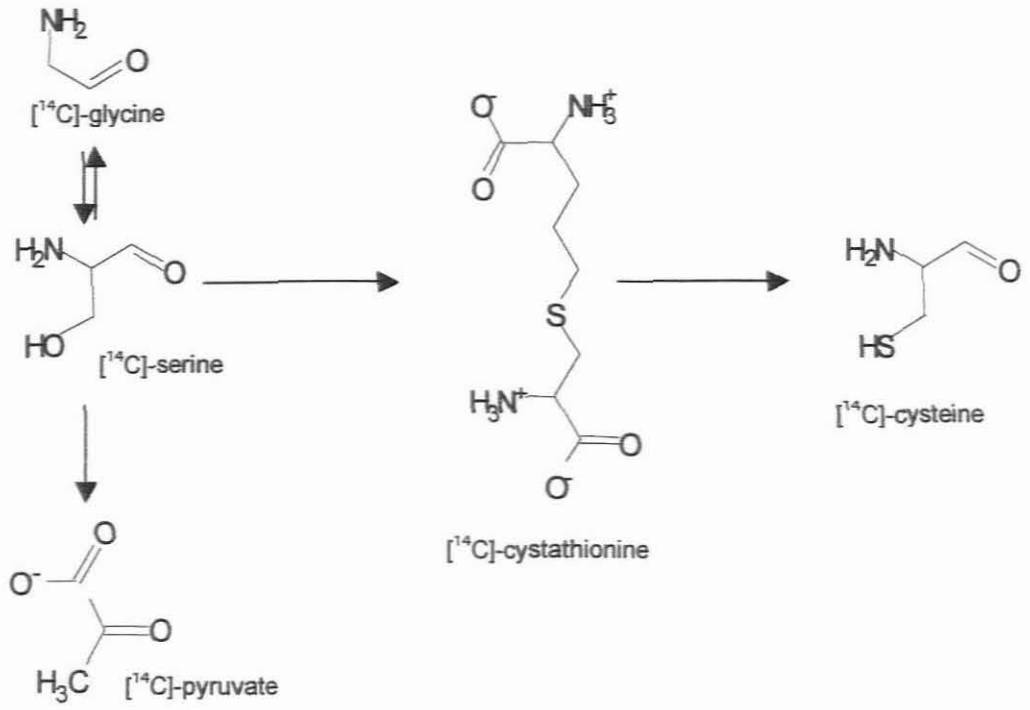


Figure 21. Possible routes for [^{14}C] radio-labelled serine.

It was important to determine that glycine and pyruvate did not elute with cystathionine and a cystathionine β-synthase assay reaction mixture was analysed on the amino acid analyzer to ascertain whether glycine and pyruvate eluted separately from cystathionine and cysteine. Fig. 22 shows that pyruvate and glycine eluted at 20 and 30 minutes respectively whereas cystathionine eluted at 45 minutes.

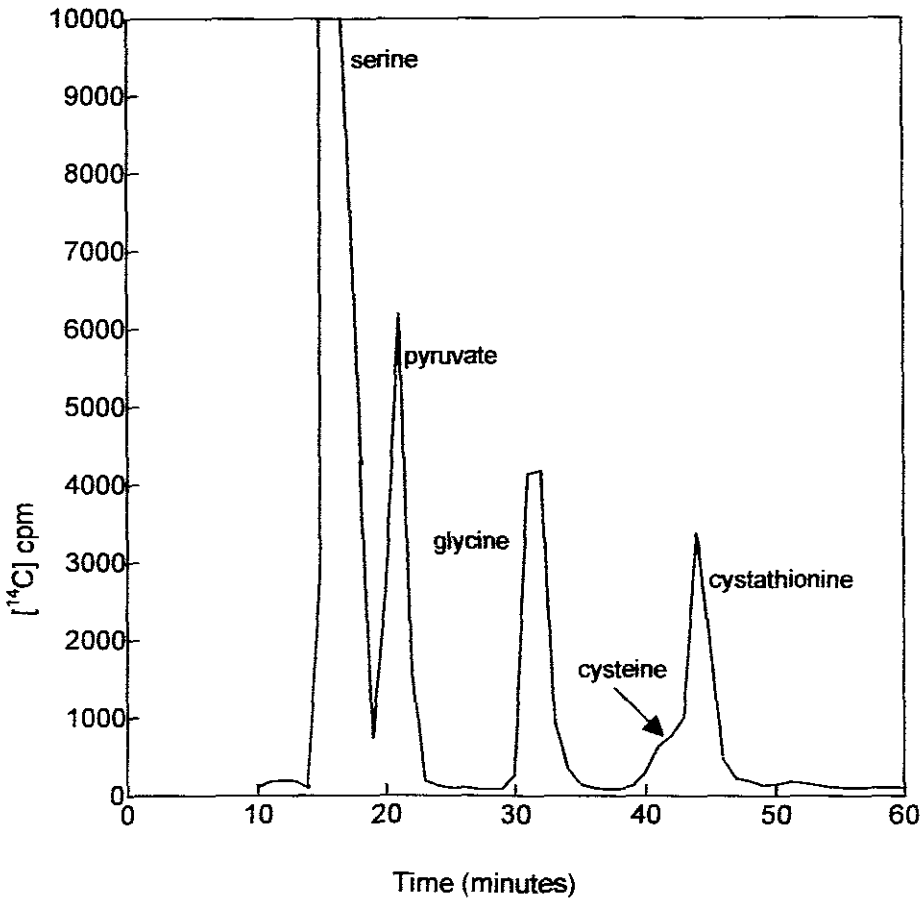


Figure 22. Possible peaks that could elute after the cystathionine β-synthase enzyme reaction mixture was analysed on an amino acid analyser. Pyruvate eluted at 20 minutes, glycine eluted at 30 minutes, cysteine eluted at 40 minutes and cystathionine eluted at 45 minutes.

The purity of the radioactive serine was confirmed by amino acid analysis of the radio labelled serine. When a cystathionine β-synthase assay reaction mixture, containing no cell extract was applied to the amino acid analyser, no radioactive counts were seen in the cystathionine peak as shown in fig. 23, confirming the purity of radio-active serine. The recovery of injected radio-active [^{14}C]-serine was 100%.

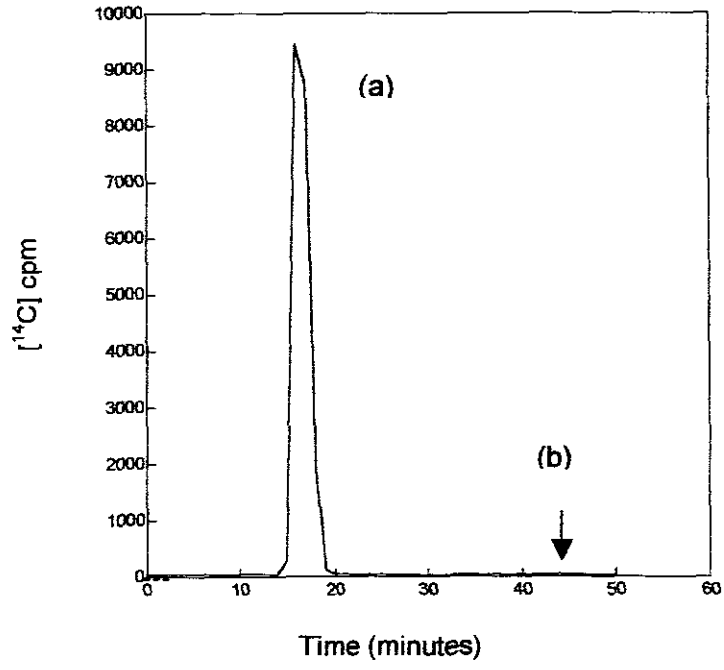


Figure 23. Chromatogram of a cystathionine β-synthase enzyme assay reaction mixture, containing no lymphoblast cell extract and 52000cpm per 100µl reaction mixture after separation on an amino acid analyser with a 50µl sample loop. (a) Serine peak (20135cpm). (b) Cystathionine peak (no cpm).

Optimization of the cystathionine β-synthase assay

In an attempt to define optimal conditions under which maximum enzyme activity could be achieved for this study, constant amounts of cell extract and [^{14}C] labelled serine was titrated with varying concentrations of serine, ranging from 0.5 to 10mmoles/L. The serine titration was performed at 15mM homocysteine and 1mM pyridoxal-5'-phosphate (PLP). Maximum enzyme activity was obtained with 10mmoles/L serine. Table 5 shows enzyme activity obtained with varying amounts of serine. Fig. 24 shows cystathionine β-synthase activity plotted as a function of serine concentration. A Michaelis Menten curve was fitted to the data using non-linear regression.

Cystathionine β-synthase activity vs serine concentration

Serine concentration (mmol/L)	Enzyme activity (nmoles/mg protein/hour)
0.5	1.0 (±0.05)
1.0	1.7 (±0.05)
2.5	3.6 (±0.05)
5.0	4.3 (±0.04)
10.0	5.7 (±0.07)

Table 5. Cystathionine β-synthase enzyme activity in nmoles/mg protein per hour obtained with different amounts of serine in mmoles/L.

The apparent K_m of Cystathionine β-synthase for serine in this study was determined to be 3.0mM with the Michaelis Menten fit. The maximal enzyme activity (V_{max}) was estimated as 7.3nmoles/mg protein/hour using the Michaelis Menten fit. (fig. 24).

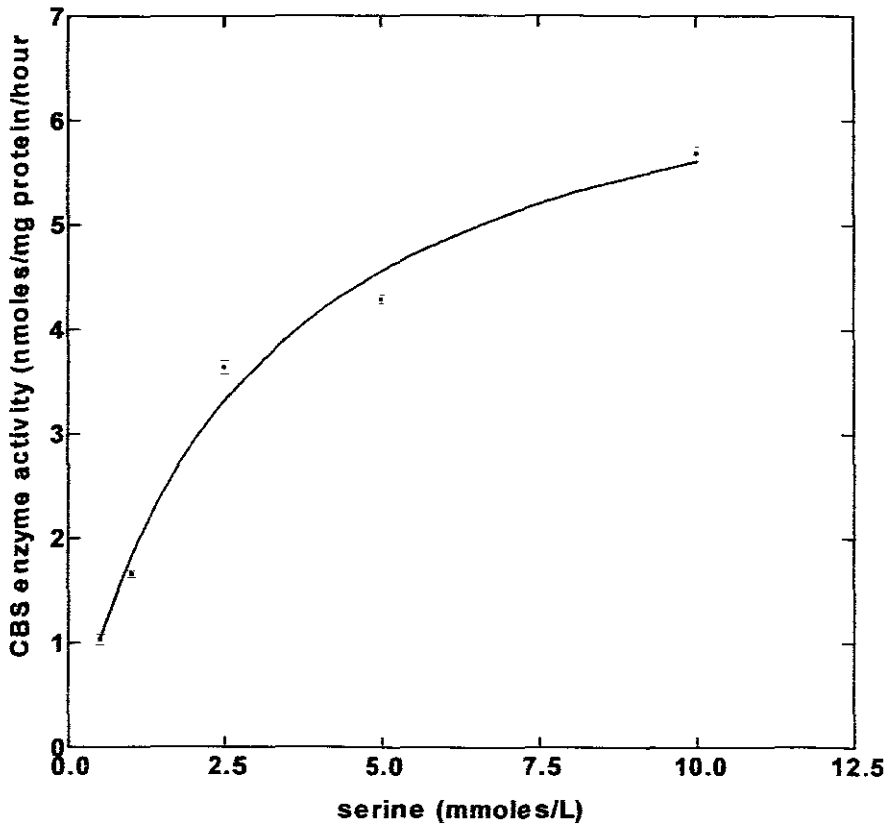


Figure 24. The effect of increasing substrate (serine) concentration on cystathionine β-synthase enzyme activity. A serine concentration of 10mM appears to yield near maximum enzyme activity. Assays were performed in duplicate and the fitted curve is described by the Michaelis-Menten equation.

When Kraus *et al.* (1978) determined cystathionine β-synthase activity in a cystathionine β-synthase rich liver homogenate, a serine concentration of 10mM, titrated to a specific

activity of 300cpm/nmole with [^{14}C] serine was used. However when they determined cystathionine β-synthase activity in cell cultures where the enzyme is not expressed fully, a lower serine concentration (2.5mM) titrated to 3000cpm/nmole with [^{14}C] serine, was used in order to improve sensitivity. In this study the specific activity was increased to 6600cpm/nmole [^{14}C] serine in order to increase the sensitivity for cystathionine detection on the amino acid analyser. To achieve this specific activity, the CβS enzyme assay was performed under sub-maximal conditions at a serine concentration of 5.0mM and not 5-10x the K_m of serine. This made the assay cost effective for diagnostic use, while little was lost in sensitivity. Previous studies by B. de Wet performed in the Inherited Metabolic Disease Unit at the University of Cape Town have shown that CβS enzyme activity obtained under conditions of maximal activity correlated well with the values obtained under conditions of sub-maximal activity.

4.1.3 Biochemical and molecular investigation of families

4.1.3.1 Biochemical investigation of family 1

The index case (patient F489) of family 1 was diagnosed with homocystinuria at the age of eight. He had lens dislocation and learning problems at school. Measurement of free homocystine in blood samples of this patient confirmed homocystinuria (see table 6). This boy died at the age of 13 from a massive cerebrovascular accident. The sister (F488) was diagnosed with dislocated lenses and homocystinuria at the age of two. She is also mentally retarded. Both grandparents have a history of ischaemic heart disease, but the mother (F486) and father (F487) are asymptomatic.

	Free Hcy (μmoles/L)	Methionine (μmoles/L)	CβS activity (nmoles/mg protein/hr)
Mother (F486)	0	-	7.2, 5.8
Father (F487)	-	-	0.9, 0.9
Daughter (F488)	26	-	0.2, 0.2
Index case (F489)	235	8488	0.2, 0.2
Reference range (men & women)	1.48 – 2.40 (Araki and Sako, 1987)	16 - 38 (Kluijtmans *et al.*, 1996)	4.0 – 22.3 (Kluijtmans *et al.*, 1996)
Reference range (wild type)			0 – 13.2 (this study)
Reference range (heterozygote)			0 – 8.7 (this study)

Table 6. Biochemical results of family 1 with homocystinuria. The cystathionine β-synthase (CβS) values represent duplicate cystathionine β-synthase enzyme determinations in cultured fibroblasts. Free Hcy denotes free homocysteine.

The free plasma homocystine, detected in the index case (F489) of this family as shown in table 8, was hundred times higher than mean free plasma homocystine found in healthy subjects (Araki and Sako, 1987). The plasma methionine in the index case (F489) was a thousand times higher than reference ranges as established by Kluijtmans *et al.* (1996). Fibroblast cystathionine β-synthase enzyme activity of the asymptomatic mother (F486) overlapped with the lower end of the reference range as detected in healthy subjects by (Kluijtmans *et al.*, 1996) and a reference range as established for this study. Cystathionine β-synthase enzyme activities detected in fibroblast cultures of the father (F487) was found to be equal to values in the lower end of the reference range for heterozygotes (0.39 – 5.4nmoles/mg protein/hr) for cystathionine β-synthase deficiency (Kluijtmans *et al.*, 1996). Both the siblings showed clinical symptoms similar to that found in patients with cystathionine β-synthase deficiency, namely optic lens dislocation, mental retardation, skeletal abnormalities and premature thrombotic events and their cystathionine β-synthase enzyme activities were similar to activities found in homozygotes for cystathionine β-synthase deficiency. On the basis of clinical symptoms and above biochemical parameters as shown in table 6, homocystinuria due to cystathionine β-synthase deficiency was confirmed in both siblings of family 1.

4.1.3.2 Molecular investigation of family 1

Cystathionine β-synthase

The cystathionine β-synthase gene of the index case (patient F489), who was diagnosed with homocystinuria, was screened for abnormalities. Fibroblast cultures from the mother (F486), father (F487) and both siblings (F489 and F488), were established from skin biopsies. mRNA was extracted from fibroblasts and reverse transcribed to cDNA. At this time the genomic sequence was not yet known and molecular studies were performed on the cystathionine β-synthase cDNA. Primers for the amplification of the cystathionine β-synthase cDNA, was initially designed, using the cystathionine β-synthase cDNA sequence from Genbank (CBS 3233.D, unpublished 1992). A semi-nested approach was followed, whereby the first amplification reaction was performed using forward primer CβS 01and reverse primer CβS 02 (fig. 25).

Amplification of cystahionine β-synthase gene with primers from incorrect sequence

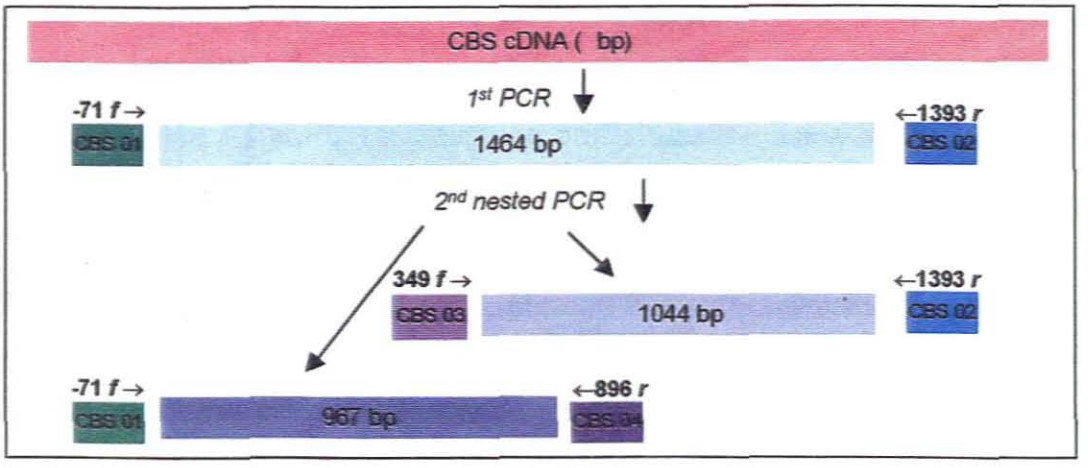


Figure 25. Schematic representation of initial primer positions and fragment sizes for the amplification of the cystathionine β-synthase gene.

The product of the first PCR (1466bp) was used as a template for a second round of amplification with primers CβS 01 and CβS 04 (967bp), CβS 03 and CβS 02 (1046bp) and CβS 03 and CβS 04 (547bp) respectively. The temperature profile comprised denaturing at 94°C for 60 seconds (s), annealing at 54°C for 60s and extension at 72°C. Extension time was increased in a stepwise manner and was 120s during the first thirteen cycles, 180s during the second set of cycles and 240s during the last thirteen cycles. No PCR product was visible on a 1.5% agarose gel either from the first or the second PCR. The PCR reaction was optimised by titration with $MgCl_2$ concentrations ranging between 1.0 and 5.0mM. Still no product was seen with any of the $MgCl_2$ titrations. We lowered the annealing temperature to 52°C in order to decrease the specificity, but still no product was detected on agarose. The next step was to re-extract and reverse transcribe mRNA to cDNA for the patient and control samples. This cDNA was checked by amplification with primers designed to amplify a fragment from the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene. This amplification resulted in the correct product. From this result it was concluded that the cDNA product for cystathionine β-synthase should be present.

The cystathionine β-synthase cDNA sequence on Genbank was assumed to be correct but when the primers were checked against the sequence published by Kraus (1993) numerous discrepancies between the two sequences were detected. The 5' untranslated region did not match and the 214 base pair insert found in some cDNA's (Kraus, 1993) was not mentioned. Matching sites in the cystathionine β-synthase sequence

(Kraus, 1993) could only be found for primers CβS 03 and CβS 04 in positions 208 forward and 498 reverse respectively. Primers CβS 01 and CβS 02 sequences were both not found on the sequence according to Kraus (1993).

New primers were designed according to the correct cystathionine β-synthase sequence of Kraus (1993). The PCR temperature profile was the same as for the initial primers except for an increase in the annealing temperature from 54°C to 58°C. The optimal $MgCl_2$ concentration for this PCR was 1.5mM (results not shown).

Previous experience in our laboratory showed that a nested primer amplification system adds sensitivity to PCR amplification of large templates and amplification of low copy number mRNA like the cystathionine β-synthase mRNA. The coding region of the cDNA was amplified, therefore using the nested primer approach. Primers CβS 1 (F-107) and CβS A (R 1955), were used to amplify CβS cDNA of 2062 base pairs (bp) as shown in fig. 23. No PCR product was detected on a 1.5% agarose gel. Subsequent amplification with nested primers CβS 2 (F-57) and CβS B (R 1928) resulted in a PCR product of 1985base pairs, which was visible on a 1.5% agarose gel as shown in figure 18. In order to improve amplification, the nested approach was applied again, whereby the 1985 base pair PCR product from the previous two amplifications, was re-amplified with primers, CβS 2 (F-32) and CβS C (R1212), and CβS 6 (F665) and CβS B (R1928), resulting in two DNA fragments of 1269 base pairs (5' end) and 1263 base pairs (3' end) respectively, with an overlapping fragment of 547 base pairs as shown in fig. 26 and fig. 27.

Amplification of cystathionine β-synthase gene with primers from correct sequence

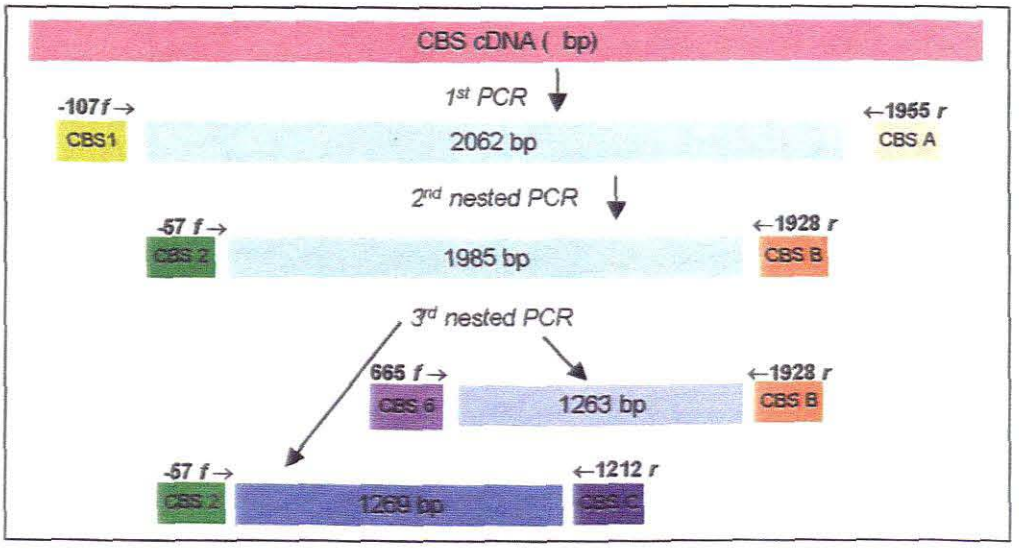


Figure 26. Schematic representation of primer positions and fragment sizes for the amplification of the cystathionine β-synthase gene.

Cystathionine β-synthase PCR

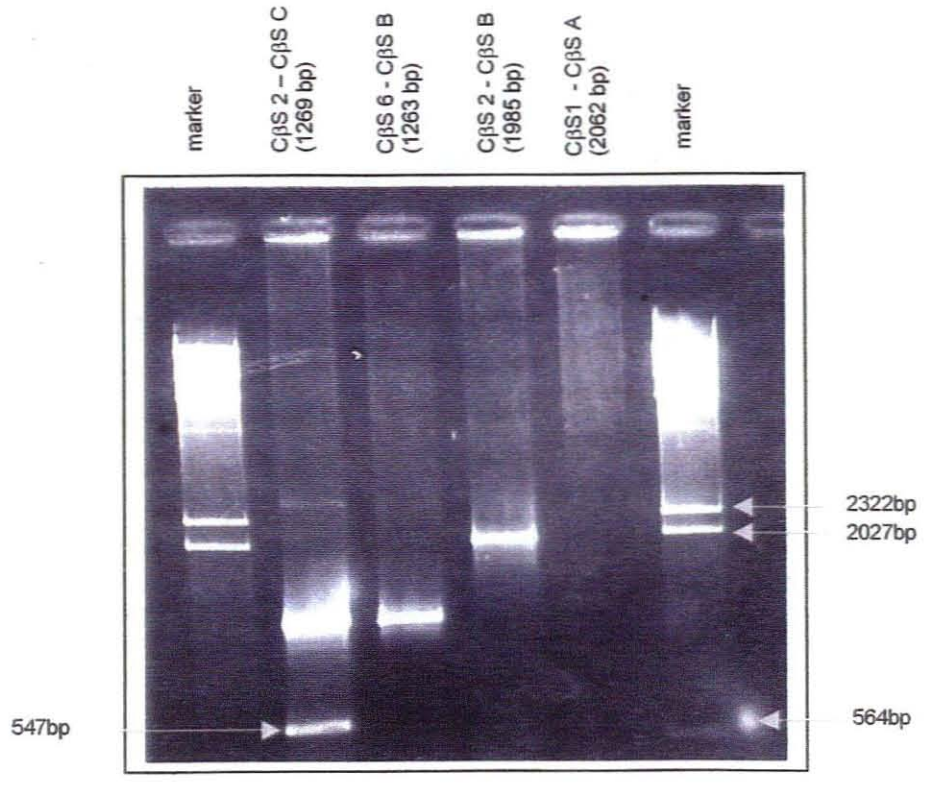


Figure 27. A 1.5% agarose gel showing fragments of the cystathionine β-synthase gene after nested PCR on cDNA of patient F489. The marker is a *Hind* III digest of lambda DNA.

In order to increase sensitivity, both fragments of, 1263 base pairs and 1269 base pairs, were first gel purified, cloned into the plasmid pGEM-T and then sequenced manually using primers from table 2. The mutations that were found were confirmed by sequencing and/or restriction enzyme analysis of the genomic DNA or cDNA. To trace the origin of the mutations that were initially identified in the cystathionine β-synthase cDNA of patient F489, genomic DNA from the sister, parents and uncles and aunts from both sides of the family were analysed.

Allele 1

8 clones from the male sibling (F489) were sequenced manually with cystathionine β-synthase sequencing primers as shown in table 2. Each sequence reaction resulted in ±200 readable base pairs. A G to A point mutation as shown in fig. 28, was detected at nucleotide 919, in 5 out of the 8 clones.

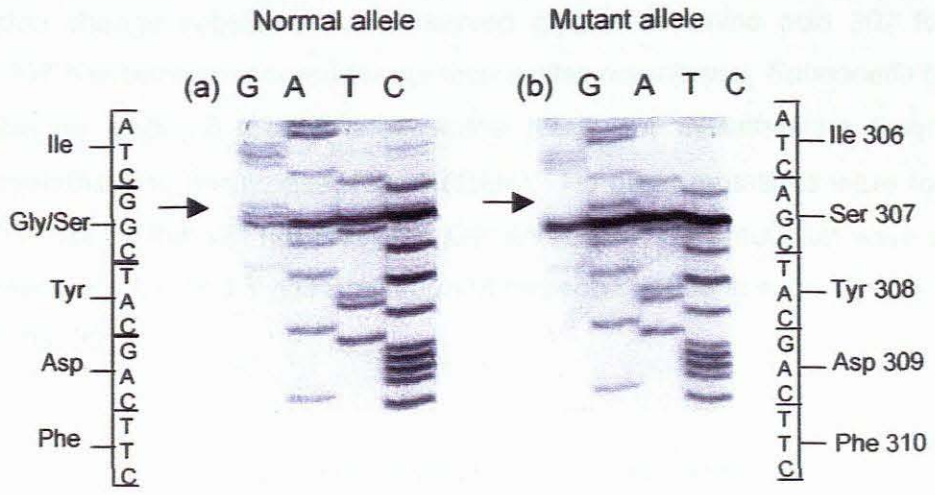
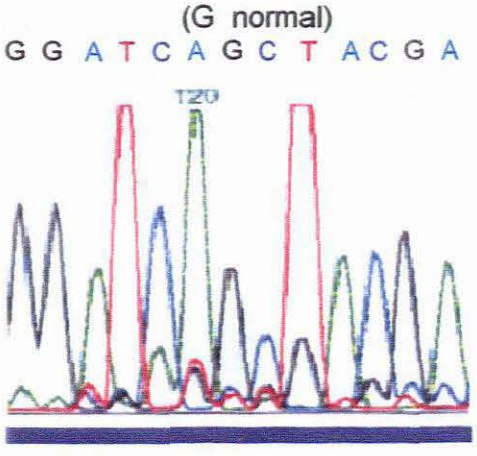


Figure 28. Autoradiograph of a sequencing reaction showing both normal and the mutant alleles for the G to A transition at nucleotide 919 in the cystathionine β-synthase cDNA obtained from patient F489 using polyacrylamide gel electrophoresis. The clones were sequenced with forward sequencing primer CβS 7 (F769) and resolved by polyacrylamide gel electrophoresis.

The G919A (Gly307Ser) mutation, occur in a GC rich region of the cystathionine β-synthase cDNA. This region was not 100% clear to read in the manual sequence as shown in fig. 28, and the G919A (Gly307Ser) mutation was later further confirmed by automated sequencing of the cDNA (fig. 29).



Exon 8 – G919A (Gly307Ser)

Figure 29. Sequence profile of the mutant allele of patient F489 carrying the G919A (Gly307Ser) transition that leads to a glycine to serine transition at amino acid 307.

The G919A (Gly307Ser) mutation resulted in a codon change from a GGC to a AGC. This codon change substituted a conserved glycine at amino acid 307 for a serine. Glycine 307 has been conserved for spinach cysteine synthase, *Salmonella typhimurium* O-acetylserine lyase, *E. coli* O-acetylserine lyase, rat cystathionine β-synthase and human cystathionine β-synthase, Kraus (1994). No other mutations were found on this allele. The clones that did not carry the G919A (Gly307Ser) mutation were checked for other mutations. A C to a T point mutation at nucleotide 1058 in exon 10 was detected as shown in fig. 30.

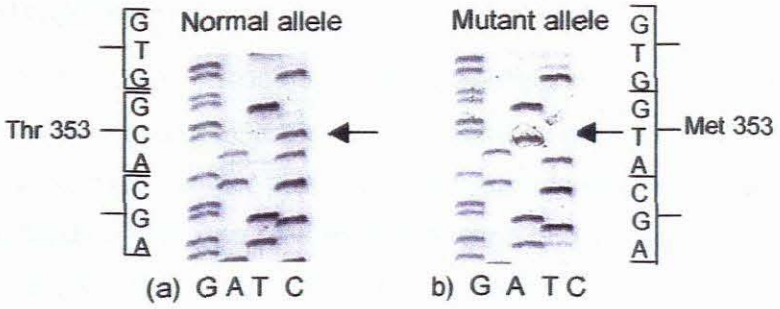
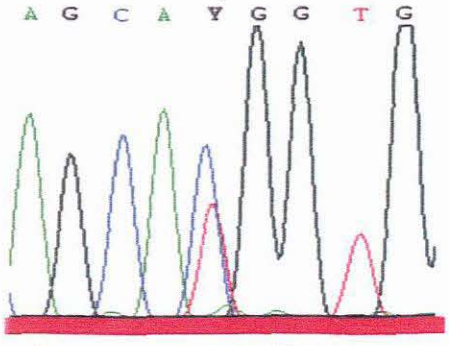


Figure 30. Autoradiograph of a sequencing reaction showing both the normal and mutant alleles for the C to T transition at nucleotide 1058 of the CβS gene. These clones were sequenced with reverse sequencing/amplification primer CβS C and resolved using polyacrylamide gel electrophoresis.

This mutation was confirmed in patient F489 by sequencing genomic DNA on an automatic sequencer in the core facility at The University of Cape Town (UCT). This result is shown in fig. 31.



Exon 10 – C1058T (Thr353Met)

Figure 31. Sequence profile of genomic DNA of patient F489 carrying the C1058T transition that leads to a threonine to methionine transition at amino acid 353. This sequence from genomic DNA contains both alleles (normal and mutant). Both bases at nt. 1058 can clearly be seen above background noise.

The C to T transition resulted in a codon change from an ACG to ATG. This codon change resulted in a change of the polar (uncharged) amino acid, threonine 353 to the non-polar amino acid, methionine. To screen a vast number of patients for a specific point mutation, by manual sequencing of the genetic material, is very labour intensive and expensive. Restriction enzyme analysis was established to screen more family members for the G919A (Gly307Ser) mutation. Several of the pathogenic mutations in the cystathionine β-synthase gene are missense mutations. Missense mutations are nucleotide changes resulting in an amino acid change. Bacterial restriction endonucleases that recognise and cleave specific nucleotide sequences have been widely used in molecular diagnosis. These enzymes can recognize mutations that occur within their target recognition sequence.

The restriction enzyme *Pvu* II, recognises the nucleotide sequence, CAGCTG. This sequence does not exist in exon 8, even when the mutation is present. By amplifying the genomic DNA with a mismatch primer that will have a complementary C rather than a T to the A at nucleotide position 923, one creates a sequence that will contain a G rather than the correct A at nucleotide 923. When the G to A mutation is present at nucleotide 919, the sequence recognised by the restriction endonuclease *Pvu* II will be present as shown in fig. 32, and the 146 base pair DNA template, when treated with *Pvu* II, will be digested, into 21, 125 and 146 base pair fragments. In obligate heterozygous patients, only 50% of the DNA will carry the specific mutation, therefore only 50% of the DNA will be digested by the restriction enzyme, thus resulting in the three fragments (two fragments from abnormal and one fragment from normal DNA).

Amplification of exon 8

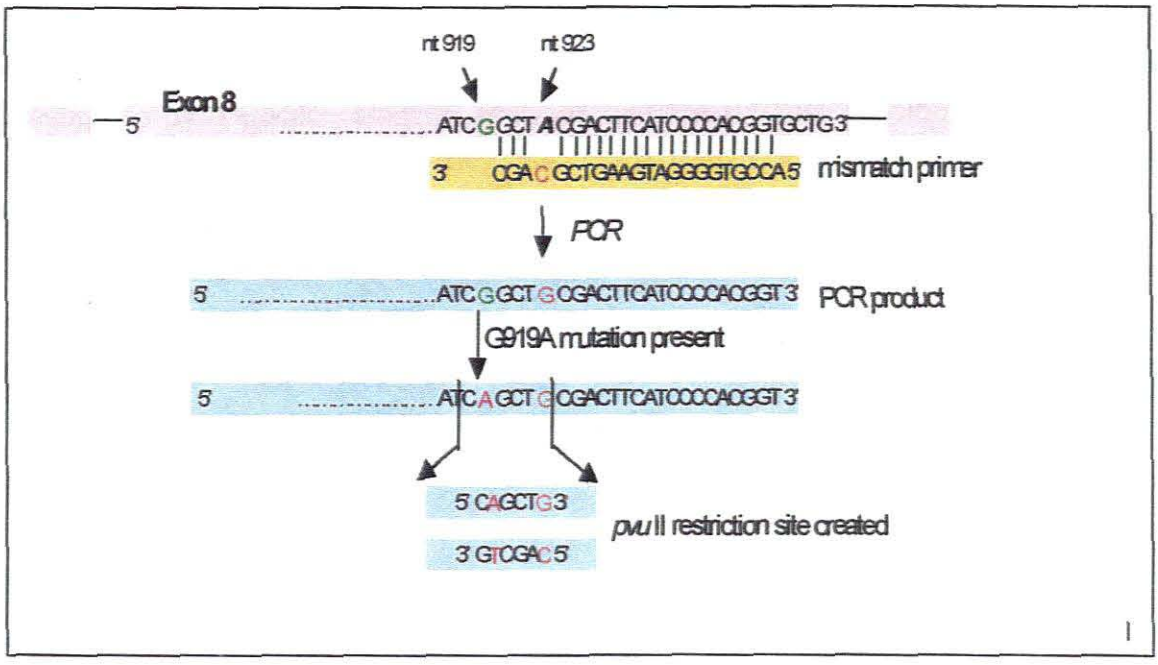


Figure 32. Schematic representation of the amplification of exon 8 of the CβS gene with mismatch primer.

The PCR reaction for the *Pvu* II restriction enzyme analysis was optimised by initially performing the amplification reaction at a range of $MgCl_2$ concentrations of 1.5, 2.0, 3.0, 4.0 and 5.0mmol/L. A $MgCl_2$ of 1.5mmol/L yielded the highest specificity as shown in fig. 33 for this reaction and was subsequently used.

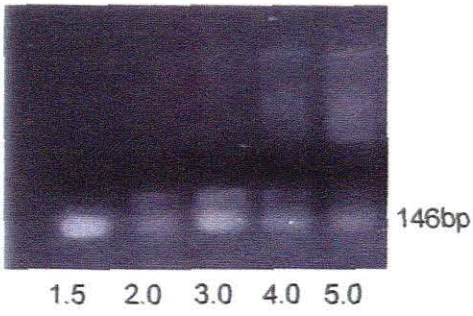


Figure 33. $MgCl_2$ titration of a 146 base pair PCR, amplifying exon 8 of the cystathionine β-synthase genomic DNA. The $MgCl_2$ concentration of each reaction is given below each lane in mM. No non-specific fragments were amplified with the 1.5mM $MgCl_2$.

The father, mother, sister and aunts and uncles from both sides were investigated for the G919A (Gly307Ser) and C1058T (Thr353Met) point mutations. Genomic DNA from the son was used as a positive control. The G919A (Gly307Ser) point mutation was investigated by restriction enzyme analyses and the C1058T (Thr353Met) mutation by sequencing. The father (F487) carried the G919A (Gly307Ser) mutation on one allele but this mutation was not detected in the mother (fig. 33). The mother carried the C1058T (Thr353Met) mutation on one allele, but the father did not carry this mutation. Both these mutations were detected in the sister. One brother of the father carried the G919A (Gly307Ser) mutation and the two sisters and two brothers of the mother carried the C1058T (Thr353Met) mutation as shown in fig. 34.

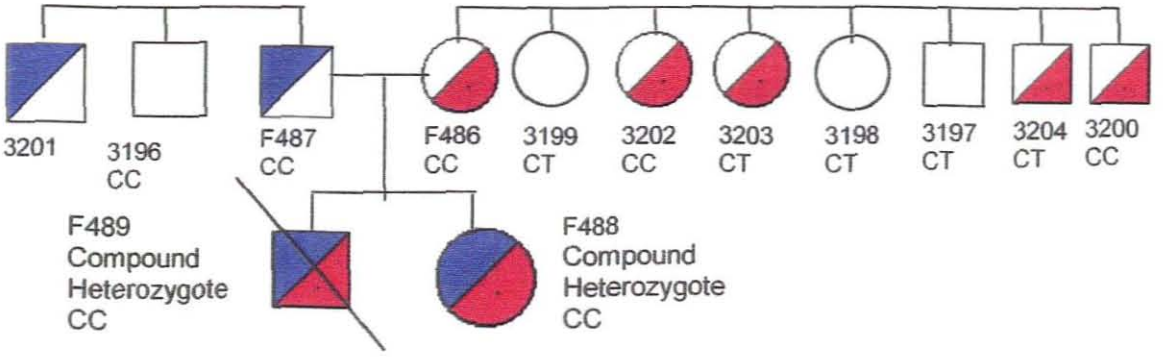


Figure 34. Genotypes of investigated family members of family 1. Patient F487 is the father and F486 is the mother of the two probands, F489 andF488. The rest are uncles and aunts of the probands. The red halves denote alleles with C1058T (Thr353met) mutation and the blue halves denote the G919A (Gly307Ser) mutation. CC denotes normal homozygosity and CT denotes heterozygosity for the C677T mutation in the methylenetetrahydrofolate reductase gene.

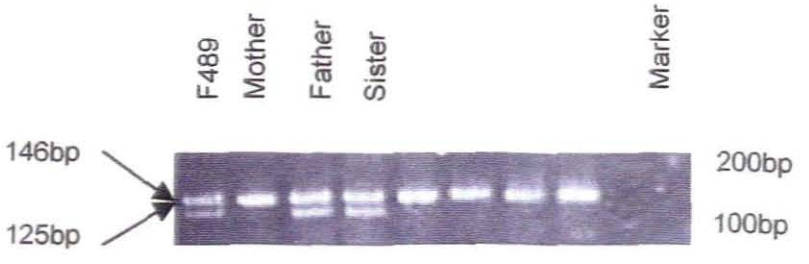


Figure 35. A *Pvu* II digests of 146bp PCR products from genomic DNA of family 1. The index case (F489), the father (F487) and the sister (F488), containing the G919A (Gly307Ser) mutation and the mother (F486) that did not carry this mutation. The PCR product is digested into two fragments, 21 and 125 base pair in the mutant sequence. The right hand lane, show a 100 base pair marker.

Methylenetetrahydrofolate reductase

Allele 1

For the purpose of screening for the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, genomic DNA was extracted from blood and a 198 base pair fragment containing the C677T mutation was amplified by PCR with primers MTHFR 1 (exonic) and MTHFR 2 (intronic) (Frosst *et al.*, 1995). The standard temperature profile for the amplification of the 198 base pair fragment resulted in the amplification of a non-specific 700 base pair fragment. To prevent non-specific annealing of primers, the temperature profile was adjusted to include a "hot start" at 94°C for 1 minute before the 35 cycles of denaturing, annealing and extension. Extension time during the 35 cycles was also shortened to 45s so as to prevent the tendency for the amplification of the 700bp fragment.

PCR reaction volumes were scaled down to 50µl. The optimum $MgCl_2$ concentration was 2.0mM. The C to T substitution creates a *Hinf* 1 recognition sequence as shown in fig.36 hich digests the 198 base pair fragment into two 175 and 23 base pair fragments respectively.

The *Hinf* I restriction site

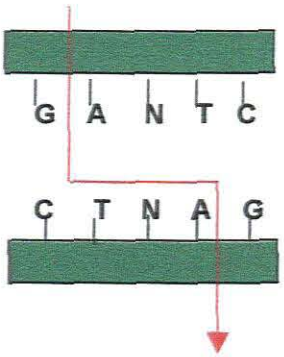


Figure 36. The recognition site of the restriction enzyme *Hinf* 1.
↓ Indicates where the enzyme cuts. N denotes any base.

The index case of family 1, his sister and their parents do not carry the C677T point mutation. Three sisters and two brothers of the mother however, carry the C677T point mutation on one allele. Results are shown in fig. 34.

4.1.3.3 Biochemical investigation of family 2

Index case

The index case of family 2, a boy (F1036), was diagnosed with homocystinuria at the age of 11. He has visual impairment and dislocated lenses, but performs well at school. He has a normal lipid profile (result not shown) but a fasting total plasma homocysteine of 208.4μmol/L, confirmed homocystinuria in this patient. The father is hypercholesterolaemic and has premature ischaemic heart disease. The father's fasting total plasma homocysteine is in the hyperhomocysteinaemic range at a value of 30μmol/L. The mother's fasting total plasma homocysteine is normal at 7.5μmol/L.

The index case (F1036) of family 2 and was treated with folate, vitamin B_6 and Vitamin B_{12}. During this period of treatment the total plasma homocysteine was measured at 6, 12, 19, 22 and 27 months after the start of the vitamin treatment. The total plasma homocysteine results after vitamin treatment are shown in fig. 37 and table 7.

Fasting total plasma homocysteine before and after vitamin treatment of family 2

Fasting total plasma homocysteine (tHcy) (μmol/L) after vitamin treatment												
months	**0**	**6**	**12**	**17**	**19**	**22**	**24**	**27**	**29**	**32**	**35**	**37**
F1036	208.4	36.0	65.0	-	59.0	55.9	-	49.3	-	34.1	84.6	46.1
F1034	29.8	-	-	34.3	55.9	-	19.9	-	25.2	-	-	-

Table 7. Fasting total plasma homocysteine (tHcy) in a homocystinuric (F1036) and a hyperhomocysteinaemic (F1034) patient.

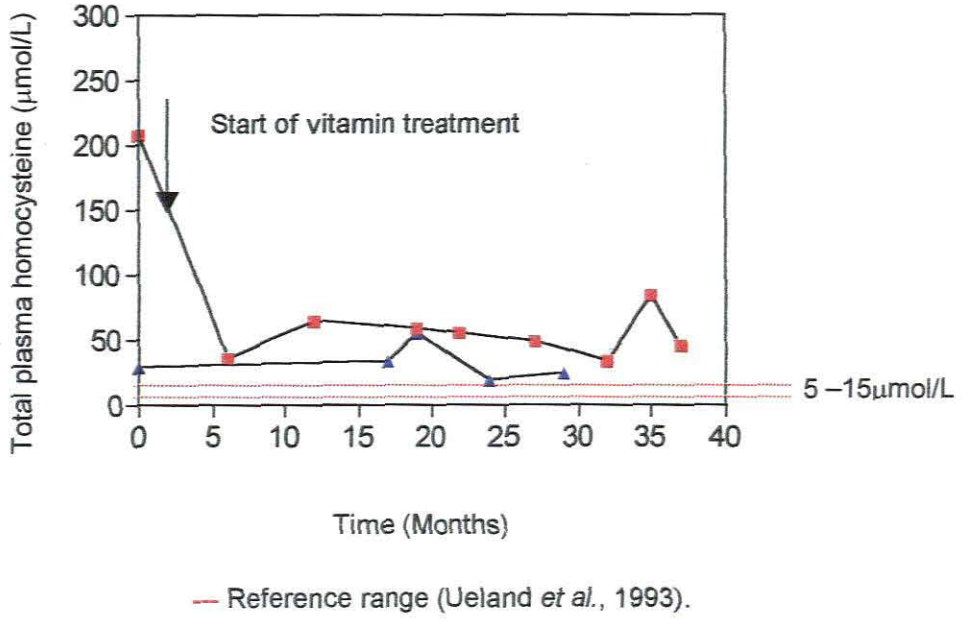
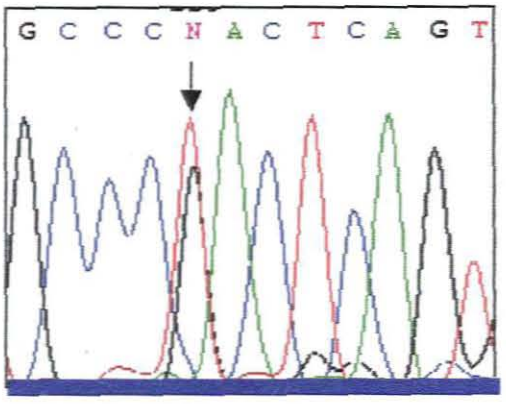


Figure 37. Fasting total plasma homocysteine (tHcy) levels of index case F1036 (■) and patient F1034 (▲) before and during vitamin treatment. Results are shown in table 7.

4.1.3.4 Molecular investigation of family 2

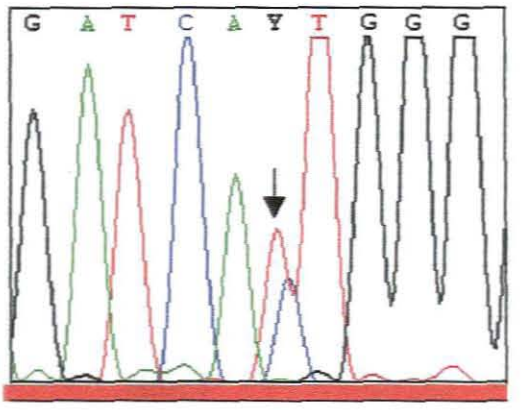
Cystathionine β-synthase

mRNA was extracted from fibroblasts of the index case and reverse transcribed. cDNA of the cystathionine β-synthase gene was screened for defects. A G to a T transition was detected at nucleotide 1126 in exon 10 on allele. This missense mutation resulted in an amino acid change of an aspartate (negatively charged) to a threonine (polar neutral) at amino acid 376. The other allele carries a T to C transition at nucleotide 833 in exon 8. This T833C mutation that changes an isoleucine (non polar) to a threonine (polar but neutral) at amino acid 278, is the second most frequently found, panethnic, mutation in patients with especially pyridoxine responsive homocystinuria. The index case was therefore a compound heterozygote for defects in the cystathionine β-synthase gene. The whole cDNA was sequenced and no other mutations were seen. Both these mutations were confirmed using primers designed for genomic DNA (fig. 38 and 39). Further molecular investigation of the cystathionine β-synthase gene in family 2, was done using genomic DNA only.



Exon 10 - G1126T (Asp376Thr)

Figure 38. Sequence profile of genomic DNA of patient F1036, showing the G to T transition at nucleotide 1126.



Exon 8 - T833C (Ile278Thr)

Figure 39. Sequence profile of genomic DNA of patient F1036, carrying the T833C transition.

The G1126T (Asp376Thr) point mutation was subsequently detected in the father (F1034). The mother carries the T833C (Ile278Thr) point mutation. The sister and one brother carry no mutations and two other brothers (F1038) (CI233) carry the G1126T (Asp376Thr) (tHcy = 11.9μmol/L) and T833C (Ile278Thr) (tHcy = 26.7μmol/L) point mutations respectively. The other brother had no mutation in the cystathionine β-synthase gene and a total plasma homocysteine of 8.0μmol/L.

Methylenetetrahydrofolate reductase

All the members of family 2 carried the C677T mutation on one allele as shown on family tree in fig. 40.

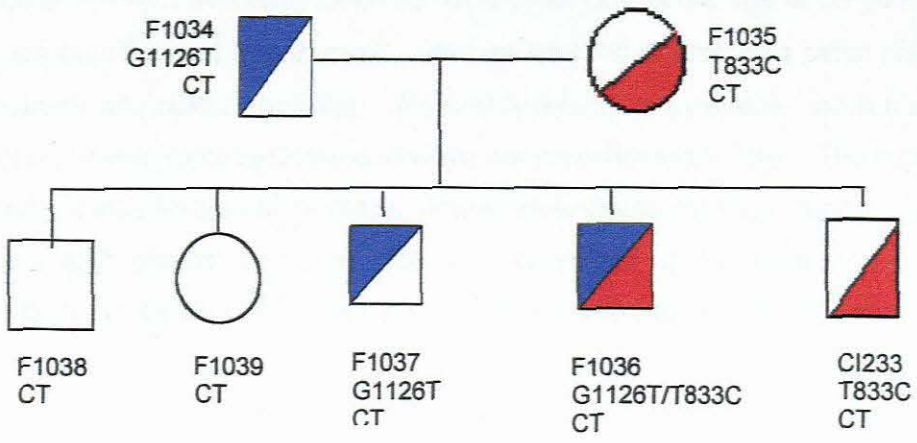


Figure 40. Genotypes of investigated members of family 2. F1034 is the father and F1035 is the mother of the index case, F1036. The rest are brothers and sister of the index case. The red halves denote alleles withT833C (Ile278Thr) mutation and the blue halves denote the G1126T (Asp376Thr) mutation. CT denotes heterozygosity for the C677T methylenetetrahydrofolate reductase mutation.

4.1.3.5 Biochemical investigation of family 3

Index case

The index case of family 3 was diagnosed as homocystinuric at the age of 35 years with a total plasma homocysteine of 226.2μmol/L. He has had visual problems since childhood, bilateral dislocation and mild retardation. No family history is available. Both his sisters had normal total plasma homocysteine and were not investigated further. The index case (F1086) of family 3 was treated with folate, vitamin B_6 and vitamin B_{12}. During this period of treatment the total plasma homocysteine was measured at 2 months and 6 months after vitamin treatment started. The total plasma homocysteine results are shown in table 8.

Total plasma homocysteine of patient from family 3 before and after vitamin treatment

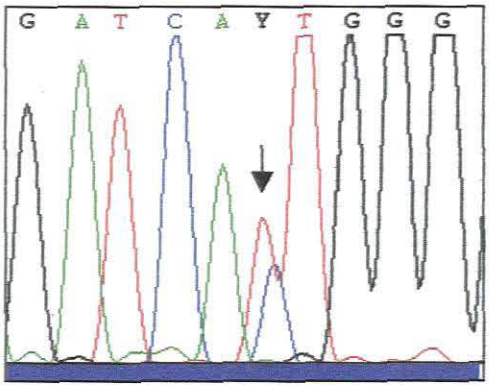
	Fasting total plasma homocysteine		
months	**0**	**2**	**6**
F1086	225.2	20.9	13.7

Table 8. Fasting total plasma homocysteine in homocystinuric index case F1086 of family 3.

4.1.3.6 Molecular investigation of family 3

Cystathionine β-synthase

mRNA was extracted from cultured fibroblasts of patient F1086 and reverse transcribed to form cDNA. The cDNA of the whole cystathionine β-synthase gene was screened for defects. The frequently found T833C (Ile278Thr) mutation was found on both alleles (fig. 39). He was therefore homozygous for cystathionine β-synthase deficiency. The T833C (Ile278Thr) mutation was confirmed in genomic DNA. Both parents carried the T833C (Ile278Thr) mutation on one allele only, as shown in fig. 41. They were therefore obligate heterozygotes for cystathionine β-synthase deficiency.



Exon 8 – T833C (Ile278Thr)

Figure 41. Sequence profile of cDNA of the mother (F1085), carrying the T833C (Ile278Thr) transition on one allele.

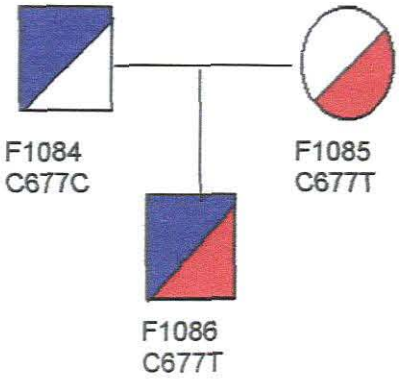


Figure 42. Genotypes of investigated members of family 3. Patient F1084 is the father and F1085 is the mother of the index case, F1086. The red halve denotes the allele from the mother with the T833C (Ile278Thr) mutation and the blue halve denotes the allele from the father with the T833C (Ile278Thr) mutation. C677T denotes heterozygosity and C677C denotes normal for the thermolabile variant of the MTHFR enzyme.

Methylenetetrahydrofolate reductase

The index case and his mother both carried the C677T mutation on one allele. They were therefore obligate heterozygotes for the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene.

4.1.3.7 Summary of the biochemical and molecular results of the investigated families

	Fasting free and tHcy (μmol/L)	CβS activity (nmoles/mg protein/hr)	CβS mutation	MTHFR C677T
Family 1				
Index case	235 (free Hcy)	0.2	C1058T/G919A	CC
Sister	26 (free Hcy)	No result-	C1058T/G919A	CC
Mother	-nil detected (free Hcy)	6.5	C1058T	CC
Father	-no result	0.9	G919A	CC
Family 2				
Index Case	208.4	-	G1126T/T833C	CT
Father	29.8	-	G1126T	CT
Mother	7.5	-	T833C	CT
Sister	9.4	-	Normal	CT
Brother 1	8.0	-	Normal	CT
Brother 2	11.9	-	G1126T	CT
Brother 3	26.7	-	T833C	CT
Family 3				
Index case	225.2	-	T833C/T833C	CT
Father	No result	-	T833C	CC
Mother	11.6	-	T833C	CT

Table 9. A summary of the biochemical (free (Hcy) and total (tHcy) homocyst(e)ine and cystathionine β-synthase (CβS) enzyme activity) and molecular (CβS mutation and C677T methylenetetrahydrofolate reductase (MTHFR) mutation) results in the families investigated for this thesis. CC denotes normal homozygosity and CT denotes heterozygosity for the C677T mutation in the methylenetetrahydrofolate reductase gene.

4.2 Hyperhomocysteinaemia in the Western Cape

The autosomal recessive inherited disorder homocystinuria initially stimulated research into mild homocysteine elevation (hyperhomocysteinaemia) in the general population. Moderate elevation of homocysteine (hyperhomocysteinemia) in plasma has been implicated as a risk factor in many (±80) conditions of which complications during pregnancy and peripheral vascular disease are just a few. The total plasma homocysteine concentration in serum/plasma reflects cellular homocysteine metabolism and is 5 – 15μmol/L (Ueland *et al.*, 1993). Patients with inborn errors in cystathionine β-synthase often have total plasma homocysteine values elevated above 200μmol/L (homocystinuria). Hyperhomocysteinaemia however refers to total plasma homocysteine levels in the range 15-30μmol/L that can sometimes be attributed to deficiencies in folate, vitamin B_6, or cobalamin metabolism (Brattström *et al.*, 1990).

The incidence, cause and relationship between hyperhomocysteinaemia and vascular disease in the Western Cape are still unknown factors. We determined total plasma homocysteine before and after oral methionine in subjects of three disease groups namely, abruptio placentae, peripheral vascular disease and pre-eclampsia, in order to establish the prevalence of hyperhomocysteinaemia in the local population with vascular disease.

Cystathionine β-synthase enzyme activity was measured in all the subjects from the abruptio placentae study in order to determine whether deficiency of this enzyme was the cause of hyperhomocysteinaemia in these patients.The effect of folate treatment on patients with peripheral vascular disease and hyperhomocysteinaemia was investigated.

Genetic deficiency of methylenetetrahydrofolate reductase is one of the rare homocystinurias leading to severe homocystinuria and vascular disease in the very young (Ueland *et al.*, 1992). The thermolabile variant of the gene, caused by the C→T substitution at base pair 677 (Frosst *et al.*, 1995) has been shown to be associated with decreased methylenetetrahydrofolate reductase enzyme activity. They found that mutant homozygotes (TT genotype, ≈ 12% of the Caucasian population) had significantly higher mean plasma homocysteine concentrations than those not carrying the mutant allele (CC genotype). The initial assumption that the C677T mutation could be a risk factor for cardiovascular disease was recently disputed by a meta-analysis done by Brattström *et al.* (1998). They also showed that total plasma homocysteine in patients with this mutation was sometimes only affected when folate levels were abnormally low. The association between the C677T mutation and abruptio placentae, peripheral vascular disease, pre-eclampsia and hypertension was also examined for this study. The association between

hyperhomocysteinaemia and the C677T mutation was also examined. This study also examined the distribution of this mutation in the three local populations namely the African, Coloured and Caucasian populations of the Western Cape.

4.2.1 Plasma homocyst(e)ine measurement

Historically, only high, free homocystine, associated with homocystinuria were measured. In the last decade a considerable interest in the relationship between hyperhomocysteinaemia and vascular disease has developed. Patients with homocystinuria, could have total plasma homocysteine of >200μmol/L, whereas an increase of as little as 5μmol/L are sometimes observed in patients with hyperhomocysteinaemia. The reference range for fasting total plasma homocysteine has been established by a variety of studies as between 5 and 15μmol/L (Ueland *et al.*, 1993). Mild, intermediate and severe hyperhomocysteinaemia (homocystinuria) have been defined as 15 to 30μmol/L., 30 to 100μmol/L and > 100μmol/L (Kang *et al.*, 1992). The narrow difference and considerable overlap between total plasma homocysteine in patients with hyperhomocysteinaemia and normal control subjects has been a major limitation in analytical procedures for the measurement of plasma homocystine in the past. Other problems are the fact that 80% of homocysteine is bound to protein in the blood, with the remaining 20% present as disulfides with itself or cysteine, and a small fraction in the free thiol form (see fig. 41). "Free homocystine" is defined as the homocysteine disulphide, homocystine. "Total homocysteine" is the homocysteine, derived from homocystine; the mixed disulphide plus the protein bound homocysteine by reduction with a reducing agent like dithiothreitol.

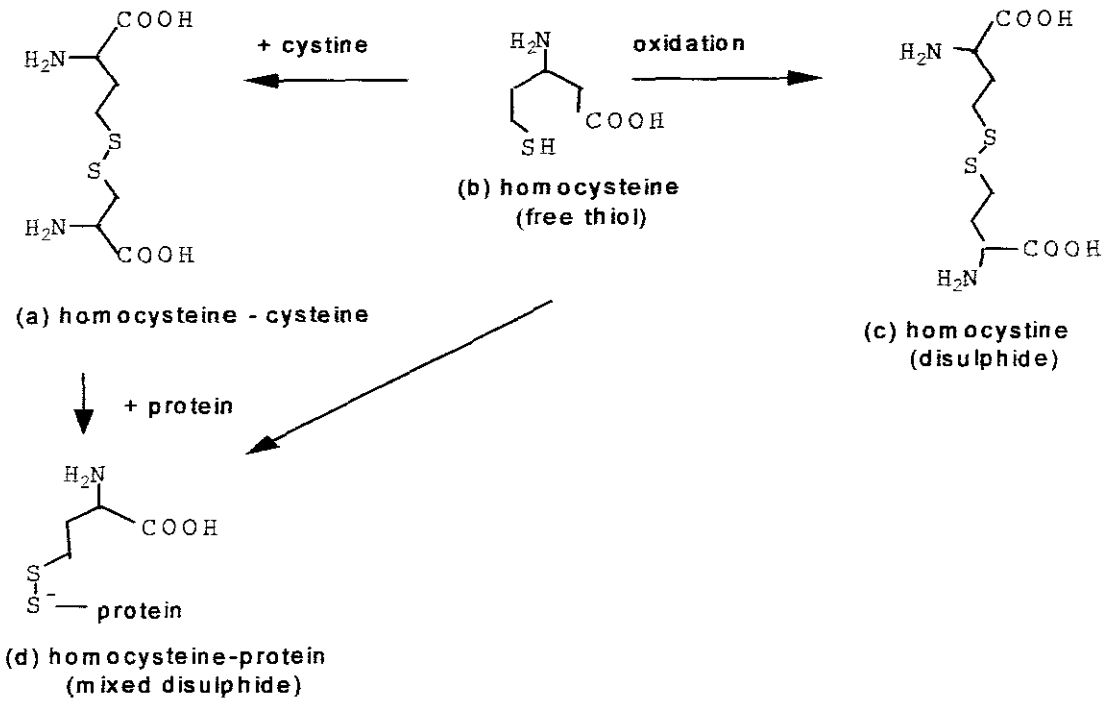


Figure 43. Homocyst(e)ine species found in deproteinised plasma, (d) represents homocysteine bound to protein, 80% of plasma homocysteine is bound to plasma albumin, (a), (b), (c), and (d) represent total homocysteine in plasma (Refsum *et al.*, 1997).

If whole blood is left standing without separating the serum/plasma from the red blood cells, false elevation in plasma homocysteine occurs due to an increase in production of homocysteine in the red cell. Redistribution between free and bound forms also occurs. Above-mentioned problems have been overcome by measuring total plasma homocysteine rather than free plasma homocystine.

4.2.2 Free plasma homocystine measurement

Initially only free plasma homocysteine was measured in the Inherited Metabolic Disease Unit at Groote Schuur Hospital as part of the analytical separation of amino acids for either plasma or urine samples. Free homocysteine measurement was adequate for the diagnosis of homocystinuria where levels can rise to above 200µmol/L. However, samples for free homocystine measurement associated with hyperhomocysteinaemia, had to be separated and deproteinised within one hour to ensure accurate measurement. In addition, the low concentration of homocysteine found in hyperhomocysteinaemia was done to the limitations of the amino acid analyser.

Sample handling for total plasma homocysteine measurement was less critical. The only precaution needed was the separation of plasma from the red blood cells within one hour.

In addition, total plasma homocysteine values were at least 10x greater than free homocysteine, bringing the values to within the accuracy of the amino acid analyser. For this thesis a method to measure total plasma homocysteine rather than free homocystine was introduced into the Inherited Metabolic Disease Unit at Groote Schuur Hospital.

4.2.3 Total plasma homocysteine measurement

Measurement of total plasma homocysteine consists of two stages;

- reduction of the different forms to homocysteine and
- separation and quantification of the amino acid

EDTA blood was collected and plasma was separated from the red blood cells within one hour. The plasma was stored at - 80°C until analysis. Reduction was adapted from Brattström *et al.* (1984). Separation and quantification of homocysteine was achieved by adapting the method of Beckman for measuring plasma amino acids, currently in use in the routine diagnostic laboratory. In the reduced form, homocysteine (16 minutes) elutes much earlier than homocystine (86 minutes), an internal standard (norleucine) that elutes earlier than the S.2-aminoethyl-L-cysteine hydrochloride standard (AEC) (102 minutes) was therefore used. This substitution of a norleucine internal standard rather than the standard AEC standard shortened the chromatographic run considerably from 102 minutes to ± 30 minutes, thus increasing the capacity of this assay.

4.2.3.1 Homocystine standard range and recovery as homocysteine

L-Homocystine standards, ranging from 0 to 200μmol/L were analysed before (fig. 44a) and after reduction (fig. 44B) with dithiothreitol. 50mMolar dithiothreitol was sufficient to completely reduce 100μmol/L L-homocystine to L-homocysteine. No homocystine was detected after a one hour incubation with 50mM dithiothreitol, thus showing that no re-oxidation of homocysteine took place during chromatography and that initial reduction was complete. Detection for total homocysteine was linear up to 200 μmol/L (fig. 44b). Homocysteine standards, at final concentrations of 50 and 200μmol/L, were processed with each subsequent run.

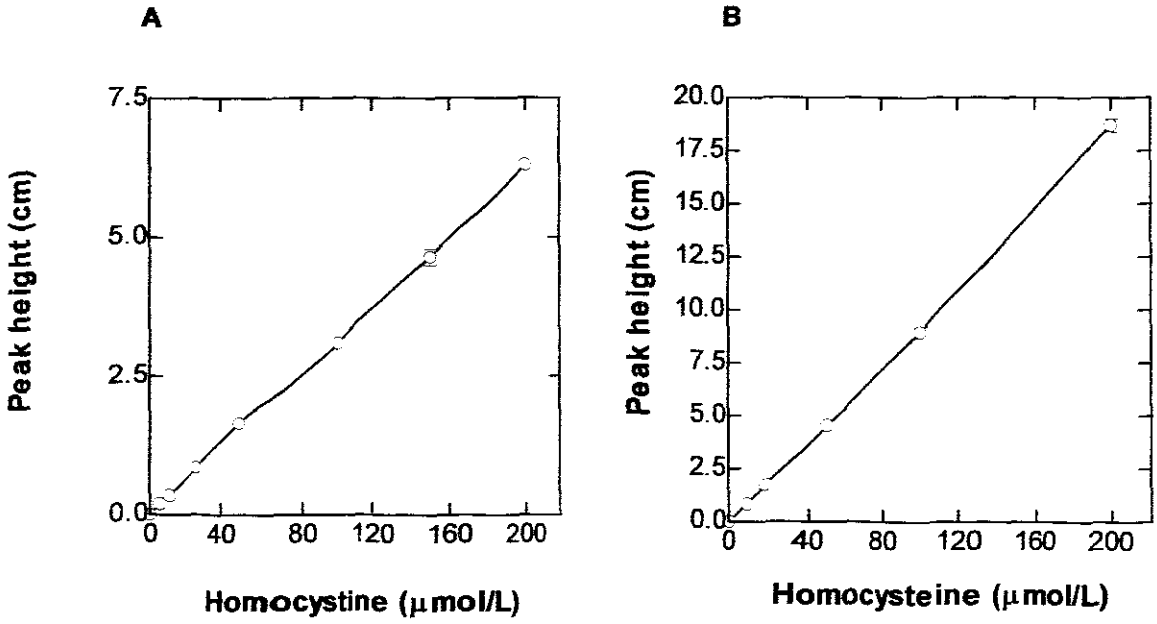


Figure 44a. Homocystine standard curve based on duplicate determinations of homocystine standards. Peak heights in cm, were plotted against homocystine concentration in μmol/L. Error bars denote mean ±SEM. Standard homocystine values were; 0 (0 ±0.0), 5 (0.2 ±0.0), 10 (0.35 ±0.05), 25 (0.85 ±0.05), 50 (1.65 ±0.05), 100 (3.10 ±0.10), 150 (4.65 ±0.15) and 200 (6.30 ±0.1). Figure before brackets denotes homocystine concentration in μmol/L and figure inside brackets denote peak height in cm ±SEM.

Figure 44b. Homocysteine standard curve based on duplicate determinations of homocysteine standards. Peak heights, in cm, were plotted against homocysteine concentration in μmol/L. Error bars denote mean ± SEM. Standard homocysteine values were 0 (0 ±0.0), 10 (0.85 ±0.15), 20 (1.75 ±0.05), 50 (4.58 ±0.02), 100 (8.95 ±0.15) and 200 (18.70 ±0.30). Figure before brackets denotes homocysteine concentration in μmol/L and figures inside brackets denote peak height in cm ± SEM.

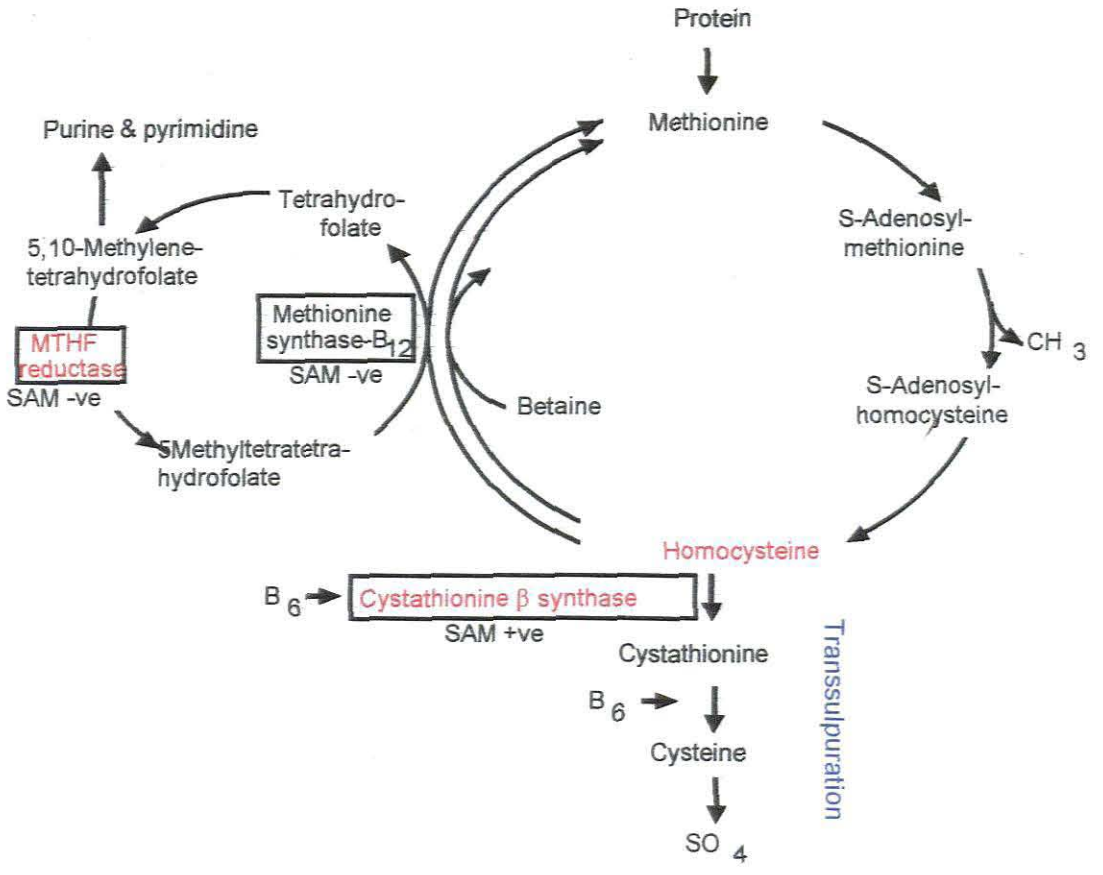


Figure 45. Methionine metabolism in the body. SAM denotes S-adenosylmethionine. Methylenetetrahydrofolate reductase (MTHFR) denotes methylenetetrahydrofolate reductase.

4.2.3.2 Oral Methionine

Homocysteine values will change in the blood according to the nutritional state of the patient. After a meal high in protein the levels of methionine will rise which in turn is rapidly converted to homocysteine during methionine metabolism (fig. 45). T o emphasize the small difference between homocysteine found in normal controls and patients with hyperhomocysteinaemia, methionine metabolism could be challenged with oral methionine. Blood samples for homocyst(e)ine (free and total) were taken after an overnight fast and after oral methionine. Methionine (100mg/kg body weight) in 200ml orange juice was then administered orally to the patient. Blood samples were taken at 2,4, 6, 8,10,12 and 24 hours after oral methionine. During this period, patients were put on a standard methionine reduced breakfast and lunch, which contained less than 14mg

of methionine. Free and total homocyst(e)ine were measured in these samples (results shown in fig. 46).

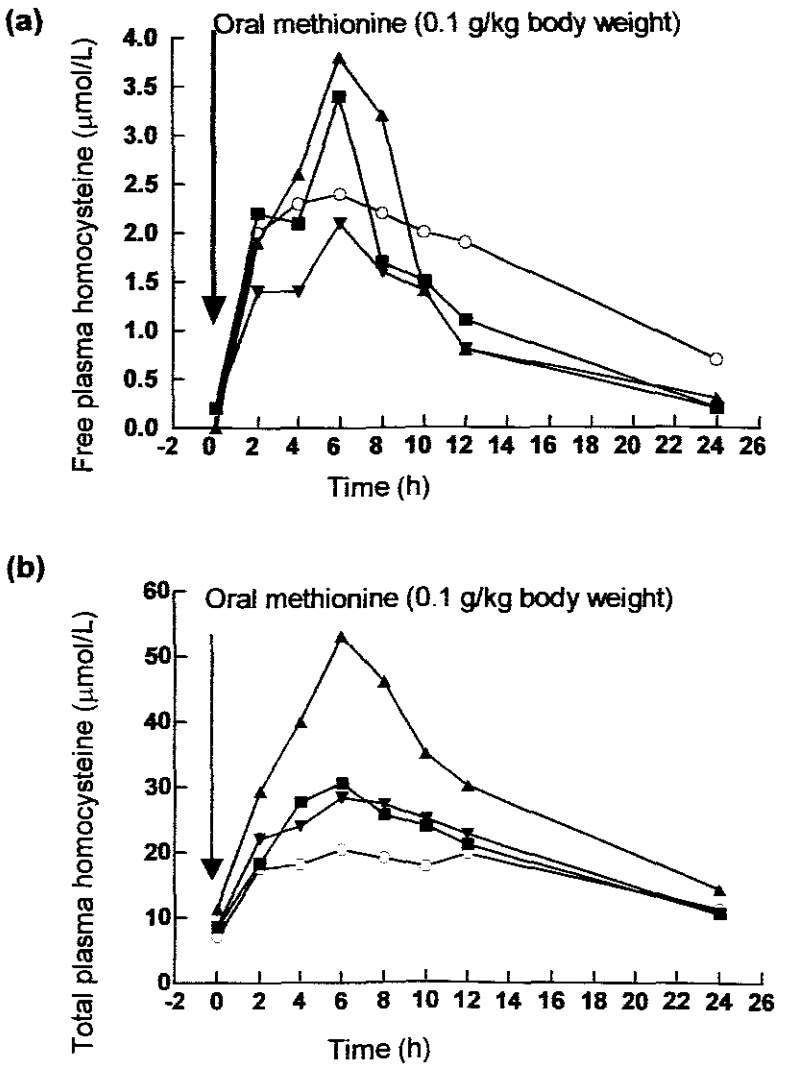


Figure 46. Plasma homocyst(e)ine levels after a methionine challenge in four patients from the abruptio placentae study. (a) Free plasma homocystine before and after oral methionine. (b) Total plasma homocysteine before and after oral methionine. -O- = L864; -▲- = L859 -▼- = L839; -■- = L863

Initially, total and free plasma homocyst(e)ine were measured in blood samples from four subjects of the abruptio placentae study, taken prior to and at 2, 4, 6, 8, 12 and 24 hours after a oral methionine. In each case the changing profile of free homocystine was reflected in the total homocysteine profile. In addition, peak homocysteine was reached at 6 hours post oral methionine in both free and total homocyst(e)ine. Consequently it was determined that total plasma homocysteine measurements taken after an overnight fast and 6 hours post oral methionine, adequately affected the ability of the methyl cycle to

control levels of homocysteine. This change reduced the number of samples from 8 to 2, the duration of the investigation from 24 to 6 hours and considerably eased sample handling.

4.2.4 Abruptio placentae study group

Parameters that were matched between abruptio placentae and control group

	Abruptio placentae group	Control group
Age	26.2±6.0	27.8±5.9
Gravida	2.23±1.6	2.23±1.4
Parity	2.1±1.1	2.1±1.4
Gestation	32.5±2.9	38.0±3.8

Table 10. Comparison of age, gravida, parity and gestation between abruptio placentae patient (n=21) and unaffected control group (n=19).

4.2.4.1 Amino acid analysis in abruptio placentae study group

Subjects from the abruptio placentae study group were screened by measuring fasting and 6 hour post oral methionine, cysteine and total plasma homocysteine. Results obtained for methionine and cysteine for both normal controls and patients from the abruptio placentae study group are shown in table 11. Methionine and cysteine were measured by analysis on the amino acid analyser (Beckman) according to an in house method, which was used to measure free homocystine as described in chapter 3, paragraph 3.2.10.1, page 51.

Amino acid results of abruptio placentae study

	Control[a] (n=19)	Abruptio Placentae[a] (n=21)	Significance (*p*)[b]
0h Methionine (μmoles/L)	59 ±41	53 ±27	NS
6h Methionine (μmoles/L)	741 ±131	911 ±43	NS
0h Cysteine (μmoles/L)	123 ±30	140 ±25	NS
6h Cysteine (μmoles/L)	123 ±29	157 ±52	0.0420
0h tHcy (μmoles/L)	5.1 ±2.1	8.0 ±3.7	0.005
6h tHcy (μmoles/L)	22.0 ±11.0	30.6 ±20	0.039
Increase tHcy (μmoles/L)	17.0 ±9.8	23.0 ±18	

Table 11. [a]Results are given in means ±SD. [b]A *p* value of <0.05 was considered significant. The 6 hour cysteine, 0h total plasma homocysteine (tHcy) (μmoles/L), 6 hour total plasma homocysteine (μmoles/L) and Increase in total plasma homocysteine (μmoles/L) was significantly higher in abruptio placentae compared to the normal control group. *P* values were assessed using the Mann Whitney U test.

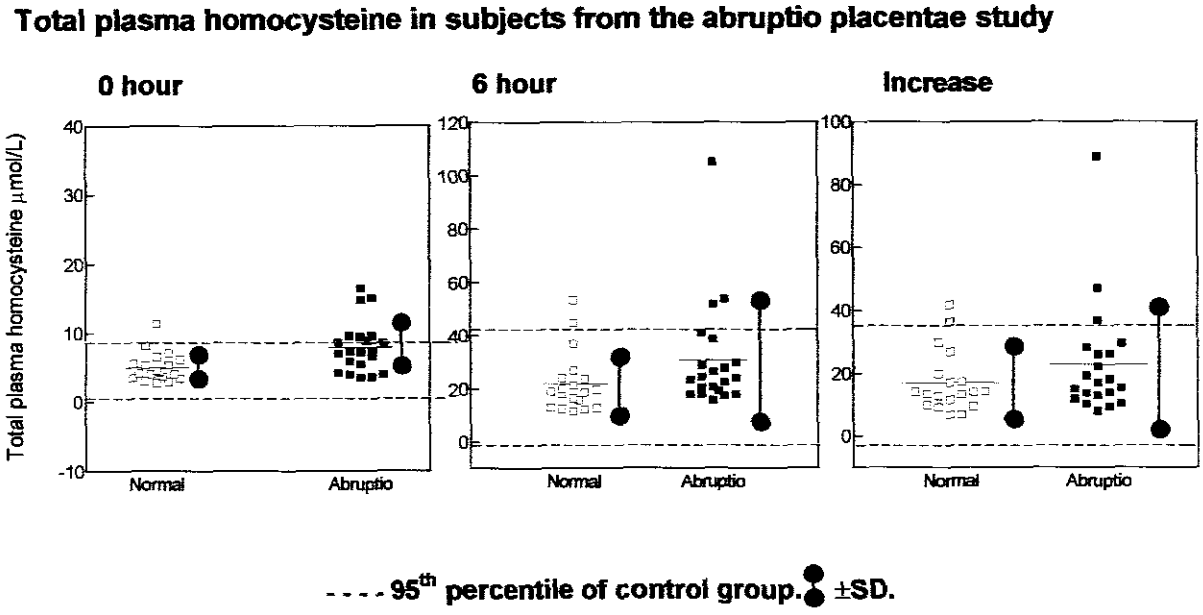


Figure 47. Fasting, 6 hour post oral methionine and change in total plasma homocysteine (tHcy) measured in normal subjects (□) and patients from the abruptio placentae study (■). Horizontal lines denote means and vertical lines denote ±SD.

28.6% (6/21) of the patients compared to 5.2% (1/19) of the normal controls had fasting total plasma homocysteine above the 95th percentile of fasting total plasma homocysteine of the control group. 14.3% (3/21) patients compared to 10.5% (2/19) of the normal controls had 6 hour post methionine total plasma homocysteine above the 95th percentile control post oral methionine total plasma homocysteine values. Similarly 14.3% (3/21) of the patients compared to 5.3% (1/19) of the normal controls, had an increase in total plasma homocysteine above the 95th percentile control post oral methionine total plasma homocysteine values (fig. 47). Oral Methionine appeared not to be necessary for the identification of hyperhomocysteinaemia in affected women. Both mean fasting and 6 hour plasma homocysteine for the patient group was significantly higher than plasma homocysteine in the control group as shown in fig. 48.

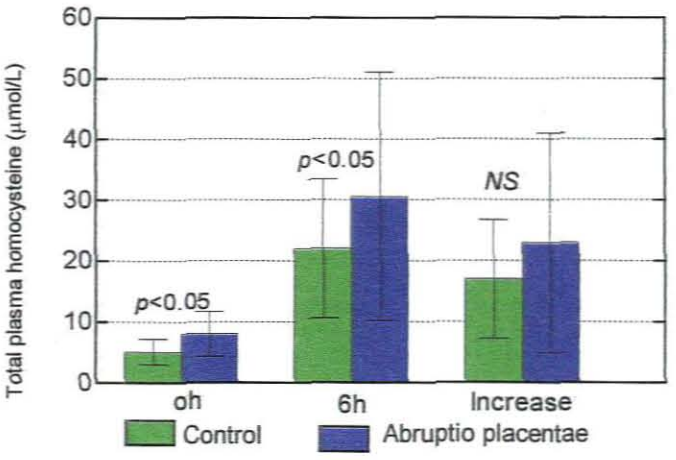


Figure 48. Bar graph showing mean fasting, 6 hour post oral methionine total plasma homocysteine and the change measured in normal controls and patients from the abruptio placentae study. The error bars denote ±1SD above the mean.

4.2.4.2 Vitamin and folate analysis of abruptio placentae study group

Vitamin B_{12}, vitamin B_6 and folate (red blood cell and serum) measurements on all the subjects of the abruptio placentae study, were performed in the Haematology Laboratory according to standard methods, at Tygerberg Hospital. These results are shown in table 12.

Vitamin B_6, B_{12} and folate values for the abruptio placentae study

	Control[a]	Abruptio placentae[a]
Vitamin B_6 (ng/ml)	2.6 ±1.3 (16)	3.2 ±1.9 (15)
Vitamin B_{12} (pg/ml)	309 ±217 (14)	273 ±176 (15)
Red blood cell folate (ng/ml)	581 ±323 (14)	481 ±152 (15)
Serum folate (ng/ml)	14 ±5.9 (14)	9.4 ±5.8 (15)

Table 12. [a]Results are given as means ±SD, with number of samples in parentheses. [b]*p* values were assessed using the Mann-Whitney U test.

No significant difference between the normal control group and abruptio placentae patients for any of the above parameters was detected. However it should be noted that the one patient in the control group with hyperhomocysteinaemia both at 0 hours and 6 hours post oral methionine had a significantly low serum folate value of 1.3ng/ml. Ubbink *et al.* (1993) have shown decreased folate, even if still within reference range, to influence plasma homocysteine.

In order to establish whether low serum folate correlated with high plasma homocysteine serum folate values, shown in fig. 49a and 49b and red blood cell folate values, shown in fig. 49c and 49d, were correlated with fasting and 6 hour post oral methionine total plasma homocysteine (tHcy) levels in both the control and patient group of the abruptio placentae study.

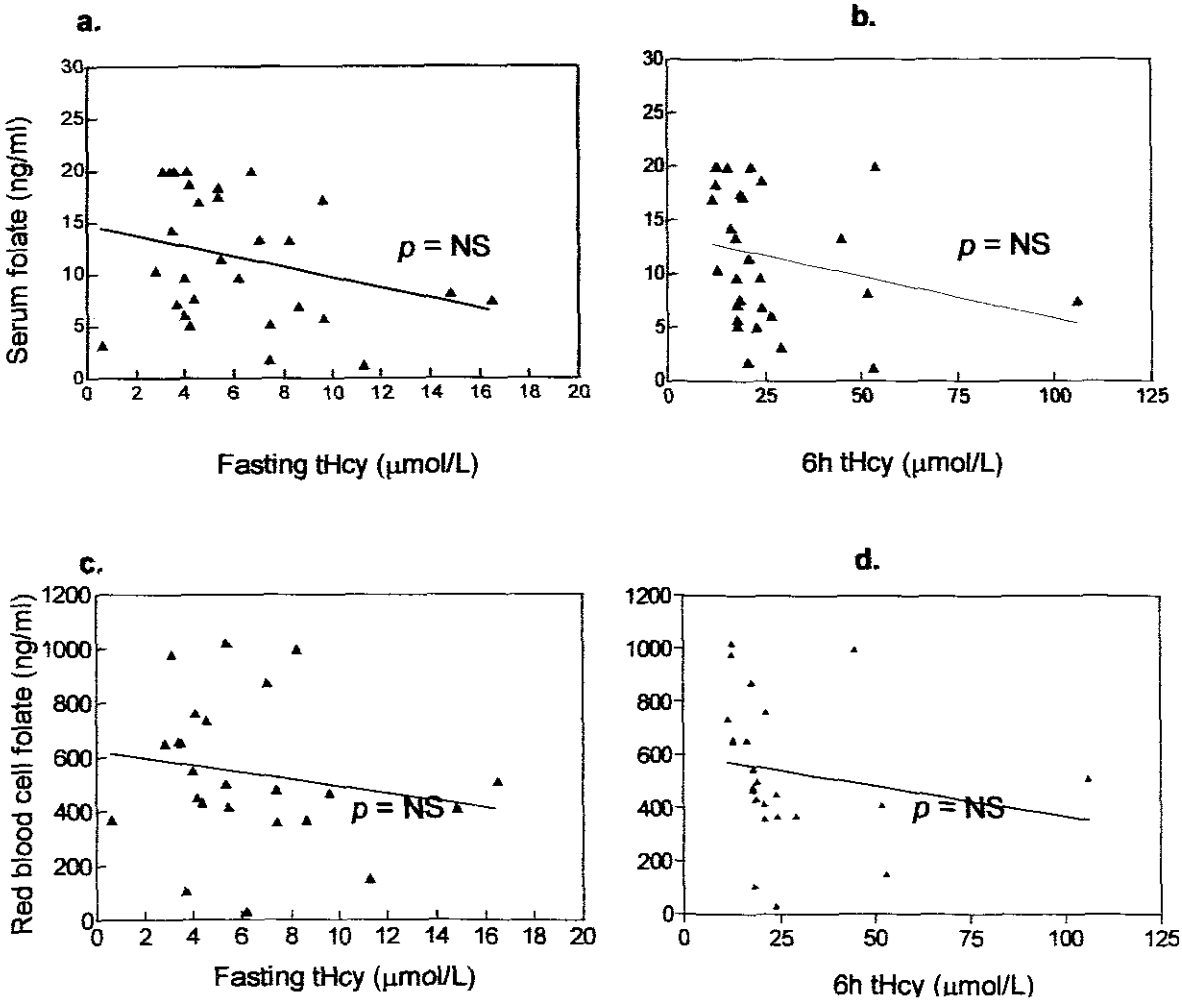


Figure 49a. Serum folate as a function of fasting total plasma homocysteine (tHcy) in normal controls and abruptio placentae patients. The straight line was drawn from an equation describing linear regression. p = NS denotes that the slope of the regression line was not significantly different from zero.

Figure 49b. Serum folate as a function of 6 hour total plasma homocysteine (tHcy) in normal controls and abruptio placentae patients. The straight line was drawn from an equation describing linear regression. p = NS denotes that the slope of the regression line was not significantly different from zero.

Figure 49c. Red blood cell folate as a function fasting total plasma homocysteine (tHcy) in normal controls and abruptio placentae patients. The straight line was drawn from an equation describing linear regression. p = NS denotes that the slope of the regression line was not significantly different from zero.

Figure 49d. Red blood cell folate as a function 6 hour total plasma homocysteine in normal controls and abruptio placentae patients. The straight line was drawn from an equation describing linear regression. p = NS denotes that the slope of the regression line was not significantly different from zero.

There was no correlation between fasting total plasma homocysteine values and serum folate in subjects from the abruptio placentae study group. There was also no correlation

between 6 hour total plasma homocysteine values and serum folate in subjects of the abruptio placentae study group. Similarly, no significant correlation existed between red blood cell folate and fasting or 6 hour total plasma homocysteine in subjects of the abruptio placentae study group. Low red blood cell folate did not always coincide with high fasting or 6 hour post oral methionine total plasma homocysteine.

4.2.4.3 Thermolabile variant of methylenetetrahydrofolate reductase in abruptio placentae study group

All the subjects from the abruptio placentae study were screened for the C677T point mutation in the methylenetetrahydrofolate reductase (MTHFR) gene. Table 13 shows the genotype frequencies detected in normal controls and affected patients from the abruptio placentae study.

Methylenetetrahydrofolate reductase genotype frequencies in abruptio placentae study group

	MTHFR C677T Genotype n (%)		
	TT	CT	CC
Patients (n=21)	0	4 (19%)	17 (89%)
Controls (n=19)	0	4 (21%)	15 (79%)

Table 13. Association for the methylenetetrahydrofolate reductase (MTHFR) phenotype with abruptio placentae. TT indicates homozygosity for the mutation; CT, heterozygosity; and CC, normal homozygosity.

No homozygotes for the C677T mutation, was detected in this study group. The CT genotype frequency was similar between the patients and normal control group. The question arose whether hyperhomocysteinaemia was associated with a particular genotype. Table 14 show mean ±SD total plasma homocysteine measured in the three genotypes from the patient group of the abruptio placentae study.

Methylenetetrahydrofolate reductase genotypes and total plasma homocysteine comparison

	MTHFR C677T Genotype		
	CC	**CT**	**TT**
n	17	4	0
0h tHcy (±SD)	8.0 (±3.77)	8.5 (± 4.87)	-
6h tHcy (±SD)	31.1 (±24.7)	31.9 (±6.27)	-
Increase (±SD)	23.1 (±21.9)	25.0 (±3.97)	-

Table 14. Average total plasma homocysteine (tHcy) (±SD) values and methylenetetrahydrofolate reductase (MTHFR) genotype in patients from abruptio placentae study.

Total plasma homocysteine was similar in the CC and CT genotype. The TT genotype was not detected in subjects from the abruptio study group. Did high total plasma homocysteine, > 95th percentile of mean total plasma homocysteine for the patients of the abruptio placentae study correlate with any particular C677T methylenetetrahydrofolate reductase genotype? Results are shown in table 15.

Methylenetetrahydrofolate reductase genotype and total plasma homocysteine percentiles

	Total plasma homocysteine percentiles	
Abruptio placentae study (n=19)	< 95%	>95%
CC	80%	80%
CT	20%	20%
TT	0%	0%

Table 15. Methylenetetrahydrofolate reductase (MTHFR) C677T genotype according to total plasma homocysteine (tHcy) (< or > 95th percentile).

No correlation existed between high total plasma homocysteine and any specific C677T MTHFR genotype in the affected patients of the abruptio placentae study.

4.2.4.4 Cystathionine β-synthase activity in abruptio placentae study group

Previously used methods for the measurement of cystathionine β-synthase activity was modified and optimized in order to measure cystathionine β-synthase activity in cell cultures from all the subjects of the abruptio placentae study. Enzyme measurements in duplicate are shown in table 16. Lymphoblast cell cultures from patients L875 and L901 and controls L880 and L913 did not grow and cystathionine β-synthase (CβS) activity was therefore not measured.

Cystathionine β-synthase (CβS) activity in abruptio placentae patients and controls

Normal control group (n=17)		Abruptio placentae group (n=19)	
Cell line	**CβS enzyme activity (nmoles/mg protein/hour)**	**Cell line**	**CβS enzyme activity (nmoles/mg protein/hour)**
L892	1.4, 1.3	L864	6.9, 6.8
L895	1.0, 0.9	L876	0.3, 0.2
L890	4.0, 3.5	L859	0.5, 0.6
L889	0.9, 0.9	L915	0.3, 0.4
L879	3.9, 4.3	L914	1.3, 1.0
L887	2.4, 2.4	L907	11.5, 13.0
L884	7.9, 5.3	L906	6.3, 5.3
L886	4.9, 4.6	L905	2.9, 2.5
L885	9.5, 9.3	L899	0.7, 0.5
L898	14.5, 15.3	L891	1.0, 0.9
L900	15.2, 16.4	L888	4.2, 3.0
L881	4.3, 5.1	L894	19.2, 17.4
L839	0.8, 0.2	L903	5.5, 4.5
L911	0.6, 0.5	L902	1.0, 1.0
L910	0.1, 0.3	L863	3.7, 3.6
L909	28.2, 28.3	L840	0.5, 0.5
L893	0.3, 0.6	L877	5.8, 5.0
		L908	1.1, 0.9
		L896	2.4, 2.3
Mean ±SD	5.8 ±7.4 (n=17)	**Mean**	3.9 ±4.8 (n=19)

Table 16. Values represent duplicate determinations of cystathionine β-synthase (CβS) activity in normal controls and affected patients from the abruptio placentae study.

There was no difference in enzyme activity between normal controls and individuals with abruptio placentae as shown in figure 50.

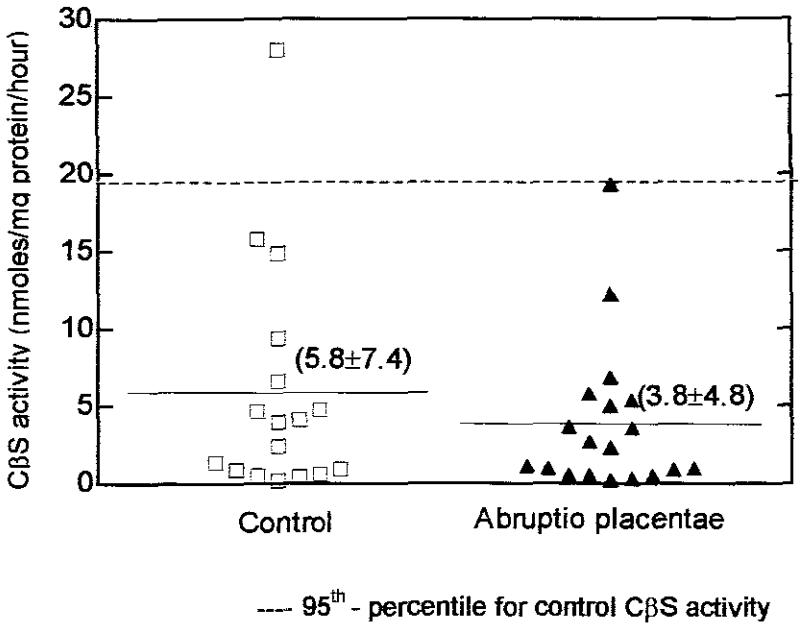


Figure 50. Cystathionine β-synthase (CβS) activity in normal controls (□) and abruptio placentae (▲) patients from apruptio placentae study.

Cystathionine β-synthase activity in 17 control subjects was 5.8 ±7.4nmoles/mg protein/h ranging from 0.10 to 28.08nmoles/mg protein/h compared to 19 patients with a mean value of 3.8 ±4.8nmoles/mg protein/h, ranging from 0.22 to 19.38nmoles/mg protein/h. No significant difference existed between cystathionine β-synthase activity of normal controls and affected patients. Nordstrom and Kjelstrom (1992) demonstrated extensive overlap in cystathionine β-synthase activity of heterozygotes and homozygotes for cystathionine β-synthase deficiency. Their study also emphasized the importance of age, matched controls.

Cystathionine β-synthase catalyses the first reaction during transsulphuration. The excess homocysteine formed after a methionine challenge is removed via the transsulphuration pathway. It is therefore more likely for a relationship to exist between 6 hour post methionine and cystathionine β-synthase activity if cystathionine β-synthase is defective. Fig. 51 shows cystathionine β-synthase activity as a function of 6 hour plasma homocysteine in all subjects of the abruptio placentae study.

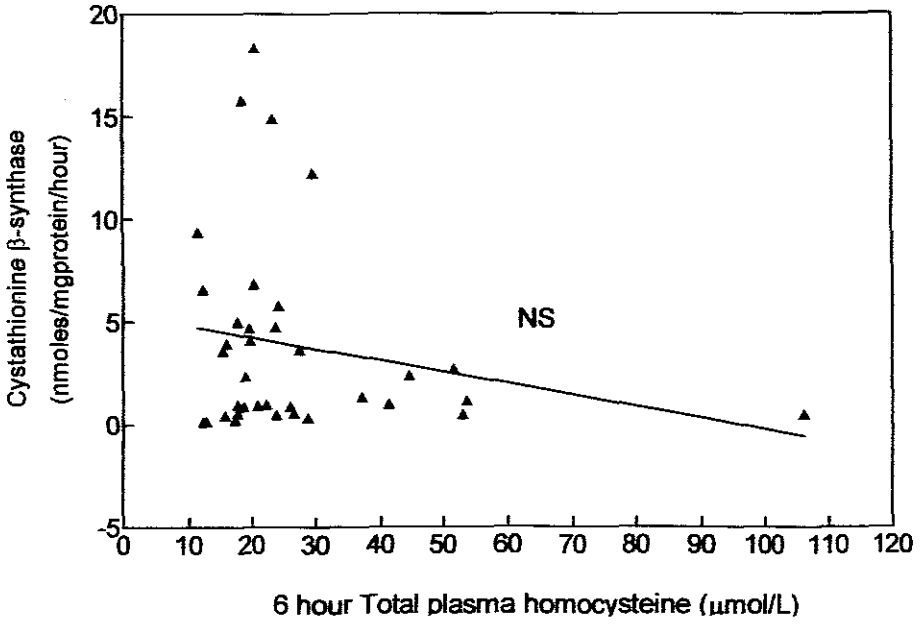


Figure 51. Cystathionine β-synthase (CβS) values versus total plasma homocysteine values at 6 hour post oral methionine, of both normal control and placentae abruptio study group. The line was obtained by linear regression of the data for the abruptio placentae study group. No correlation existed between cystathionine β-synthase activity and total plasma homocysteine.

The cystathionine β-synthase assay was initially set up for this study in order to determine enzyme activity in a family with homocystinuria and subsequently to determine if low enzyme activity correlated with raised homocysteine in subjects from the study groups investigated for this thesis. No correlation existed between high total plasma homocysteine and low cystathionine β-synthase activity in both patients and controls of the abruptio placentae group, indicating that hyperhomocysteinaemia in patients with abruptio placentae was not caused by deficient cystathionine β-synthase enzyme activity. Measurement of cystathionine β-synthase activity needs tissue culture facilities and radio-activity and is therefore not a suitable assay for the routine diagnostic laboratory.

4.2.5 Pre-eclampsia study group

Subjects from the pre-eclampsia study group were screened for fasting and 6 hour post oral methionine, total plasma homocysteine, in order to establish the prevalence of hyperhomocysteinaemia among local African and Coloured pregnant females from the Western Cape.

Fasting total plasma homocysteine levels in 53 control pregnant subjects was 6.1 ±2.8μmoles/L range (2.1 to 15.7), compared to 8.3 ±3.2μmoles/L, range (2.9 to 15.9) in 44 pre-eclampsia patients. 6 Hour total plasma homocysteine levels in 53 control subjects was 19.3 ±7.4μmoles/L, (range 7.8 to 40.6), compared to 28.7 ±13.4μmoles/L, range (12.8 to 81.2) in 44 pre-eclampsia patients. The increase of total plasma homocysteine in 53 controls was 13.2 ±6.3μmoles/L range (3.6 to 30.8), compared to 20.5 ±11.8μmoles/L in 44 pre-eclampsia patients (range 7.1 to 68.1). Total plasma homocysteine (tHcy) was significantly higher in pregnant women with pre-eclampsia than in normal pregnant women with $p < 0.001$ (see table 17).

4.2.5.1 Amino acid analysis of pre-eclampsia study group

Total plasma homocysteine in subjects from pre-eclampsia study group

	Control[a] (n=53)	Pre-eclampsia[a] (n=44)	Significance[b]
0h tHcy (μmoles/L)	6.1 ±2.8	8.3 ±3.2	< 0.001
6h tHcy (μmoles/L)	19.3 ±7.4	28.7 ±13.4	< 0.001
Increase in tHcy (μmoles/L)	13.2 ±6.3	20.5 ±11.8	< 0.001

Table 17. [a]Results are given as means ±SD, with number of samples in parentheses. [b]*p* values were assessed using the Mann-Whitney U test. A *p* value of < 0.05 was considered significant.

18.2% (8/44) of the patients compared to 3.8% (2/53) of the normal controls had fasting total plasma homocysteine above the 95th percentile of fasting total plasma homocysteine in pregnant control group. 22.7% (10/44) of the patients compared to 1.9% (1/53) of the normal controls had 6 hour post oral methionine, total plasma homocysteine above the 95th percentile of control post oral methionine values. Similarly, 20.5% (9/44) of the patients compared to 3.8% (2/53) of the normal controls, had an increase in total plasma homocysteine above the 95th percentile of control post oral methionine values. Results shown in fig. 52.

Total plasma homocysteine in subjects from pre-eclampsia study

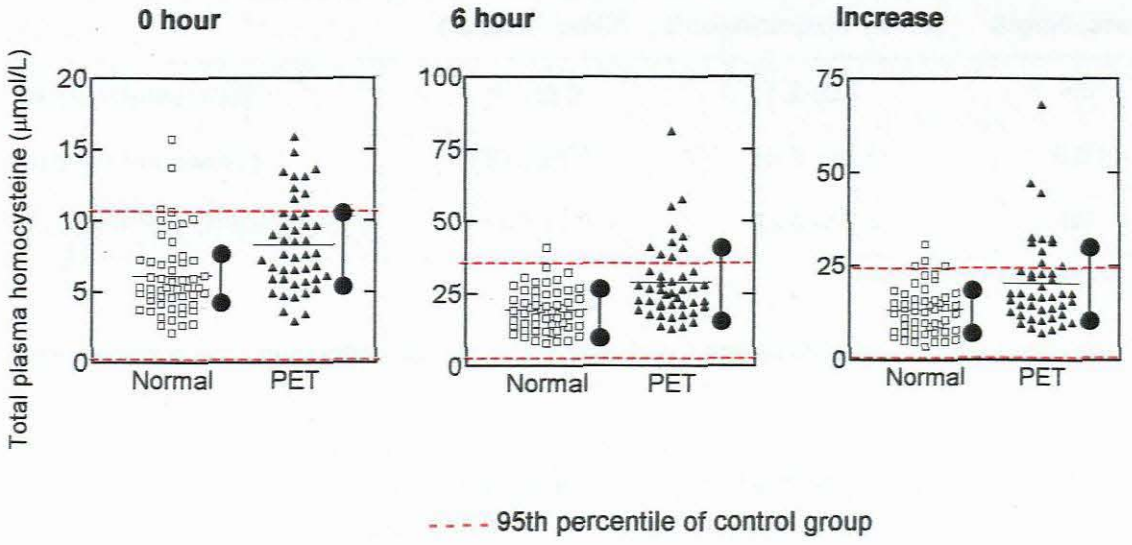


Figure 52. Fasting, 6 hour post oral methionine and change in total plasma homocysteine (tHcy) measured in normal pregnant subjects (□) and patients from the pre-eclampsia (PET) study (▲). Horisontal lines denote means and vertical lines denote ±SD. $p < 0.001$ between normal pregnant subjects and pre-eclampsia (PET) patients total plasma homocysteine (tHcy) at 0 hour, 6 hour and for the increase after oral methionine. The p value was calculated using the Mann Whitney U test.

Hyperhomocysteinaemia was significantly more common in pre-eclamptic women than in controls (p <0.001). Oral methionine allowed identification of an extra five affected women than screening by fasting total plasma homocysteine levels alone. No significant difference was observed when these parameters were compared between Coloured and African females of the normal control group {(5.6±3.2) vs (6.1±2.8) for 0 hour, (20.0±8.6) vs (19.0±7.4) for 6 hour and (14.3±7.2) vs (13.0±6.3)} for the increase after oral methionine and Coloured and African females of the pre-eclampsia group {(7.2±2.7) vs (8.3±3.2) for 0 hour, (30.8±18.3) vs (28.7±13.4) for 6h and (23.6±16.4) vs (20.5±11.8) for 6 hours after oral methionine (table 18).

Total plasma homocysteine in Coloured females from pre-eclampsia study

(a)

	Control[a] (n=17)	Pre-eclampsia[a] (n=11)	Significance[b]
0h tHcy (μmoles/L)	5.6 ±3.2	7.2 ±2.7	NS
6h tHcy (μmoles/L)	20.0 ±8.6	30.8 ±18.3	0.03
Increase in tHcy (μmoles/L)	14.3 ±7.2	23.6 ±16.4	NS

Total plasma homocysteine African females from pre-eclampsia study

(b)

	Control[a] (n=53)	Pre-eclampsia[a] (n=44)	Significance[b]
0h tHcy (μmoles/L)	6.1 ±2.8	8.3 ±3.2	< 0.01
6h tHcy (μmoles/L)	19.3 ±7.4	28.7 ±13.4	< 0.01
Increase in tHcy (μmoles/L)	13.2 ±6.3	20.5 ±11.8	< 0.01

Table 18a and 18b. [a]Results are given in means ±SD. [b]A *p* value of <0.05 was considered significant. A comparison between normal control subjects and pre-eclampsia subjects of both race groups showed a significant difference between fasting (0h), 6 hours after oral methionine (6h) and the increase 6 hours after oral methionine. Similar results were obtained when the African female control and pre-eclampsia groups were compared. When the Coloured female control and pre-eclampsia groups were compared, a significant difference was only observed at 6 hours after oral methionine.

4.2.5.2 Thermolabile variant of methylenetetrahydrofolate reductase in pre-eclampsia study

All the subjects from the pre-eclampsia study were screened for the C677T point mutation in the methylenetetrahydrofolate reductase (MTHFR) gene. Table 19 shows the genotype frequencies found in normal controls and affected patients from the pre-eclampsia study.

Methylenetetrahydrofolate reductase genotype in pre-eclampsia study group

	MTHFR C677T Genotype frequencies n (%)		
	TT	**CT**	**CC**
Patients (n=44)	0	3 (6.8%)	41 (93.2%)
Controls (n=53)	0	5 (9.4%)	48 (90.6%)

Table 19. Association for the methylenetetrahydrofolate reductase (MTHFR) genotype with pre-eclampsia. TT indicates homozygosity for the mutation; CT, heterozygosity; and CC, normal homozygosity.

No homozygotes (TT) for the C677T mutation, were detected in this study group. The CT genotype frequency was similar between the patients and normal control group. The question arose whether hyperhomocysteinaemia was associated with a particular genotype. Table 20 shows mean ±SD total plasma homocysteine (tHcy) measured in the three genotypes from the patient group of the pre-eclampsia study.

Methylenetetrahydrofolate reductase genotype and total plasma homocysteine

	MTHFR C677T Genotype vs tHcy		
	CC	**CT**	TT
n	41	3	0
0 hour tHcy (±SD)	8.18 (±3.2)	9.3 (±3.3)	-
6 hour tHcy (±SD)	29.3 (±13.8)	21.0 (±3.09)	-
Increase (±SD)	21.1 (±2.0)	11.7 (±2.58)	-

Table 20. Average total plasma homocysteine values and methylenetetrahydrofolate reductase (MTHFR) genotype in patients from the pre-eclampsia study.

Total plasma homocysteine was similar in the CC and CT genotype. The TT genotype was not detected in the pre-eclampsia study group. Did high total plasma homocysteine (tHcy) > 95th percentile of mean total plasma homocysteine (tHcy) for all patients of the

pre -eclampsia study correlate with any particular C677T MTHFR genotype? Results are shown in table 21.

Total plasma homocysteine percentiles and methylenetetrahydrofolate reductase genotypes

	Total plasma homocyteine percentiles	
Pre-eclampsia study (n=44)	< 95%	>95%
CC	94.7%	78.0%
CT	5.3%	12.0%
TT	0%	0%

Table 21. Methylenetetrahydrofolate reductase (MTHFR) C677T genotype according to total plasma homocysteine (tHcy) (< or > 95th percentile).

No correlation existed between high total plasma homocysteine (tHcy) and any specific C677T Methylenetetrahydrofolate reductase (MTHFR) genotype in the affected patient group of the pre-eclampsia study.

4.2.6 Peripheral vascular disease study group

Patients with early onset peripheral vascular disease (PVD), recruited from the Surgical Department at Groote Schuur Hospital, were screened for fasting and 6 hour post oral methionine, total plasma homocysteine, in order to establish whether hyperhomocysteinaemia was prevalent amongst these patients. European normal ranges were used to classify patients as hyperhomocysteinaemic (Ueland *et al.*, 1993). Previous studies used different reference ranges for men and women, especially between the ages of 30 to 50 years. Total plasma homocysteine between men and women with peripheral vascular disease were compared as shown in table 22, in order to determine if a similar difference existed.

4.2.6.1 Amino acid analysis in patients with peripheral vascular disease

Total plasma homocysteine in male and females of the peripheral vascular disease study group

	Male [a] (n=39)	Female[a] (n=31)	Significance[b]
0h tHcy (μmol/L)	13.0 ±11.8	10.0 ±6.0	NS
6h tHcy (μmol/L)	37.1 ±17.7	32.6 ±12.5	NS
Increase (μmol/L)	23.2 ±12.8	22.6 ±10.3	NS

Table 22. Total plasma homocysteine (tHcy) values in males and females of peripheral vascular disease study. [a]Results are given in means ±SD. A *p* value of <0.05 was considered significant.

No significant difference in total plasma homocysteine (tHcy) existed between males and females with peripheral vascular disease. Total plasma homocysteine in 70 peripheral vascular disease patients after an overnight fast was 12.0 ±9.8μmoles/L (range 2.2 – 21.8) compared to reference range of (5.5 – 15.9μmoles/L (Andersson *et al.*, 1992). Total plasma homocysteine in 70 peripheral vascular disease patients, 6 hours post oral methionine, was 35.0 ±16.0μmoles/L (range 19.0 – 51.0μmoles/L) compared to a reference range of (15 – 43.9μmoles/L) (Andersson *et al.*, 1992). The increase in total plasma homocysteine (tHcy), 6 hours post oral methionine was 23.0 ±12.0μmoles/L (range 11.0 - 35.0) compared to reference range of (10 - 28μmol/L) (Andersson *et al.*, 1992).

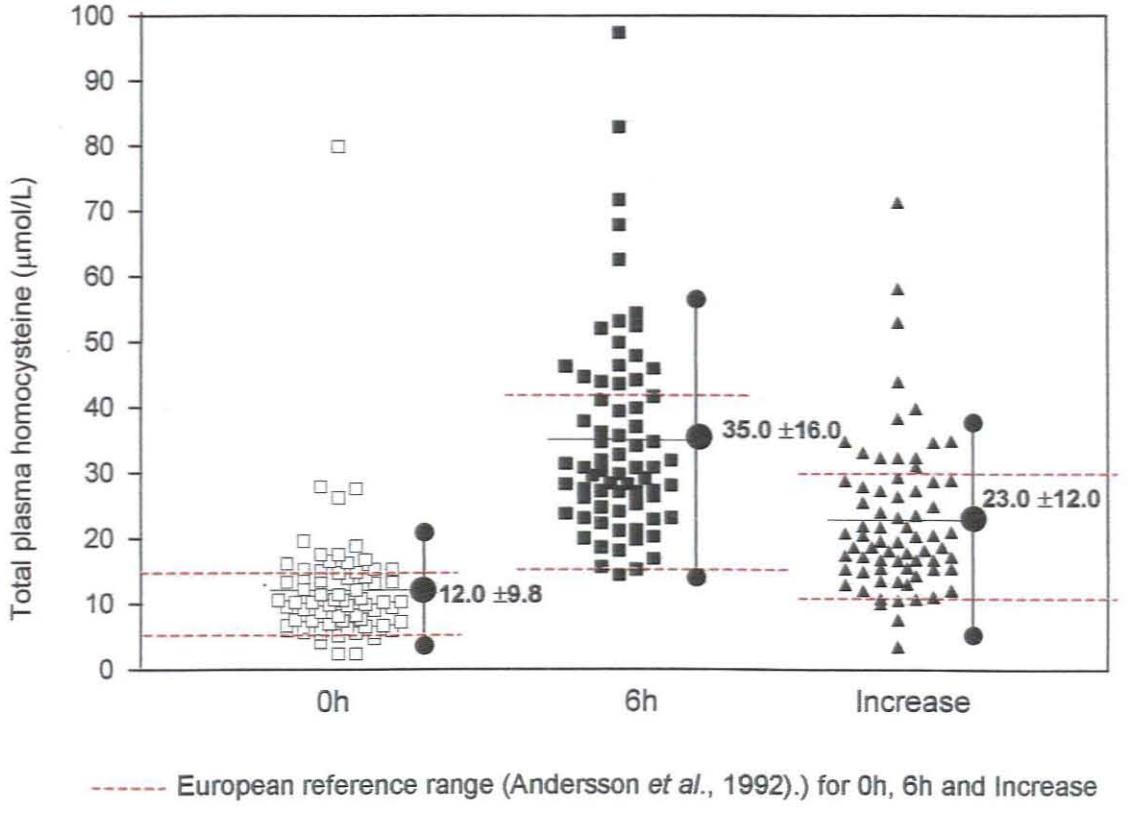


Figure 53. Total plasma homocysteine before and after oral methionine in 70 patients with peripheral vascular disease. (Referance range, 0h = 5.5 – 15.9; 6h = 15 – 43.9; increase = 10 - 28µmol/L (Andersson *et al.*, 1992).

17% (12/70) of the patients had hyperhomocysteinaemia according to fasting total plasma homocysteine (tHcy) measurements and 24% (17/70) of the patients had hyperhomocysteinaemia according to 6 hour post methionine measurements as shown in figure 51. 27% (19/70) of the patients had abnormal increase in total plasma homocysteine after oral methionine. 13% of the patients had a normal fasting total plasma homocysteine but an abnormal total plasma homocysteine 6 hours after oral methionine. Indicating that oral methionine was necessary for the diagnosis of hyperhomocysteinaemia in some patients.

4.2.6.2 Folate treatment in patients with peripheral vascular disease

Fourteen patients with peripheral vascular disease and post oral methionine total plasma homocysteine values above the upper limit of the reference range of Andersson *et al.* (1992) were given oral folic acid and vitamin B_6 (5mg folate and 25mg vitamin B_6) daily for 6 weeks. After folate and vitamin B_6 treatment, these patients were given a second oral methionine test and retested for total plasma homocysteine at 0 and 6 hours post oral

methionine. Total plasma homocysteine measurements before and after folate treatment are shown in table 23.

Total plasma homocysteine before and after vitamin treatment in PVD patients

	Before folate[a]	After folate[a]	Significance[b]
0 hour tHcy (μmol/L)	14.2 ±4.5	7.6 ±1.4	< 0.01
6 hour tHcy (μmol/L)	57.1 ±15.7	29.2 ±4.7	< 0.01
Increase after 6 h (μmol/L)	37.2 ±25.2	22.3 ±5.9	< 0.01

Table 23. Total plasma homocysteine (tHcy) values in hyperhomocysteinaemic peripheral vascular (PVD) disease patients before and after folate treatment (n=14). [a]Results are given in means ±SD. [b]A *p* value of <0.05 was considered significant.

Both fasting and 6 hour post oral methionine total plasma homocysteine after folate treatment was significantly lower than total plasma homocysteine before folate treatment. Both fasting and 6 hour total plasma homocysteine (tHcy) values were lowered to within the reference range as shown in fig. 54.

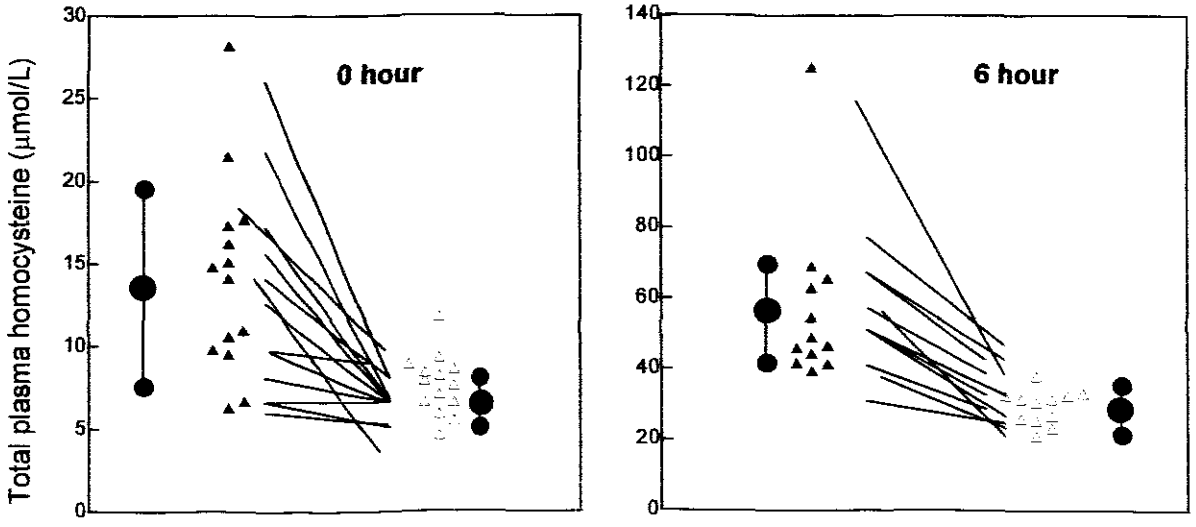


Figure 54. Fasting (0h) and 6 hour total plasma homocysteine (tHcy) values (±SD.) before (▲) and after (△) folate treatment.

The mean value for the fasting total plasma homocysteine (tHcy) before folate treatment was 14.2 ±6.0μmol/L compared to 7.6 ±1.8μmol/L after folate treatment. The mean post oral methionine, total plasma homocysteine value before folate treatment was 57.1μmol/L ±15.7 compared to 29.2 ±4.7μmol/L after folate treatment. There was a significant difference between mean fasting ($p < 0.01$) and 6 hour ($p < 0.01$) total plasma homocysteine before and after folate treatment.

4.2.6.3 Thermolabile variant of methylenetetrahydrofolate reductase in the peripheral vascular disease study group

All the subjects from the peripheral vascular disease study were screened for the C677T point mutation in the methylenetetrahydrofolate reductase MTHFR gene. Table 24 shows the genotype frequencies found in randomly selected patients that visited Groote Schuur Hospital for minor ailments and had no sign of vascular disease and affected patients from the peripheral vascular disease study.

Methylenetetrahydrofolate reductase genotype in peripheral vascular disease study group

	MTHFR Genotype n (%)		
	TT	**CT**	**CC**
Patients (n=70)	4 (5.6%)	16 (22.5%)	50 (71.8%)
Controls (n=247)	1 (0.4%)	46 (18.6%)	200 (81%)

Table 24. Distribution of the methylenetetrahydrofolate reductase (MTHFR) genotype amongst patients from the peripheral vascular disease study group and subjects from control group from the general population.

The question arose whether hyperhomocysteinaemia was associated with a particular genotype. Table 25 shows mean ±SD total plasma homocysteine measured in the three genotypes from the patient group of the peripheral vascular disease study. More patients than controls carried the TT genotype. The population was however not large enough for statistical analysis.

Total plasma homocysteine and methylenetetrahydrofolate reductase genotype frequencies in patients with peripheral vascular disease.

	MTHFR n(%) Genotype		
	CC	**CT**	**TT**
n	51	16	4
0h tHcy (±SD)	10.5 (±4.87)	12.8 (±5.13)	29.8 (±34.8)
6h tHcy (±SD)	33.7 (±14.2)	35.4 (±11.9)	51.8 (±31.6)
Increase (±SD)	23.1 (±12.8)	22.6 (±8.7)	22.0 (±4.3)

Table 25. Average total plasma homocysteine as mean ±SD, values and methylenetetrahydrofolate reductase (MTHFR) genotype in patients from the peripheral vascular disease study.

The mean total plasma homocysteine (tHcy) in patients with the TT genotype were higher than mean total plasma homocysteine values of patients with the CC and CT genotypes, but not significantly different.

4.2.7 Methylenetetrahydrofolate reductase genotype in the different local race groups

In order to determine if there was genetic variation between the different local ethnic groups of the Western Cape regarding the methylenetetrahydrofolate reductase (MTHFR) gene, the genotype frequency of the C677T mutation was compared amongst the two local ethnic populations, namely African and Coloured women. Does the C677T methylenetetrahydrofolate reductase variant associate with a particular ethnic group? Results shown in table 26.

Methylenetetrahydrofolate reductase genotype frequencies in the local race groups

MTHFR	CC n (%)	CT n (%)	TT n (%)
Caucasians (n=91)	60 (65.9)	31(34.1)	0
Coloureds (n=195)	153 (78.5)	35 (17.9)	7 (3.6)
Africans (n=68)	65 (95.6)	3 (4.4)	0

Table 26. Methylenetetrahydrofolate reductase (MTHFR) genotype frequencies in Caucasians, Coloureds and Africans. CC denotes normal MTHFR genotype. CT denotes heterozygosity for the C677T thermolabile variant. TT denotes homozygosity for the C677T thermolabile variant. Significance was calculated using the Mann Whitney test. Caucasians vs Coloured, $p = 0.0009$, Caucasians vs Africans, $p = 0.0001$, Coloured vs African, $p = 0.040$.

The T allele appeared to be more prevalent amongst the Caucasian population with the CT genotype at a frequency of 34.1% compared to 17.9% in the Coloured population and only 4.4% in the African population.

4.3 An association between peripheral vascular disease, hypertension and variation in the fibrillin-1 and elastin genes

4.3.1 Introduction

During this study an association between hyperhomocysteinaemia and peripheral vascular disease in the local population of the Western Cape Region, South Africa was confirmed (paragraph 4.2, page 93), but no definite genetic link could be established. Previous investigators have used polymorphic markers (microsatellites) to link specific genes to certain disease states. For this kind of study however large sample populations are needed. Hypertension is one of the most common chronic diseases affecting the general population and the Inherited Metabolic Disease Unit at Groote Schuur Hospital regularly measures total plasma homocysteine in patients from the Hypertension Clinic. For this study it was therefore decided to use the patient population from the Hypertension Clinic to perform linkage analysis on. An association between hypertension and hyperhomocysteinaemia has been reported (Nygard, 1995). Powell *et al.* (1996) investigated patients with abdominal aortic aneurysms and found, by using microsatellite analysis of a tandem repeat polymorphism located within the fibrillin-1 gene, a significant association between a fibrillin-1 genotype and abdominal aortic aneurysm existed. Subsequently they also reported an association between the fibrillin-1 genotype and blood pressure in a healthy population of 245 men aged between 50 to 61 years. The product of the elastin gene is intimately associated with fibrillin-1 in the construction of the microfibrillar structure of elastic tissue. Deletions in the elastin gene have been reported in patients with Williams' syndrome, a disease characterized by vascular abnormalities.

This study investigated the possible association of hypervariable markers (microsatellites) in the (a) the fibrillin-1 gene and (b) the elastin gene in 156 patients with hypertension and 77 patients with peripheral vascular disease. A control group (n=79), consisting of patients who were admitted to Groote Schuur Hospital for minor ailments and were normotensive were investigated simultaneously. Their blood pressures were recorded and only subjects with systolic blood pressures below 160mm Hg were included in statistical analysis. The subjects from the control group with systolic blood pressures higher than 160mm Hg were however included for race comparisons.

Each subject from both the normotensive and hypertensive study groups was typed for polymorphic markers (i.e., determining the number of repeats) found in two loci in the fibrillin-1 gene and one locus in the elastin gene. One of the loci in the fibrillin-1 gene was the $(TAAAA)_n$ repeat used by Powell *et al.* (1996). Powell used a search for a genotypic association. Rather than the obvious search for an allele association. Although it is not

clear what the advantage of this approach is, it was used as well for this study in case compound heterozygotes or homozygotes for certain alleles in a recessive situation are associated with disease.

A genotype represents the genetic factors inherited from both parents. Therefore it comprises two alleles, one from the father and one from the mother. An allele is an alternative form of a gene, which represents a certain trait or characteristic (inherited from either the mother or the father) e.g. it may carry a specific number of repeats for a given microsatellite.

4.3.2 Microsatellite analysis

Fragments of genomic DNA spanning the three microsatellites (two in the fibrillin-1 gene and one in the elastin gene was amplified by PCR. The alleles were separated by electrophoresis on a vertical polyacrylamide gel. These gels have sufficient resolution to separate alleles that differ by as little as one base pair. This is necessary as the dinucleotide tandem repeats could differ by one repeat. A size marker was run on each gel at regular intervals so that microsatellites were scored reproducibly.

A common complication associated with the amplification of dinucleotide repeats is the "stutter bands" visible on the polyacrylamide gel, usually underneath the main allele band (see fig. 55). These are amplification artifacts caused most likely by slippage events during the PCR replication process, in which the template DNA and the newly synthesized strand misalign during elongation. This results in the amplification of additional truncated products (Tautz, 1989). Generally the stutter bands appear fainter on the gel than the main alleles, and can be disregarded fairly easily.

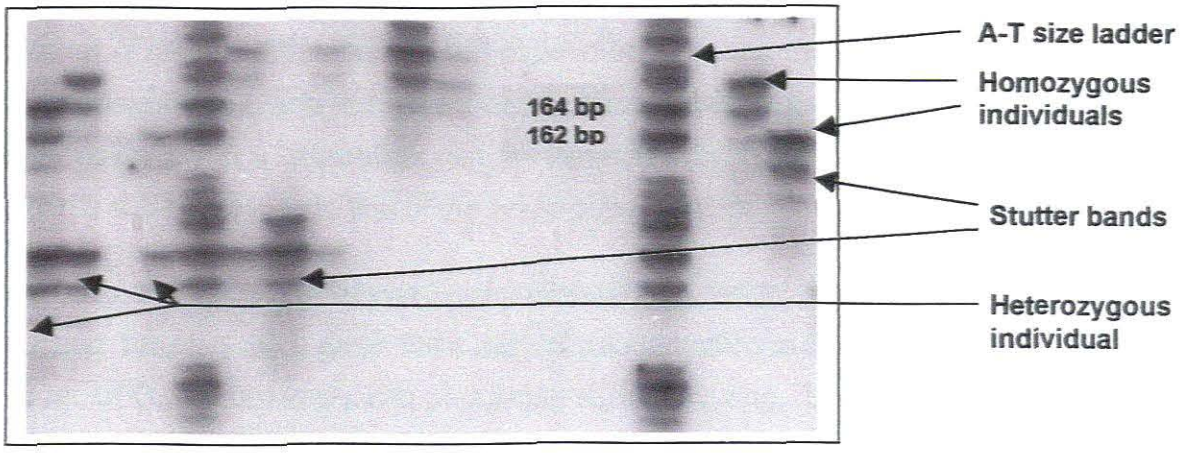


Figure 55. Example of a polyacrylamide gel of the fibrillin-1 f1 microsatellite locus, showing homozygous and heterozygous individuals and slippage products ("stutter bands").

4.3.3 Statistical Analysis

Statistical analysis of allele frequencies was performed to establish whether population differentiation could be established between these disease groups and the normal population. Chi^2 (distribution) was performed to determine whether certain genotypes associated with an increase in systolic blood pressure and pulse pressure. Statistical analysis to determine whether specific alleles co-segregated with a disease was also performed.

Two previously identified loci in intron 5 (21495) and intron 28 (21496) respectively (Perreira *et al.*, 1994), in the fibrillin-1 gene and a locus in intron one in the elastin gene (Urban *et al.*, 1997) were selected for microsatellite analysis of the subjects from the peripheral vascular disease and hypertensive study groups. The microsatellite at locus 21495 was called fibrillin-1 f1 and the microsatellite at locus 21496 was called fibrillin-1 f5, in order to distinguish between the two loci in the fibrillin-1 gene for the purpose of this thesis.

To determine whether a significant difference in genotype and allele frequencies existed between disease states like peripheral vascular disease, hypertension and normotensive subjects, amplifications of DNA that spanned each repeat were generated by PCR, separated and scored according to size. Association between blood pressures and genotypes and the hypertensive disease group and genotypes were assessed by chi^2 (distributions) or analysis of variation (one way ANOVA) (means). Results from this study were compared with results from a previous study by Powell *et al.* (1996).

Allele frequencies, heterozygosity, and population differentiation statistical analysis was performed on data obtained in order to examine population differentiation according to allele frequencies rather than genotype frequencies. Populations are often divisible into sub-populations, which are no longer part of a single randomly mating population. The amount of genetic differentiation will depend on how long they have been apart, the size of the populations, and the extent of migration between the populations. The level of subdivision within a population can be measured by comparing the diversity of alleles within a sub-population to that of the entire population (or to that of other sub-populations).

We used two values to measure population differentiation. The G_{st} value, introduced by Nei and Tateno (1975) gives the extent of differentiation in the sub-population(s). Small values of G_{st} (<0.05) imply little population sub-structuring. Large values of G_{st} (0.1 to 1) imply much substructuring. Another measure of population differentiation was developed which is specifically tailored for use with microsatellites. This is called R_{st} (Slatkin, 1995), and takes into account the model of evolution which is thought to be followed by

microsatellites: whereas G_{st} assumes the "infinite allele model" (all mutations generate a unique new allele), R_{st} assumes a single step model whereby the repeat unit changes in size most of the time by one repeat unit. R_{st} can also be defined as the fraction of the total variance in allele size, which is found between populations. Another feature of R_{st} is that it takes into account the high mutation rate ($\sim 10^{-3}$) which is what generates the remarkable number of alleles commonly found in microsatellites, whereas G_{st} assumes that the mutation rate is insignificant over the time scale of the observed data (i.e. only genetic drift is assumed to be operating. Both these values have been included in the results to examine population differentiation and as an example of the different values they can produce. Both values of G_{st} and R_{st} range from 0 to 1, with 0 being no differentiation between populations in question, and 1 representing completely differentiated populations (completely unrelated). A value of 0.05 or higher is usually considered to represent significant population differentiation. R_{st} and G_{st} values were calculated using AGARST software (Harley, 1998).

Study groups were investigated initially without separating the three race groups, after which statistical analysis was performed on the individual race groups, namely African, Coloured and Caucasian. Statistical analysis was performed individually for each microsatellite at fibrillin-1 f1 locus in intron 5 and fibrillin-1 f5 locus in intron 28 and the locus in intron 17 in the elastin gene.

4.3.4 Genotype comparisons

For this study, primers (chapter 3, table 4, page 63) that resulted in allele sizes, of 183, 178, 173 and 168 base pairs were designed for the amplification of the microsatellite at the fibrillin-1 f5 locus, $(TAAAA)_n$. DNA from a subject with the 2-2 (178 – 178) genotype, was sequenced after amplification. The sequence as shown in fig. 56 confirmed 6 inserts of the TAAAA repeat, and the total length of the amplified sequence was confirmed as 178 in our study, showing that our size measurement of amplified alleles was correct.

4.3.4.1 Fibrillin-1 f5 microsatellite

(a)

gaaagataag taaaagtagcg atgaaaacaa aagtccagag tactagagtg ttttagggaga gatgaaat taaaataaaa taacataaca taacataaca taaaataaag $(TAAAA)_6$ aagaacttac caacacaaaa tagcctatcg ggagttgaat ggtagccagg gttgcaggca cactgatact tccctatgag g

(b)

gaaagataag taaaagtagcg atgaaaacaa aagtccagag tactagagtg ttttagggaga gatgaaat taaaataaaa taacataaca taacataaca taaaataaag $(TAAAA)_n$ aagaacttac caacacaaaa tagcctatcg ggagttgaat ggtagccagg gttgcaggca cactgatact tccctatgag g

Figure 56: DNA sequence flanking the fibrillin-1 f5 microsatellite polymorphisms in the fibrillin-1 gene. The red letters indicate the location of the sense and antisense primers, used to amplify the fragment of DNA that carries the TAAAA microsatellite in this study (a) and in Powell's study (b) respectively.

An allele of 171 base pair was the most commonly found allele according to Powell. This was also his second largest allele. This study however showed allele 178 [$(TAAAA)_6$ penta nucleotide repeat insert)] to be the most prevalent allele. Allele 178 was the second largest allele detected in this study. The size differences can be in part be explained by the use of different primers.

Fibrillin-1 f5 TAAAA repeat sequence

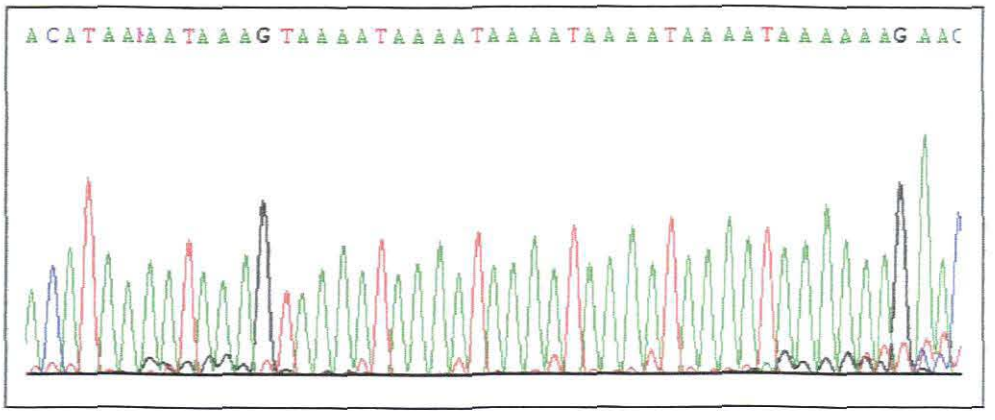


Figure 57. Sequence profile of the genomic DNA fragment amplified from the fibrillin-1 f5 locus, showing the six inserts of the TAAAA repeat.

During this study only three common genotypes were observed in the general population as shown in table 27.

Common fibrillin-1 f5 genotypes

Genotype	Name
178 - 178	2-2
178 - 173	2-3
178 – 168	2-4

Table 27. The three commonly found genotypes at the fibrillin-1 f5 microsatellite. The single number naming was used throughout the text to facilitate comparison with Powell's data.

Six other genotypes (shown in table 28) occurred less frequently (< 5%) and were pooled for statistical analysis.

Rare fibrillin-1 f5 genotypes

Genotype	Name
183 - 178	1-2
183 –173	1-3
183 – 168	1-4
173 – 173	3–3
173 – 168	3-4
168 – 168	4-4

Table 28. The fibrillin-1 f5 genotypes that occurred less frequently are shown here. The single number naming was used throughout the text.

The allele size of 178 [$(TAAAA)_6$ penta nucleotide repeat insert)] was the most prevalent allele size. Allele 178 was the second largest allele detected for this study. Chi^2 (distributions), were calculated with the rare alleles included and excluded (-rare). Different *p* values were obtained in each case. Genotype distributions for the fibrillin-1 f5 locus were compared between patients with hypertension, patients with peripheral vascular disease and the normotensive group (table 29).

Fibrillin-1 f5 distribution between the normotensive and disease groups

	Control	HPT	PVD
	n (%)	n (%)	n (%)
2-2	40 (50.6)	77 (49.4)	32 (41.6)
2-3	7 (8.9)	12 (7.6)	12 (15.6)
2- 4	22 (27.8)	39 (25.0)	26 (33.8)
p value (-rare)		0.954 (NS)	0.282 (NS)
rare	10 (12.7)	28 (17.9)	7 (9.1)
n	79	156	77
p value		0.758 (NS)	0.385 (NS)

Table 29. The distribution of the fibrillin-1 f5 genotypes in normotensive subjects (Control), patients with hypertension (HPT) and patients with peripheral vascular disease (PVD). *p* Values are for chi ^{2} (distribution). 2-2 denotes the 178-178 genotype, 2-3 denotes the 178-173 genotype, 2-4 denotes the 178-168 genotype. "Rare" denotes all the genotypes present at frequencies less than 5%.

Fibrillin-1 f5 genotype distributions in both disease groups were similar to that in the normotensive group, no association was seen between fibrillin-1 f5 genotype and hypertension or peripheral vascular disease. No significant difference for mean systolic blood pressure or pulse pressures was detected between the different genotypes. Both hypertensive patients and normotensive subjects, who carried the 2-3 genotype, had the highest mean pulse pressure as shown in fig. 58.

Distribution about mean systolic blood pressure and pulse pressure for the fibrillin-1 f5 genotypes

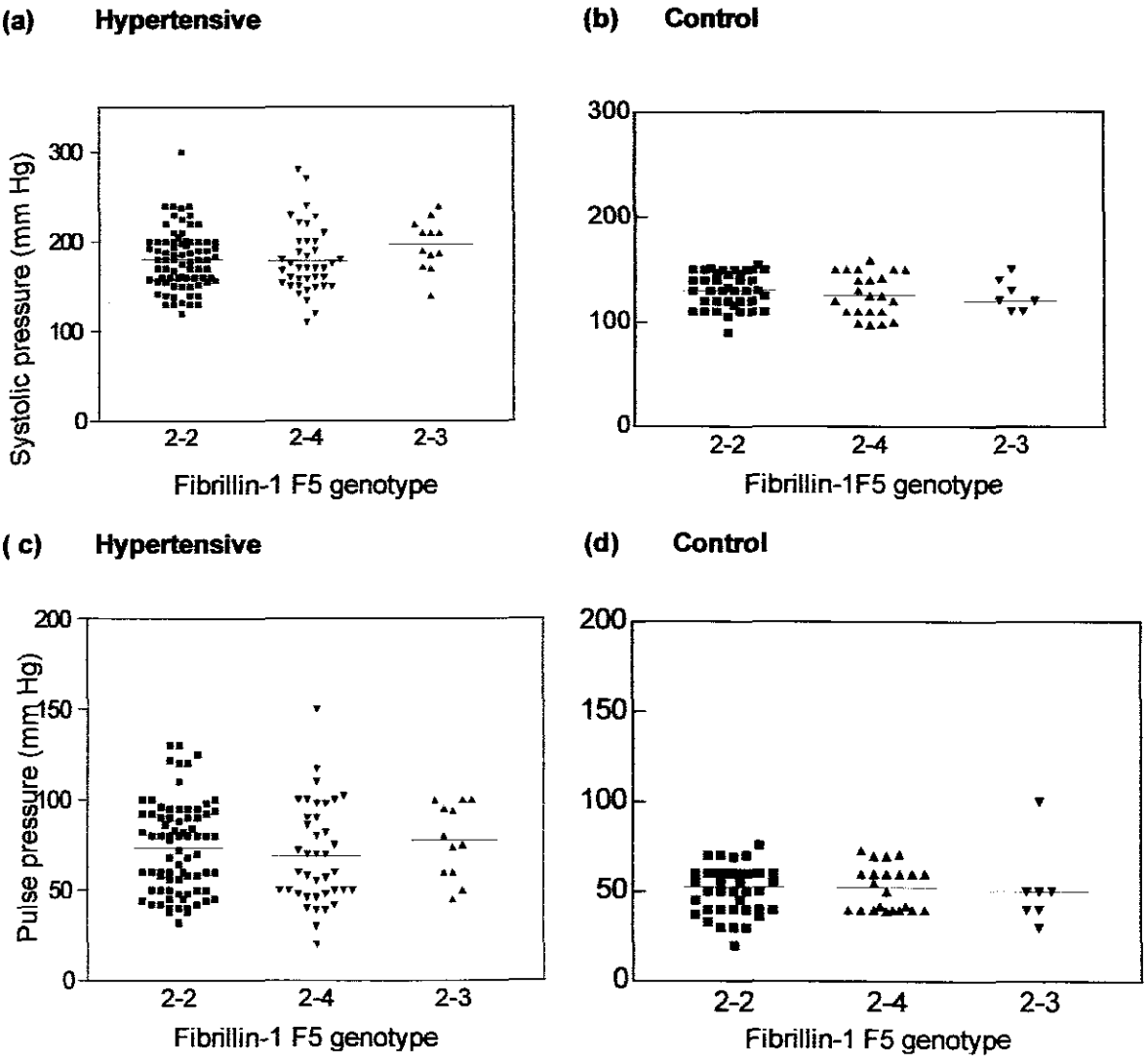


Figure 58a and 58b. Systolic blood pressure and fibrillin-1 f5 genotype in hypertensive patients (a) and normotensive subjects (b). Systolic blood pressures of the three common fibrillin-1 f5 genotypes only are shown. Differences between groups were assessed by Mann Whitney U test. (a) *p* Values were 2-2 vs 2-4 = 0.518; 2-4 vs 2-3 = 0.0534 (b) *p* Values 2-2 vs 2-3 = 0.922, 2-3 vs 2-4 = 0.981.

Figure 58c and 58d. Pulse pressure and fibrillin-1 f5 genotype in hypertension patients (c) and normotensive subjects (d). Pulse pressures of the three common fibrillin-1 f5 genotypes only, are shown. Differences between groups were assessed by Mann Whitney U test.(c) *p* Values were 2-2 vs 2-4 = .412; 2-3 vs 2-4 = 0.210 (d) *p* Values were 2-2 vs 2-3 = 0.370; 2-4 vs 2-3 =0.771.

The 2-2 fibrillin-1 f5 genotype was the most prevalent genotype in both normotensive subjects and patients with hypertension.

Distribution of fibrillin-1 f5 genotypes according to blood pressure

	Normotensive subjects					
	2-2	**2-3**	**2-4**	**rare**	***p* value**	***p* (-rare)**
syst <130 n (%)	18 (45.0)	2 (28.6)	9 (40.9)	3 (30.0)		
syst >130 n(%)	22 (55.0)	5 (71.4)	13 (59.1)	7 (70.0)	0.423	0.001
Mean BP (±SD)	130.3 (±16.7)	125.7 (±15.1)	126.6 (±20.4)	130.9 (±14.4)	0.797 (NS)	
pp <50 n(%)	20 (50.0)	6 (85.7)	11 (50.0)	4 (40.0)		
pp >50 n(%)	20 (50.0)	1 (14.3)	11 (50.0)	6 (60.0)	0.119	0.029
Mean pp (±SD)	50.8 (±13.2)	51.4 ± (22.7)	52.4 ± (12.4)	55.1 (±14.3)	0.944 (NS)	
n	40	7	22	10		

	Hypertensive patients					
	2-2	**2-3**	**2-4**	**rare**	***p* value**	***p* (-rare)**
syst<178 n(%)	34 (44.2)	3 (25.0)	24 (61.5)	14 (50.0)		
syst>178 n(%)	43 (55.8)	9 (75.0)	15 (38.5)	14 (50.0)	0.1162	0.003
Mean BP (±SD)	180.7 (±32.8)	197.0 (±28.4)	178.7 (±37.5)	179.8 (±38.9)	0.327 (NS)	
pp <69 n(%)	34 (44.2)	4 (33.3)	20 (51.3)	13 (46.4)		
pp >69 n(%)	43 (55.8)	8 (66.7)	19 (48.7)	15 (53.6)	0.727	0.085
Mean pp ± (SD)	73.4 (±25.0)	77.8 (±20.3)	69.2 (±27.8)	72.4 (±32.9)	0.924 (NS)	
n	77	12	39	28		

Table 30. Distribution of fibrillin-1 f5 genotypes according to blood pressure (bp) in subjects from the normotensive control group (n=79) and hypertensive patients (n=156). Blood pressure and pulse pressure are given in mm Hg. The distribution about the median is shown, followed by mean (±SD). *p* Values are for chi^2 (distributions) or one way ANOVA (means). Median systolic bp and pulse pressures for the normotensive subjects were 130mmHg and 55mm Hg respectively. Median systolic bp and pulse pressures for the hypertensive subjects were 178mmHg and 69mm Hg respectively. *p* Values for chi^2 (distributions) were calculated with and without rare alleles.

The median systolic blood pressure in hypertensive patients was 178mm Hg and 130mm Hg in normotensive subjects. Median pulse pressure in hypertensive patients was 69mm Hg compared to 50mm Hg in normotensive subjects. The chi^2 test for distribution of the genotypes around these medians were calculated in order to determine whether an association exist between a particular genotype and an increase in systolic and pulse pressure.

A significant association between fibrillin-1 f5 genotype and systolic blood pressure and between fibrillin-1 f5 genotype and hypertensive patients with high pulse pressure were detected when rare alleles was excluded with *p* values of 0.003 and 0.037 respectively. The 2-3 genotype was present at a frequency of 75% in hypertensive patients with systolic blood pressures above 178mm Hg compared to 25 % in hypertensive patients with systolic pressures below than 178mm Hg. Similarly the 2-3 genotype was present at a higher frequency of 66% in the hypertensive patients with pulse pressures higher than 69mm Hg than the frequency of 33% in hypertensive patients with pulse pressures lower than 69mm Hg. A similar association was seen in normotensive subjects. This significance however was caused by an increase of the 2-3 genotype in the normotensive patients with pulse pressures lower than 50mm Hg, which is contradictory to the increase of this genotype in patients with higher systolic blood pressures and pulse pressures in hypertensive patients.

Although no significant differences between mean systolic blood pressures and mean pulse pressures were observed in the different genotypes, mean systolic blood pressures and mean pulse pressures were higher in patients carrying the 2-3 genotype. The patient with the highest systolic blood pressure however, carried the 2-2 genotype as shown in fig. 58. Statistical analysis of the control group showed no difference in pulse or systolic blood pressure between the genotypes. No genotype was significantly associated with an increase in pulse or systolic blood pressure.

4.3.4.2 Fibrillin-1 f1 microsatellite

Patients from the hypertensive study group and subjects from the normotensive group were also genotyped for the fibrillin-1 f1 microsatellite. Primers (chapter 3, table 4, page 63) flanking the fibrillin-1 f1 microsatellite (fig. 59) were used to amplify the microsatellite at the fibrillin-1 f1 locus. Alleles ranging from 166 base pairs to 136 base pairs were observed at the fibrillin-1 f1 locus after fractionation by electrophoresis on a 6% polyacrylamide gel. The genotype with identical alleles 144-144, was sequenced to confirm allele sizes as shown in fig. 60.

atcttgcaga gtctccaatt aatcatggca cacgagtact cttcattaaa ggaaaagaga aagaaggaaa
aaaaata$(ca)_{19}$ tcttaga gtcctagagg gcagaaataa tcttggggag ctttgcaag acgtatatgc ctggg

Figure 59. DNA sequence flanking the fibrillin-1 f5 microsatellite polymorphism in the fibrillin-1 gene at locus 21945. The red letters indicate the location of the sense and antisense primers, used to amplify the fragment of DNA that carries the $(CA)_{19}$ repeat.

Fibrillin-1 f1 (CA) repeat sequence in a 144-144 (13-13) genotype

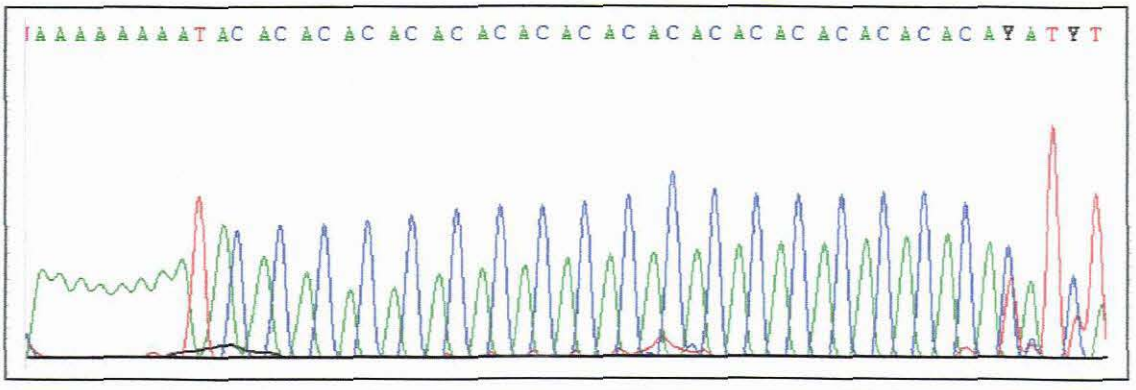


Figure 60. Sequence profile of the genomic DNA fragment amplified from the fibrillin-1 f1 locus, showing the 19 inserts of the (CA) repeat.

The dinucleotide repeat in intron 5 of the fibrillin-1 gene has seventeen alleles, of which three genotypes as shown in table 31 were observed the most frequently. The largest allele was designated as 1 and subsequent smaller alleles were designated consecutive numbers.

Common fibrillin-1 f1 genotypes

Genotype	Name
156 - 144	7-13
144 - 144	13-13
146 – 144	12-13

Table 31. The three commonly found genotypes at the fibrillin-1 f1 microsatellite. The single number naming was used throughout the text.

For statistical analysis these genotypes were analysed individually. Thirty-six other genotypes (not shown) occurred less frequently (< 5%) and were pooled for statistical analysis, and were called "rare". Genotype distributions for the fibrillin-1 f1 locus were compared between patients with hypertension, patient with peripheral vascular disease and the control normotensive group (results shown in table 32).

Fibrillin-1 f1 genotype distribution between disease groups and normotensive subjects

	Control	HPT	PVD
	n (%)	n (%)	n (%)
7-13	3 (3.8)	15 (9.6)	3 (3.9)
12-13	1 (1.3)	6 (3.8)	2 (2.6)
13-13	5 (6.3)	7 (4.5)	2 (2.6)
***p* value (-rare)**		0.232 (NS)	0.499 (NS)
rare	70 (88.6)	128 (82.0)	70 (90.9)
***p* value**		0.250 (NS)	0.660 (NS)
n	79	156	77

Table 32. Distribution of the fibrillin-1 f1 genotypes in normotensive subjects (Control), patients with hypertension (HPT) and patients with peripheral vascular disease (PVD). *p* Values are for chi^2 (distribution).

The majority of patients carried the genotypes that appeared less frequently. This locus was clearly not suitable for analyses of chi^2 (distribution), because of the small population sizes for even the more commonly found genotypes.

Distribution about the mean systolic blood pressure and pulse pressure for the fibrillin-1 f1 genotypes

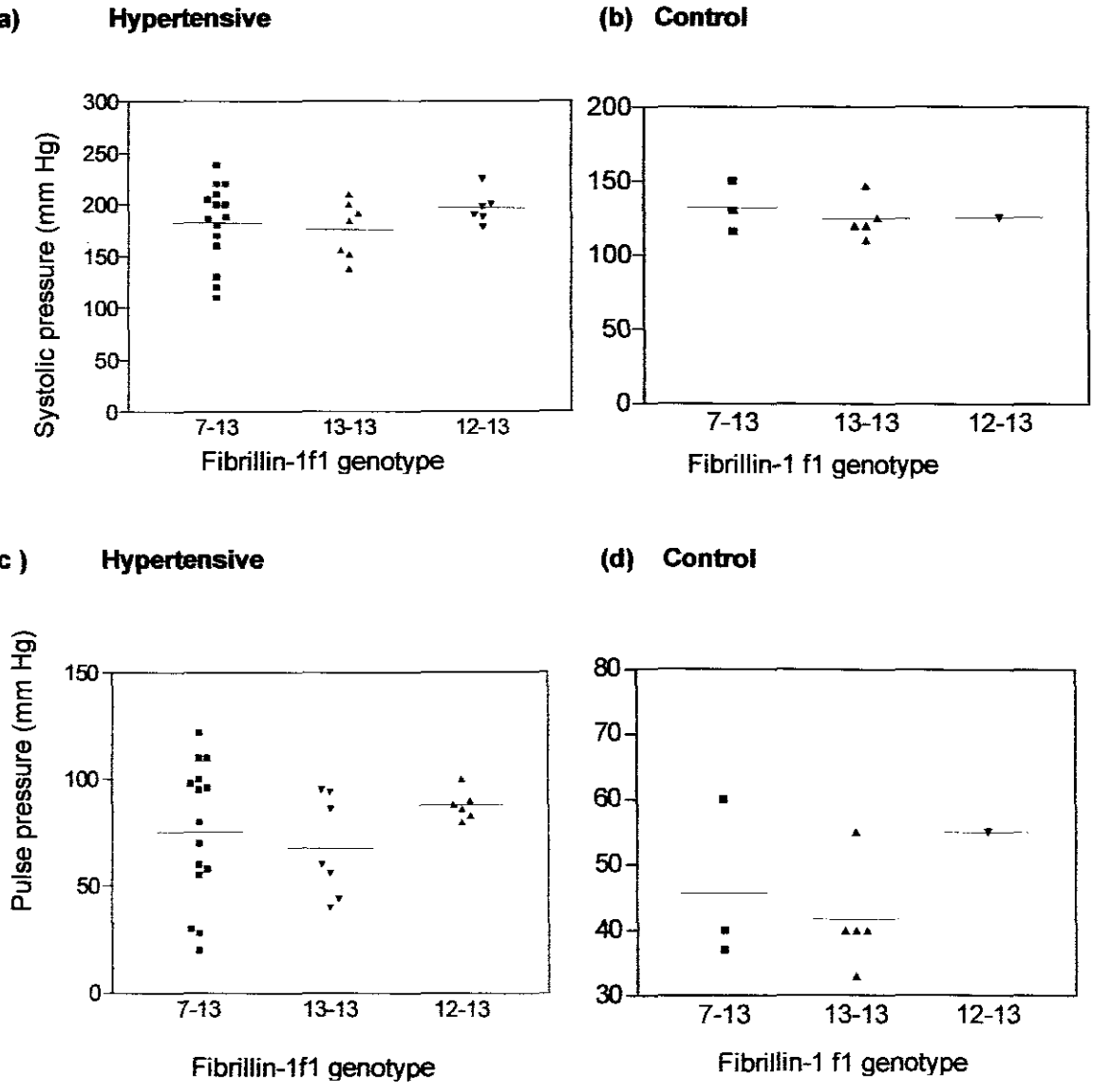


Figure 61a and 61b. Systolic blood pressure and fibrillin-1 f1 genotype in patients (a) with hypertension and normotensive subjects (b). Systolic blood pressures of the three common fibrillin-1 f1 genotypes only are shown. Differences between groups were assessed by Mann Whitney U test. *p* Values were 7-13 vs 13-13 = 0.549 and 13-13 vs 12-13 = 0.295. *p* Value = NS

Figure 61c and 61d. Pulse pressure and fibrillin-1 f1 genotype in patients with hypertension and normotensive subjects (d). Pulse pressures of the three common fibrillin-1 f1 genotypes only, are shown. Differences between groups were assessed by Mann Whitney U test. No significant difference existed between the three groups. *p* Values were 7-13 vs 13-13 = 0.668 and 13-13 vs 12-13 = 0.234. *p* Value = NS.

The 7-13 genotype was the most prevalent genotype in hypertensive patients. Mean systolic blood pressures appeared to be higher in patients carrying the 12-13 genotype. The control population was too small for statistical analysis.

Distribution of fibrillin-1 f1 genotypes according to blood pressure in the normotensive group and hypertensive patients

	Non-hypertensive subjects					
	7-13	**13-13**	**12-13**	**rare**	***p* value**	***p* (-rare)**
syst <130	2 (50)	5 (66.7)	1 (100)	40 (51.9)		
syst >130	1 (50)	0 (33.3)	0	30 (48.1)	0.230 (NS)	0.324 (NS)
Mean BP (±SD)	132.0 (17.1)	124.4 (13.8)	125.0 (0)	129.2 (17.7)	N/a	
pp <55	2 (50)	4 (66.7)	0 (0)	35 (44.4)		
pp >55	1(50)	1 (33.3)	1 (100)	35 (55.6)	0.431 (NS)	0.301 (NS)
Mean pp (± SD)	45.7 (±12.5)	41.6 (±8.1)	55.0 (±0.0)	52.7 (±14.2)	N/a	
n	3	5	1	70		

	Hypertensive patients					
	7-13	**13-13**	**12-13**	**rare**	***p* value**	***p* (-rare)**
syst <178 n (%)	5 (33.3)	3 (42.9)	0 (0)	63 (49.2)		
syst >178 n (%)	10 (66.7)	4 (57.1)	6 (100.0)	65 (50.8)	0.084	0.083
Mean BP (±SD)	182 (±38.2)	176.0 (±27.3)	196.5 (±16.0)	180.7 (±35.6)	0.516	
pp <69 n (%)	6 (40%)	4 (57.1%)	0 (0%)	21 (16.4%)		
pp >69 n (%)	9 (60%)	3 (42.8%)	6 (100%)	107 (83.6%)	0.006	0.195
Mean pp (±SD)	75.5 (±32.6)	67.9 (±23.4)	87.8 (±6.9)	71.7 (±26.8)	0.424	
n	15	7	6	128		

Table 33. Distribution of fibrillin-1 f1 genotypes according to blood pressure in patients from hypertensive study (n=156) and normotensive controls (n=79). Blood pressure and pulse pressure are given in mm Hg. The distribution about the median is shown, followed by mean (±SD). *p* Values are for chi^2 (distributions) or analysis of variance (one way) ANOVA (means).

4.3.4.3 Elastin microsatellite

Patients from the hypertensive study group, peripheral vascular disease study group and subjects from the normotensive group were also genotyped for the microsatellite in intron 17 of the elastin gene. The dinucleotide (AC) repeat (Foster *et al.*, 1993) in intron 17 of the elastin gene has nine alleles ranging from 158 to 174 base pairs. The largest allele was designated as 1 and subsequent smaller alleles were designated consecutive numbers. During this study only five common genotypes for the elastin microsatellite were observed in the general population as shown in table 34. Primers from Foster *et al.* (1993) were used to amplify the DNA fragment containing the elastin (AC) repeat in intron 17. The reverse primer was used to sequence the 162-162 genotype as shown in fig. 62.

Elastin (AC) repeat sequence in a 162-162 (7-7) genotype

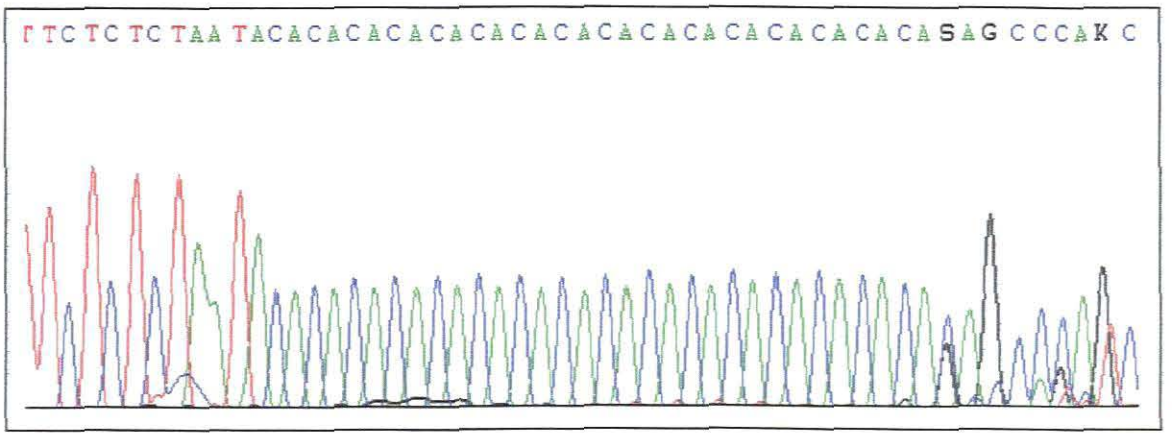


Figure 62. Sequence profile of the genomic DNA fragment amplified from the fibrillin-1 f1 locus showing the 19 inserts of the (AC) repeat.

Common elastin genotypes

Genotype	Name
172-162	2-7
168-162	4-7
166-162	5-7
164-162	6-7
162-162	7-7

Table 34. The five commonly found genotypes at the elastin microsatellite. The single number naming was used throughout the text.

Nine other genotypes (shown in table 35) occurred less frequently (< 5%) and were pooled for statistical analysis.

Rare elastin genotypes

Genotype	Name
174 - 162	1 -7
172 - 172	2-2
172 - 170	2-3
172 - 166	2-5
170 - 166	3-5
170 - 162	3-7
168 - 164	4-6
166 - 166	5-5
162 - 158	7-9

Table 35. The elastin genotypes that occurred less frequently. The single number naming was used throughout the text.

Elastin genotype distribution between disease groups and normotensive subjects

	Control	HPT	PVD
2-7	5 (6.3)	9 (5.8)	0 (0)
4-7	10 (12.7)	22 (14.0)	14 (18.2)
5-7	19 (24.0)	31 (19.9)	15 (19.5)
6-7	5 (6.3)	13 (8.3)	11(14.3)
7-7	20 (25.3)	46 (29.5)	22 (28.6)
***p* value (-rare)**		0.895 (NS)	0.078 (NS)
rare	20 (25.3)	35 (22.4)	15 (19.5)
***p* value**		0.932 (NS)	0.102 (NS)
n	79	156	77

Table 36. *Distribution of the elastin genotypes in non-hypertensive (control) subjects,* patients with hypertension (HPT) and patients with peripheral vascular disease (PVD). *p* Values are for chi^2 (distribution).

Elastin genotype distribution was the same in patients with hypertension and non-hypertensive subjects (results shown in table 36). The elastin genotype distribution in patients with peripheral vascular disease however showed a trend towards a possible association in normotensive and hypertensive subjects with a *p* value of 0.078 (rare excluded). No 2-7 genotypes were observed in the patients with peripheral vascular disease. Genotype 6-7 was present in the peripheral vascular disease group at a frequency of near double, at 14.3% compared to 8.3% and 6.3% in the hypertensive and normotensive groups.

Distribution about the mean systolic blood pressure and pulse pressure for the elastin genotypes

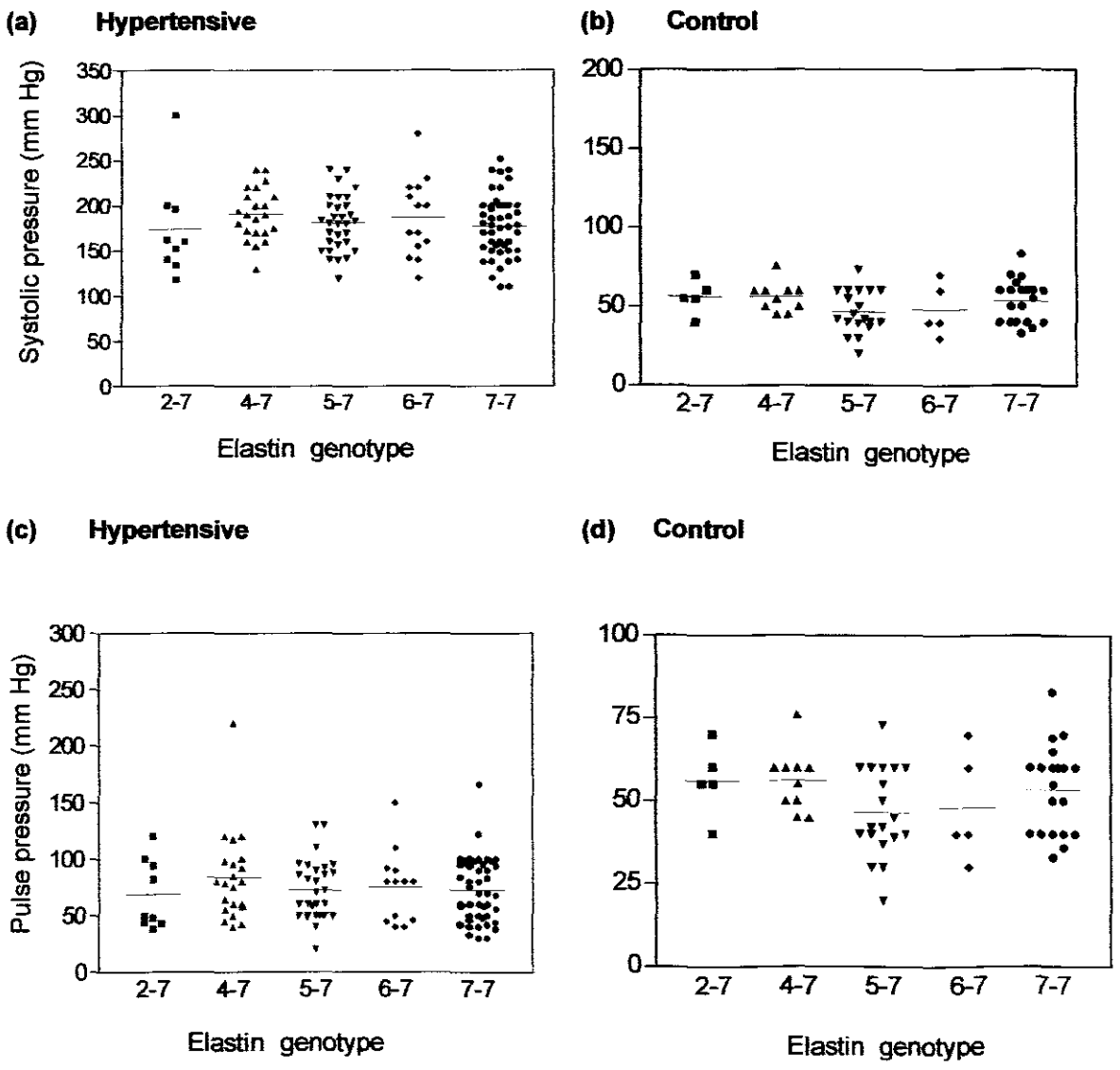


Figure 63a and 63b. *Systolic blood pressure and elastin genotype in patients with hypertension (a) and* normotensive subjects (b). Systolic blood pressures of the three common elastin genotypes only, are shown. Differences between groups were assessed by Mann Whitney U test. *p* Values were (2-7 vs 4-7 =0.085, 4-7 vs 5-7 = 0.210, 5-7 vs 6-7 = 0.722, 6-7 vs 7-7 = 0.479.

Figure 63c and 63d. *Pulse pressure and elastin genotype in patients with hypertension* (a) and normotensive subjects (b). Pulse pressures of the three common elastin genotypes only, are shown. Differences between groups were assessed by Mann Whitney U test. No significant difference existed between the three groups. *p* Values were 2-7 vs 4-7 = 0.327, 4-7 vs 5-7 = 0.441, 5-7 vs 6-7 = 0.796 and 6-7 vs 7-7 = 0.949.

Distribution of elastin genotypes according to blood pressure in the normotensive group and hypertensive patients

	Normotensive subjects							
	2-7	**4-7**	**5-7**	**6-7**	**7-7**	**rare**	***p***	***p* -rare**
Syst<130	3 (60.0)	5 (50.0)	15 (78.9)	3 (60.0)	9 (45.0)	12 (60.0)		
Syst>130	2 (40.0)	5 (50.0)	4 (21.1)	2 (40.0)	11 (55.0)	8 (40.0)	0.403	0.277
mean BP (±SD)	132.0 (±17.54)	131.3 (±18.1)	121.4 (±17.7)	130.0 (±18.7)	133.5 (±14.5)	128.3 (±19.6)	0.403	
pp<55	1 (20.0)	4 (40.0)	12 (63.2)	3 (60.0)	9 (45.0)	8 (40.0)		
pp>55	4 (80.0)	6 (60.0)	7 (36.8)	2 (40.0)	11 (55.0)	12 (60.0)	0.491	0.416
mean pp (±SD)	56.0 (±10.8)	56.1 (±9.3)	46.5 (±13.5)	48.0 (±16.3)	53.6 (±13.4)	52.4 (±16.8)	0.440	
n	5	10	19	5	20	20		

	Hypertensive patients							
	2-7	**4-7**	**5-7**	**6-7**	**7-7**	**rare**	***p***	***p* -rare**
syst>178	4 (44.4%)	13 (59.0)	18 (58.1)	4 (25)	23 (50.0)	20 (57.1)		
syst<178	5 (55.6%)	9 (41.0)	13 (41.9)	9 (75)	23 (50.0)	15 (42.9)	0.549	0.479
mean BP (±SD)	173.6 (±54.5)	190.9 ± (28.8)	181 (±30.5)	189.0 (±44.6)	178.6 (±33.7)	180.7 (±33.3)	0.558	
pp>62	4 (44.4)	14 (63.6)	23 (51.6)	8 (61.5)	24 (52.2)	13 (37.1)		
pp<62	5 (55.5)	8 (36.3)	15 (48.4)	5 (38.5)	22(47.8)	22 (62.9)	0.302	0.788
mean pp (± SD)	68.8 (±30.5)	79.1 (±26.8)	73.1 (±25.4)	75.6 (±32.0)	72.7 (±28.3)	70.8 (±25.4)	0.659	
n	9	22	31	13	46	35		

Table 37. Distribution of elastin genotypes according to blood pressure in subjects from normotensive control group (n=79) and hypertensive patient group (n=156). Blood pressure and pulse pressure are given in mm Hg. The distribution about the median is shown followed by mean (±SD). *p* Values are for chi^2 (distributions) or analysis of variance (one way ANOVA) (means).

Although no significant difference existed between the mean systolic blood pressures of the patients with the different elastin genotypes and subjects from the normotensive study,

a definite increase in mean systolic blood pressure was noted in the 4-7 and 6-7 genotypes. The patient with the highest systolic blood pressure however carried the 2-7 genotype as shown in fig. 63. No association between systolic blood pressure or pulse pressure and elastin genotype was seen in both hypertensive patients and normotensive subjects.

4.3.4.4 Genotype distribution amongst disease groups, separated according to race group

For this study, analysis of the fibrillin-1 f5, and elastin genotype distribution between peripheral vascular disease and control group and hypertensive disease group and control group was performed after separation of the two disease groups and the control group into the three separate ethnic race groups from the local population in the Western Cape, namely Caucasian, Coloured and African, in order to determine whether different distribution patterns are present in the different race groups. The microsatellite at fibrillin-1 f1 was excluded because of the wide variety of genotypes at this locus.

Fibrillin-1 f5 and elastin distribution amongst disease groups in Caucasians

Caucasian

(a) Fibrillin-1 f5 locus

	Control	HPT	PVD
	n (%)	n (%)	n (%)
2-2	12 (60.0)	12 (63.2)	6 (66.7)
2-3	2 (10.0)	2 (11.8)	1 (11.1)
2- 4	4 (20.0)	0 (0.0)	1 (11.1)
p value (-rare)		0.169 (NS)	0.844 (NS)
rare	2 (10.0)	3 (15.8)	1 (11.1)
p value		0.263 (NS)	0.952 (NS)
n	20	17	9

(b) Elastin locus

	Control	HPT	PVD
2-7	3 (15.0)	3 (17.6)	1 (11.1)
4-7	0 (0)	0 (0.0)	3 (33.3)
5-7	5 (25.0)	3 (17.6)	2 (22.2)
6-7	1 (5.0)	0 (0.0)	1 (11.1)
7-7	7 (35.0)	8 (47.0)	0 (0.0)
p value (-rare)		0.837 (NS)	0.035
rare	4 (20.0)	3 (17.6)	2 (22.2)
p value		0.915 (NS)	0.068 (NS)
n	20	17	9

Table 38. Distribution of the fibrillin-1 f5 (a) and elastin (b) genotypes in Caucasian non-hypertensive subjects, patients with hypertension and patients with peripheral vascular disease. *p* Values are for chi ^{2} (distribution).

A possible association between peripheral vascular disease and elastin gene was observed when $p = 0.035$, was calculated without rare alleles.

Fibrillin-1 f5 and elastin distribution amongst disease groups in the Coloureds

Coloured

(a) Fibrillin f5 locus

	Control	HPT	PVD
	n (%)	n (%)	n (%)
2-2	22 (50.0)	56 (47.5)	23 (42.6)
2-3	1 (2.3)	7 (5.9)	10 (18.5)
2- 4	12 (27.3)	35 (29.7)	17 (31.5)
***p* value (-rare)**		0.896 (NS)	0.116 (NS)
rare	9 (20.5)	16 (13.6)	4 (7.4)
***p* value**		0.712 (NS)	0.044
n	44	118	54

(b) Elastin locus

	Control	HPT	PVD
2-7	1 (2.3)	6 (5.1)	0 (0.0)
4-7	8 (18.2)	19 (16.1)	11 (20.4)
5-7	11 (25.0)	21 (17.8)	10 (18.5)
6-7	1 (2.3)	12 (10.2)	7 (13.0)
7-7	10 (22.7)	33 (28.0)	17 (30.5)
***p* value (-rare)**		0.522 (NS)	0.213 (NS)
rare	13 (29.5)	27 (22.9)	9 (16.7)
***p* value**		0.672 (NS)	0.194 (NS)
n	44	118	54

Table 39. Distribution of the fibrillin f5 and elastin genotypes in Coloured non-hypertensive subjects, patients with hypertension and patients with peripheral vascular disease. *p* Values are for chi ^{2} (distribution).

Statistical analysis of the Coloured group showed no difference in genotype distribution between the hypertensive group and the control group and the peripheral vascular disease group and control group. No particular genotype was associated with a particular disease group.

Fibrillin-1 f5 and elastin distribution amongst disease groups in Africans

African

(a) Fibrillin f5 locus

	Control	HPT	PVD
	n (%)	n (%)	n (%)
2-2	7 (46.7)	9 (42.9)	3 (21.4)
2-3	3 (20)	3 (14.3)	2 (14.3)
2- 4	1 (6.7)	3 (14.3)	7 (50.0)
p value -rare		0.693 (NS)	0.072 (NS)
rare	4 (26.7)	6 (28.6)	2 (14.3)
p value		0.850 (NS)	0.0565 (NS)
n	15	21	14

(b) Elastin locus

	Control	HPT	PVD
2-7	0 (0.0)	0 (0.0)	1 (7.1)
4-7	2 (13.3)	3 (14.3)	0 (0.0)
5-7	3 (20.0)	7 (33.3)	3 (21.4)
6-7	2 (13.3)	2 (9.5)	3 (21.4)
7-7	4 (25.0)	4 (19.0)	6 (42.9)
p value (-rare)		0.912 (NS)	0.334 (NS)
rare	4 (26.7)	5 (23.8)	1 (7.1)
p value		0.958 (NS)	0.278 (NS)
n	15	21	14

Table 40. Distribution of the fibrillin-1 f5 (a) and elastin (b) genotypes in African non-hypertensive subjects, patients with hypertension and patients with peripheral vascular disease. *p* Values are for chi ^{2} (distribution).

No significant difference between genotype frequencies of the fibrillin-1 f5 microsatellite was observed between the peripheral vascular disease or hypertensive and control group. The population size however was very small and this result might change with a larger population.

4.3.5 Allele frequencies

Previous investigators have used either only genotypic frequencies (Powell *et al.*, 1996) or both genotypic frequencies and allelic frequencies (Perreira *et al.*, 1994) to investigate linkage between hypervariable markers and a disease. Genotyping is useful when inheritance patterns are studied in families. Powell *et al.* (1996) used, instead of the obvious search for an allelic association, a search for a genotypic association. Although it is not clear what the advantage of this approach is, we used the allelic approach as well in case compound heterozygotes or homozygotes for certain alleles in a recessive situation are associated with disease.

We compared tables of allele sizes across the normotensive, hypertensive and peripheral vascular disease study groups for the three microsatellites independently. Allele frequency patterns were also observed. (fig. 64) for the fibrillin-1 f1, fibrillin-1 f5 and elastin microsatellites, in the normotensive, hypertensive (HPT) and peripheral vascular disease (PVD) study groups. R_{st} and G_{st} values were determined as measures of population differentiation. The control and disease groups comprised subjects from diverse racial origin.

Allele numbers per locus were similar for the different groups, even though population size varied between peripheral vascular disease (PVD) (n=77), normotensive control group (n=79) and hypertensive group (n=156). Figure 64 shows allele frequency patterns at microsatellites, fibrillin-1 f1, fibrillin-1 f5 and elastin for the peripheral vascular disease, hypertensive and control groups.

Allele frequency patterns in the normotensive group, and HPT and PVD patients

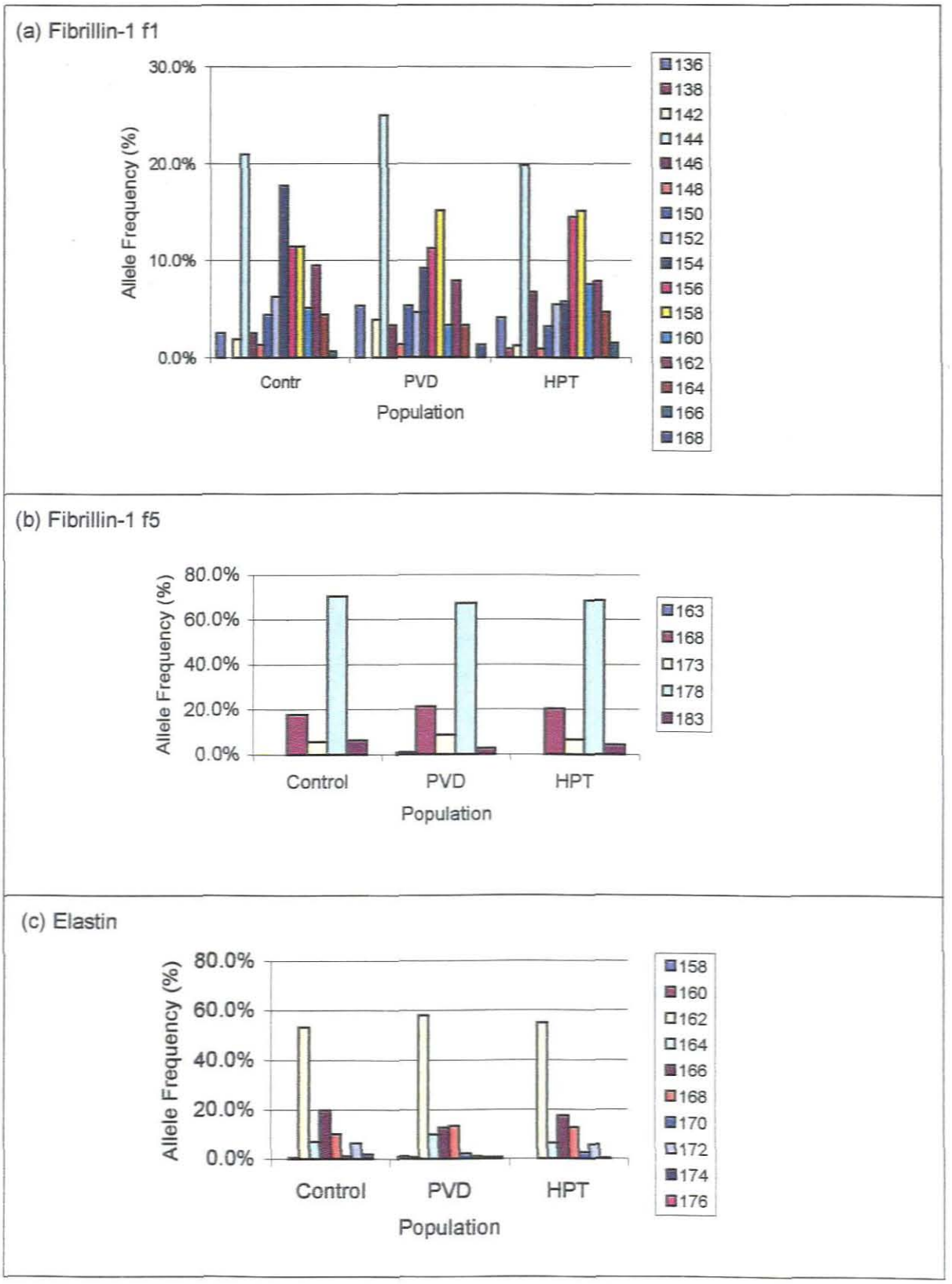


Figure 64. Comparison of allele frequencies of microsatellites in the fibrillin-1 and elastin genes between disease populations and normotensive control group of diverse racial origin. (a) fibrillin-1 f1 (locus 21495), (b) fibrillin-1 f5 (locus 21496) , (c) elastin gene.

R_{st} and G_{st} values for pairwise comparisons between disease groups at the fibrillin-1 f1, f5 and elastin loci

	Fibrillin-1 f1	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0089 (NS)	0.0037 (NS)
Control vs HPT	0.0044 (NS)	0.0072 (NS)
PVD vs HPT	0.0073 (NS)	0.0055 (NS)
	Fibrillin-1 f5	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0090 (NS)	0.0016 (NS)
Control vs HPT	0.0001 (NS)	0.0060 (NS)
PVD vs HPT	0.0016 (NS)	0.0058 (NS)
	Elastin	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0112 (NS)	0.0052 (NS)
Control vs HPT	0.0044 (NS)	0.0037 (NS)
PVD vs HPT	0.0094 (NS)	0.0092 (NS)

Table 41. G_{st} and R_{st} estimates for pairwise population comparisons for the fibrillin-1 f1, the fibrillin-1 f5 and the elastin loci for the normal control population (n=79), peripheral vascular disease study group (n=77) and the hypertensive study group (n=156) of diverse racial origin.

Both R_{st} and G_{st} values are very low for the fibrillin-1 f1, f5 and elastin microsatellite pairwise population comparisons (table 41). This indicates little differentiation between subjects with peripheral vascular disease and hypertension disease states and normotensive subjects of diverse racial origin.

R_{st} and G_{st} values for population comparisons of fibrillin-1 f1 (locus 21945) microsatellite, showed that no significant difference existed between disease groups and the normal control group when the study populations consisted of individuals of diverse racial origin. A R_{st} and G_{st} value of > 0.05 is considered significant. This result was similar to that found when genotype frequencies were compared amongst these study groups of diverse racial origin.

Comparison of race groups

Statistical analysis of allele frequencies on data obtained from microsatellite analysis of fibrillin-1 and elastin genes was also performed on the different race groups in the local population of the Western Cape region in South Africa, in order to determine whether a difference in allele frequencies existed between the different race groups (fig. 65). The two disease groups and normal control group were combined and divided into the three race groups of the local population, namely African, Coloured and Caucasian populations. Statistical analysis was performed for each marker in the fibrillin-1 and elastin gene individually (table 42).

Allele frequency patterns for the fibrillin-1 f1, f5 and elastin microsatellites in the three race groups

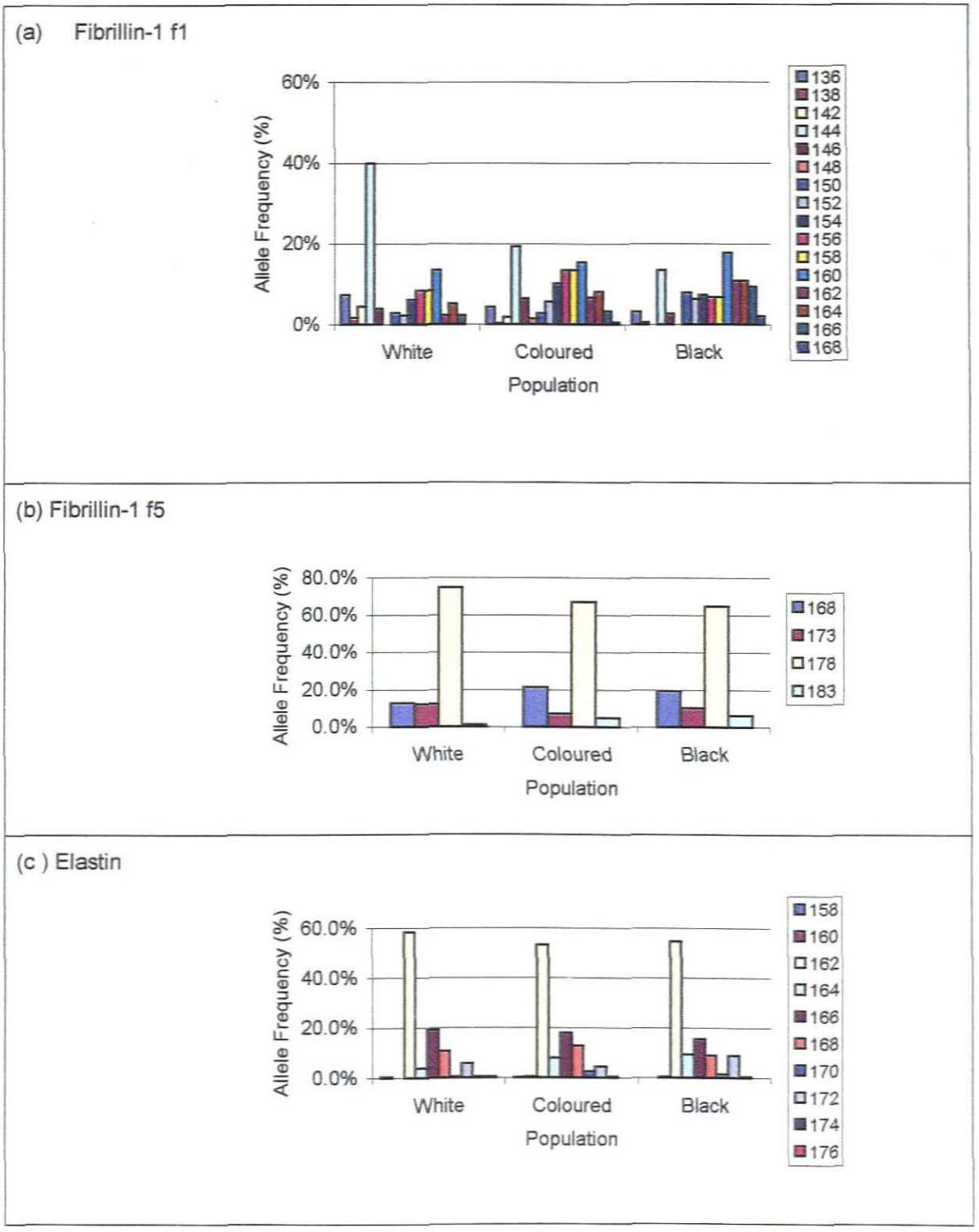


Figure 65. Comparison of allele frequencies of microsatellite in fibrillin-1 f1, fibrillin-1 f5 and elastin genes between race groups in the general population (diseased and healthy) (a) Fibrillin-1 f1 , (b) fibrillin-1 f5l and (c) elastin gene.

R_{st} and G_{st} values for pairwise comparisons between the race groups at the fibrillin-1 f1, f5 and elastin loci

	Fibrillin-1 f1	
Population comparison	**Rst value**	**Gst value**
Caucasians vs Coloured	0.0999	0.0397 (NS)
Caucasian vs African	0.2474	0.0303 (NS)
Coloured vs African	0.0542	0.0024 (NS)

	Fibrillin-1 f5	
Population comparison	**Rst value**	**Gst value**
Caucasians vs Coloured	0.0058 (NS)	0.0600
Caucasian vs African	0.0019 (NS)	0.0135 (NS)
Coloured vs African	0.0023 (NS)	0.0159 (NS)

	Elastin	
Population comparison	**Rst value**	**Gst value**
Caucasians vs Coloured	0.0033 (NS)	0.0239 (NS)
Caucasian vs African	0.0035 (NS)	0.0038 (NS)
Coloured vs African	-0.0030 (NS)	0.0039 (NS)

Table 42. G_{st} and R_{st} estimates for pairwise population comparisons at fibrillin-1 f1, fibrillin-1 f5 and elastin loci for the Caucasian population (n=89), Coloured population (n=318) and the African population (n=95).

The differences that were observed in R_{st} values when allele frequencies of fibrillin-1 f1 microsatellite was compared between the different race groups, can be seen in the allele frequency in fig. 65. Pairwise comparisons for the fibrillin-1 f1 microsatellite between Caucasian and Coloured populations and Caucasian and African population (table 42) showed R_{st} values of 0.0999 and 0.2474 respectively, which indicates a significant difference. The G_{st} values of 0.0397 and 0.0303 however did not support this finding. No significant difference was therefore demonstrated between Caucasians and Coloured or Coloured and African race groups when allele frequencies of the fibrillin-1 f1 microsatellite were compared. The G_{st} value for the Caucasian versus the Coloured group indicated a difference but the R_{st} value did not confirm this. Pairwise comparisons of allele frequencies for the elastin microsatelite showed no significant difference between the three race groups existed at this locus.

Allele frequency comparison within the local Caucasian race group

The R_{st} values for the pairwise comparisons of the fibrillin-1 f1 microsatellite between Caucasians and Africans and Caucasians and Coloured population, indicated that a difference between these populations may exist. It was therefore deemed necessary to separate the disease groups and normal control group into race groups before statistical analysis was applied to determine population differentiation.

Allele frequency patterns in the Caucasian population

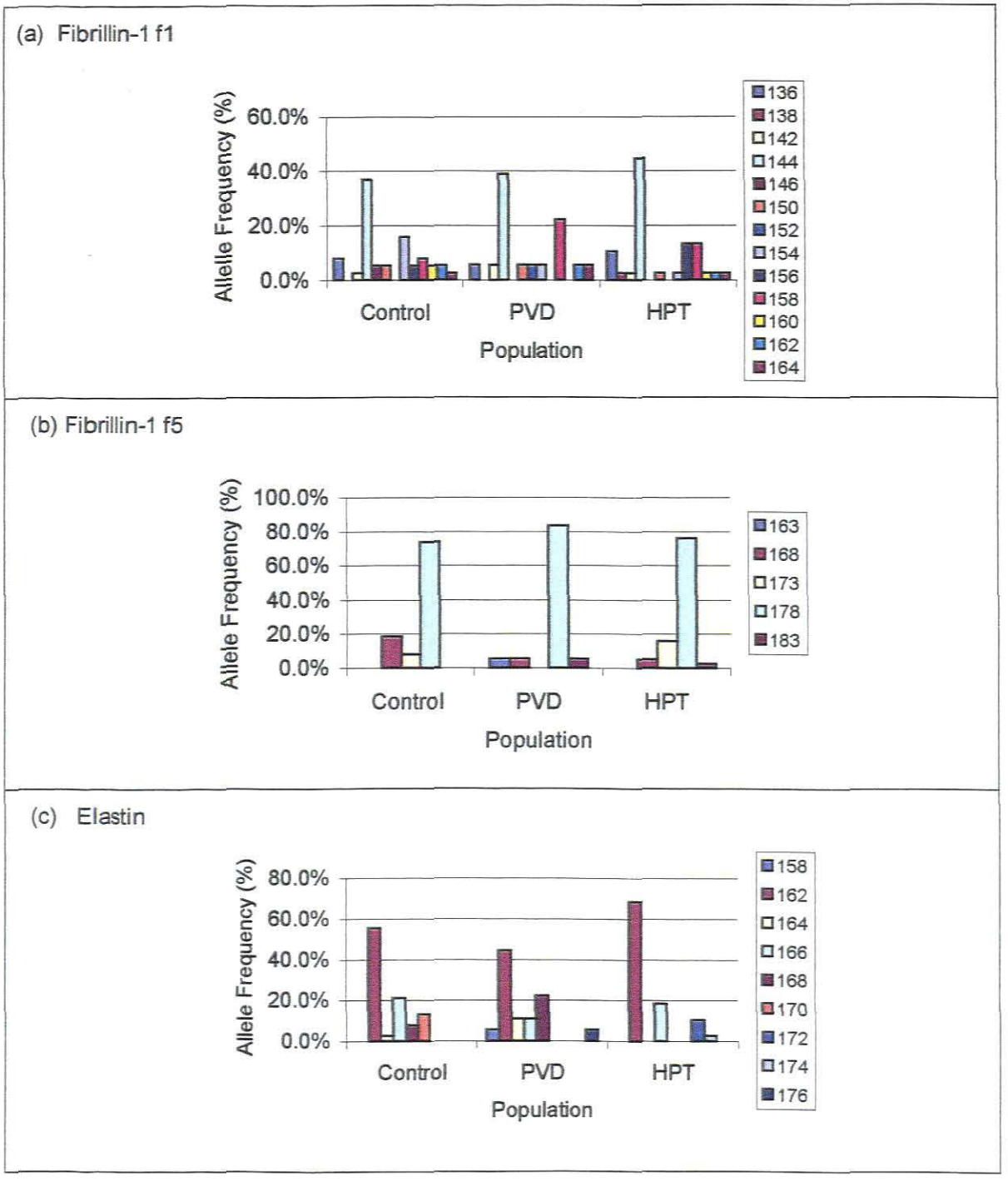


Figure 66. Comparison of allele frequencies of microsatellites in the fibrillin-1 and elastin genes in the normotensive group and the disease groups in the Caucasian population (a) Fibrillin-1 f1, (b) fibrillin-1 f5, and (c) elastin gene.

R_{st} and G_{st} values for pairwise comparisons amongst the Caucasian disease groups

	Fibrillin-1 f1	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0401 (NS)	0.0196 (NS)
Control vs HPT	0.0159 (NS)	0.0123 (NS)
PVD vs HPT	0.0171 (NS)	0.0048 (NS)

	Fibrillin-1 f5	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0063 (NS)	0.0773
Control vs HPT	0.0186 (NS)	0.0152 (NS)
PVD vs HPT	0.0485 (NS)	0.0653

	Elastin	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0452 (NS)	0.0019 (NS)
Control vs HPT	0.0135 (NS)	0.0117 (NS)
PVD vs HPT	0.0361 (NS)	0.0187 (NS)

Table 43. Gst and Rst estimates for pairwise population comparisons for the fibrillin-1 f1, fibrillin f5 and elastin markers for the disease groups and the non-hypertensive Caucasian population (n=20), PVD study group (n=9) and the HPT study group (n=17).

Pairwise comparison at the fibrillin-1 f1 locus (table 43) showed that no differences existed between peripheral vascular disease and hypertension patients and control subjects from the Caucasian population. Pairwise comparison of microsatellite analysis in the fibrillin-1 f5 gene between the normal control group and the peripheral vascular disease group showed a G_{st} value of 0.0773, indicating that a possible variation may exist when allele frequencies of the microsatellite at the fibrillin-1 f5 locus in the fibrillin-1 gene were compared between these two populations.

Allele frequency comparison of disease groups and normal healthy subjects within the local Coloured race group

Allele numbers were similar between the Coloured disease and control groups, although population size varied between peripheral vascular disease (n=54), control group (n=44) and Hypertensive (n=118) (fig. 67).

Allele frequency patterns in the Coloured population

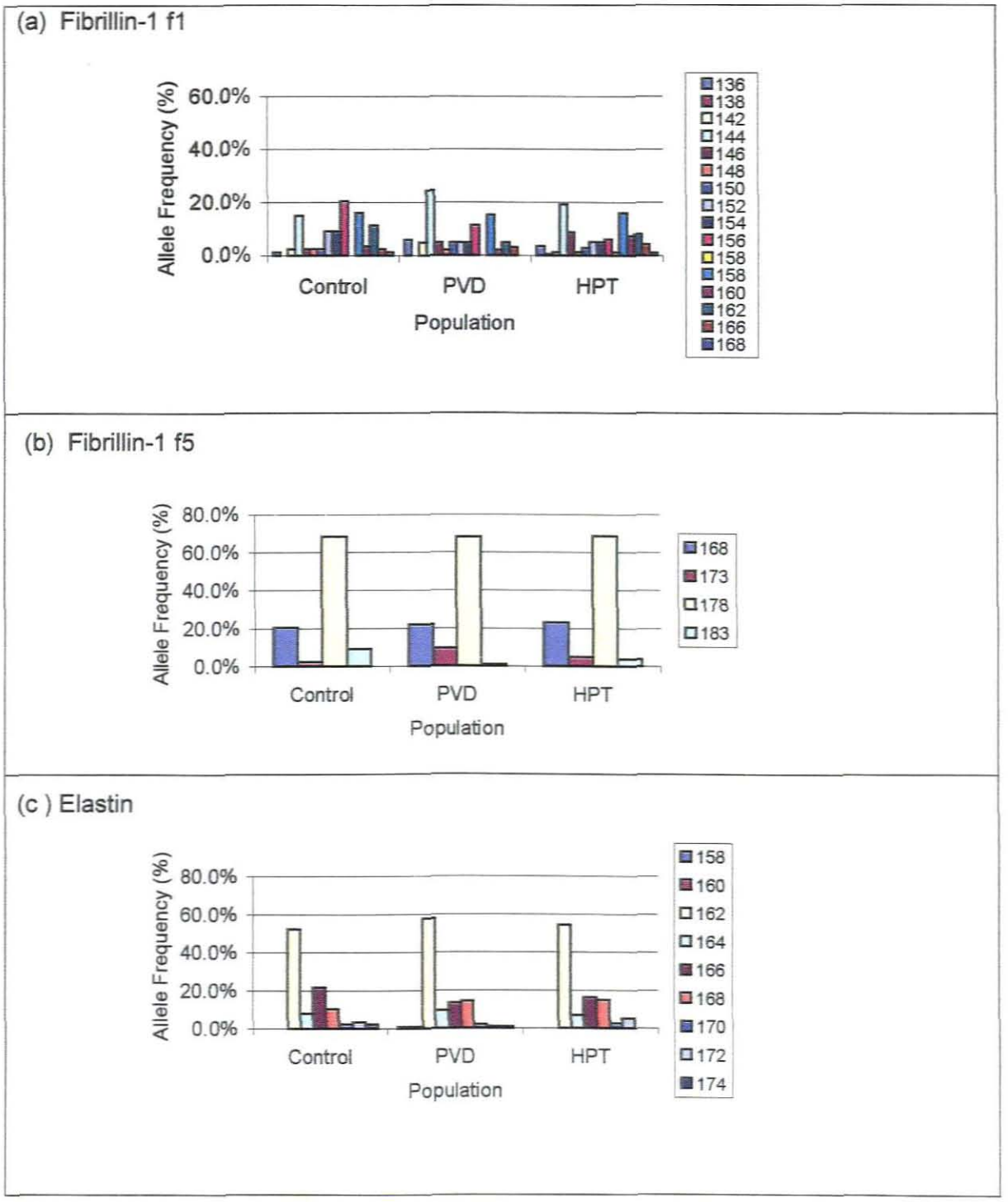


Figure 67 Comparison of allele frequencies of microsatellites in the fibrillin and elastin genes in normotensive and disease groups in the Coloured population. (a) Fibrillin locus 21495, (b) fibrillin locus 21496, (c) elastin gene.

R_{st} and G_{st} values for pairwise comparisons amongst the Coloured disease groups at the fibrillin-1 f1, f5 and elastin loci

	Fibrillin-1 f1	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0675	0.0081 (NS)
Control vs HPT	0.0017 (NS)	0.0106 (NS)
PVD vs HPT	0.0258 (NS)	0.0062 (NS)

	Fibrillin-1 f5	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0101 (NS)	0.0059 (NS)
Control vs HPT	0.0026 (NS)	0.0023 (NS)
PVD vs HPT	0.0049 (NS)	0.0012 (NS)

	Elastin	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0074 (NS)	0.0020 (NS)
Control vs HPT	0.0077 (NS)	0.0024 (NS)
PVD vs HPT	0.0110 (NS)	0.0110 (NS)

Table 44. G_{st} and R_{st} estimates for pairwise population comparisons at the fibrillin-1 f1, fibrillin-1 f5 and elastin markers for the Coloured control population (n=44), Coloured PVD study group (n=54) and the Coloured HPT study group (n=118).

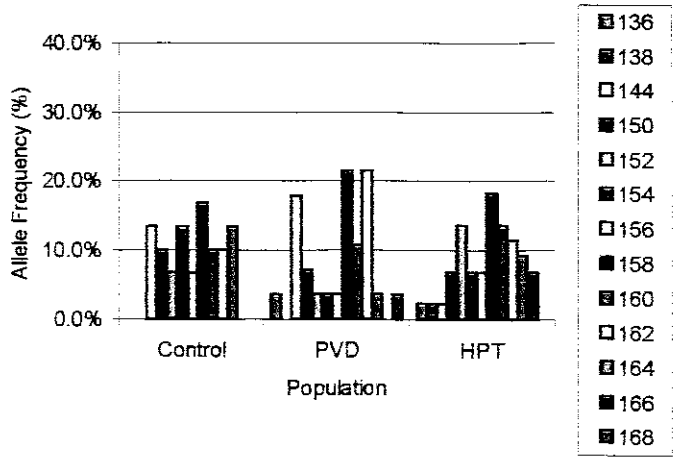
Pairwise comparison of allele frequencies of the fibrillin-1 f1 marker as shown in table 44 amongst the disease groups and the normal population in the local Coloured population showed that no significant difference existed between the normal controls and disease groups. Pairwise comparison of allele frequencies of the fibrillin-1 f1, f5 and elastin markers, amongst the disease groups and the normal population in the local Coloured population showed no significant difference between the normal controls and disease groups.

Alelle frequency comparison within the local African population

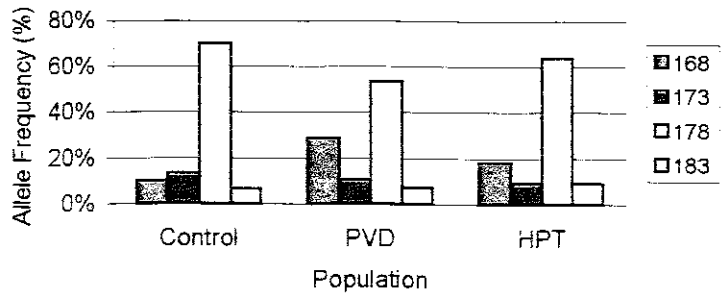
Allele numbers were similar between the African disease groups, although population size varied between PVD (n=14), control group (n=15) and Hypertensive (n=21) (fig. 68).

Allele frequency patterns for the Fibrillin-1 f1, f5 and elastin microsatellites in the Control group, and HPT and PVD patients from the African population

(a) Fibrillin-1 f1



(b) fibrillin-1 f5



(c) Elastin

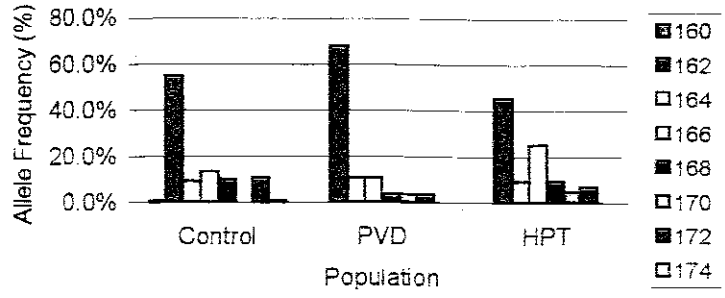


Figure 68. Comparison of allele frequencies at microsatellites in the fibrillin and elastin genes between disease populations and normal control population of the African population. (a) Fibrillin-1 f1 locus 21495, (b) fibrillin-1 f5 locus 21496, (c) elastin locus.

Allele frequencies at the fibrillin-1 f1 locus appeared to be quite different between the control and two disease groups even though the G_{st} and R_{st} values did not confirm this, indicating that the population comparisons should be used in combination with the allele frequencies.

R_{st} and G_{st} values for pairwise comparisons amongst the disease groups from the African population at the fibrillin-1 f1, f5 and elastin loci

	Fibrillin-1 f1	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0350 (NS)	0.0124 (NS)
Control vs HPT	0.0135 (NS)	0.0094 (NS)
PVD vs HPT	0.0118 (NS)	0.0201 (NS)

	Fibrillin-1 f5	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0391 (NS)	0.0235 (NS)
Control vs HPT	0.0205 (NS)	0.0184 (NS)
PVD vs HPT	0.0033 (NS)	0.0045 (NS)

	Elastin	
Population comparison	**R_{st} value**	**G_{st} value**
Control vs PVD	0.0182 (NS)	0.0239 (NS)
Control vs HPT	0.0269 (NS)	0.0087 (NS)
PVD vs HPT	0.0619	0.0608

Table 45. G_{st} and R_{st} estimates for pairwise population comparisons for the fibrillin-1 f1, fibrillin-1 f5 and elastin in the African normal control population (n=15), peripheral vascular disease study group (n=14) and the HPT study group (n=21).

Pairwise comparison of microsatellite analysis of the fibrillin-1 (f5 and f1) gene (table 45) amongst the African disease groups and the African normotensive group showed that no significant difference existed between the normal controls and disease groups. Pairwise comparison of microsatellite analysis of the elastin microsatellite, showed a possible G_{st} and R_{st} values of 0.0608 and 0.0619 respectively when the African hypertensive and peripheral vascular disease groups were compared.

CHAPTER 5 DISCUSSION

5.1 Homocystinuria

Carson *et al.* (1962) first described homocystinuria in mentally retarded individuals from Northern Ireland. This condition is the result of defective methionine metabolism that is most commonly caused by a defect in cystathionine β-synthase, the enzyme that catalyses the first step during the transsulphuration of homocysteine. Clinical symptoms, in patients with homocystinuria, are diverse and could involve four major organ systems, namely, the eye, (high myopia and ectopia lentis), the skeleton (osteoporosis, scoliosis, Marfanoid features), the vascular system (premature arteriosclerosis, thromboembolism) and the central nervous system (mental retardation, convulsions and psychiatric disturbances) (Kluijtmans *et al.*, 1999). Patients with homocystinuria are diagnosed biochemically by severe hyperhomocysteinaemia with free plasma homocysteine >10.0μmol/L and total plasma homocysteine >100μmol/L, homocystinuria, hypermethioninaemia and a decrease in plasma cysteine. Cystathionine β-synthase enzyme activity in cultured fibroblasts and phytohaemaglutinin stimulated lymphoblasts of these patients, is usually severely deficient or completely absent (Fowler *et al.*, 1978; Goldstein, 1973). Three unrelated families with clinical symptoms typically of that found in homocystinuric patients were investigated.

5.1.2 Patient details

Family 1

An eight year old Coloured boy presented with clinical symptoms of dislocated lenses and mental retardation, indicative of homocystinuria, which was confirmed with quantitative measurement of homocysteine and methionine in the Inherited Metabolic Disease Laboratory at Groote Schuur Hospital. Excessive levels of free homocystine, 24μmol/L (reference range; 1.48 – 2.40μmol/L) and methionine, 8488μmol/L (reference range; 26 - 30μmol/L) was detected in this patient. His sister also had homocystinuric symptoms and was confirmed to have homocystinuria. The grandparents had a history of ischaemic heart disease although the parents were asymptomatic.

Family 2

A total plasma homocysteine of 208.4μmol/L was measured in an 11 year old Coloured boy with dislocated lenses and visual impairment but no learning disability. The father's total plasma homocysteine was in the heterozygous range of 30μmol/L and the mother's total plasma homocysteine was normal at 7.5μmol/L. The son had a normal lipid profile but the father was hypercholesterolaemic and had ischaemic heart disease.

Family 3

The index case was a 35 year old Coloured male with a total plasma homocysteine of 226.2μmol/L. Clinical symptoms in this patient were visual problems with bilateral lens dislocation and mental retardation. The mother, father and two sisters had normal total plasma homocysteine and no clinical history for them was available.

5.2 Cystathionine β-synthase enzyme assay

5.2.1 Optimisation of cystathionine β-synthase assay

Cystathionine β-synthase is found in the cytoplasm of cells. Three methodologies that were used by previous investigators to release cytoplasmic enzymes were compared. These were sonication by Fowler *et al.* (1978), freezing and thawing by Fleisher *et al.* (1973) and digitonin release by Mackall *et al.* (1979). Mackall reported that techniques like sonication and freezing and thawing lead to incomplete cell rupture or extensive leakage of mitochondrial and lysosomal enzymes. Digitonin produces perforations in the plasma membrane, causing selective release of cytoplasmic constituents without lysing mitochondria and other organelles. For this study the sonication procedure gave the highest yield of cystathionine β-synthase enzyme activity. Reproducibility of enzyme activity was however very variable. Freezing and thawing gave the lowest yield. Digitonin release of cystathionine β-synthase was reproducible during an assay and between assays using the same cell lines in duplicate. Cystathionine β-synthase enzyme activities obtained using digitonin release were lower than activities obtained with sonication, but they were in agreement with values found by Kluijtmans *et al.* (1996) (see paragraph 4.1.2). Cystathionine β-synthase activity in the cell lysates was measured by the conversion of ^{14}C-[3]-serine to ^{14}C-cystathionine and expressed as nmoles/mg protein/hour.

The cystathionine β-synthase enzyme is present in fibroblasts/lymphocytes as a full-length 63kDa tetramer. In liver cells this tetramer is subject to tryptic cleavage, resulting in an increased active dimeric core of 45kDa. Cystathionine β-synthase activity in cultured lymphoblast cells is only 10% of the activity found in liver cells. Kery *et al.* (1998) reported the 63kDa tetramer to have a Km of 3.0mM for serine and the 45kDa a Km of 2.7mM for serine under maximal homocysteine concentration. Kraus, and Rosenberg (1983), found similar results.

The kinetic properties of the cystathionine β-synthase enzyme extracted from lymphoblasts by digitonin treatment, was investigated to establish optimal assay conditions. The Km for serine at maximal homocysteine in the assay was determined to be 3.0mM using the Michaelis Menten fit as shown in fig. 24. This result was in accordance with a Km of 3.0mM for serine, reported by Kery *et al.* (1998), for the full-length 63kDa cystathionine β-synthase tetramer and a Km of 2.7mM for serine for the dimeric active core (45kDa), formed after tryptic cleavage. Both these measurements were also performed at maximal homocysteine concentration.

Cystathionine β-synthase activity measured in cultured cells is only 10% of activity found in liver cells. For this study unlabelled serine at a concentration of 5mM and ^{14}C-[3]-serine, with a final specific activity of 6600cpm/nmole ^{14}C-[3]-serine was used compared to specific activities of 300cpm/nmole serine used by other investigators (Kraus, 1978) when measuring cystathionine β-synthase in liver cells. This specific activity rendered the assay sensitive enough in order to measure cystathionine β-synthase activities as low as 2% of the reference range.

Conditions for the chromatographic separation of ^{14}C-cystathionine formed after cell lysates were incubated with ^{14}C-[3]-serine, was optimised on the amino acid analyser by ensuring that possible additional products, like cysteine, pyruvate and glycine, did not co-elute with cystathionine. It was determined that these products eluted at 20, 30 and 40 minutes respectively and therefore did not interfere with cystathionine that eluted at 45 minutes.

5.2.2 Cystathionine β-synthase activity in family 1

Both the homocystinuric index case of family 1 and his sister had cystathionine β-synthase activities of 0.2nmoles/mg protein/hour, which was well below the reference range of 4.0 to 22.32nmoles/mg protein/hour (Kluijtmans *et al.*, 1996) and similar to that of activities found in homozygotes for cystathionine β-synthase deficiency. Cystathionine β-

synthase activity of 6.5 ±0.7nmoles/mg protein/hour in the asymptomatic mother was at the lower end of the reference range for healthy individuals (Kluijtmans *et al.*, 1996). The father, had cystathionine β-synthase activity well below the reference range at 0.9nmoles/mg protein/hour. The biochemical finding of high free homocystine and low cystathionine β-synthase activity confirmed homocystinuria due to cystathionine β-synthase deficiency in the affected siblings of this family.

5.2.3 Cystathionine β-synthase activity in family 2 and 3

Family 1 was first investigated for homocystinuria before the molecular sequence of the cystathionine β-synthase gene was known. The only methodology to confirm cystathionine β-synthase deficiency was to measure the enzyme activity. Familes 2 and 3 presented some time after family 1 by which time the sequence of the cystathionine β-synthase gene had been determined and family one had been investigated at the molecular level for defects in this gene. Defects of the cystathionine β-synthase gene are almost always the cause of classical homocystinuria. The enzyme assay requires expensive tissue culture, radioactivity and does not always detect heterozygotes as in the mother of family 1. For a complete diagnosis, extensive family investigations and possible prenatal diagnosis, it was decided that enzyme activity alone was inadequate and that if possible the molecular defect should be determined. Familes 2 and 3 were not investigated for enzyme activity and were only investigated at the molecular level for defects in the cystathionine β-synthase gene.

5.3 Molecular investigation of the cystathionine β-synthase gene

Mutation in the cystathionine β-synthase gene, causing either complete or partial deficiency of cystathionine β-synthase activity, is the major cause of homocystinuria (Mudd *et al.*, 1985). These mutations have been detected in exons 1 to 14 and exon 16. A recent survey of all the mutations, deletions and insertions previously found in the cystathionine β-synthase gene is shown in fig. 72 (Kraus *et al.*, 1999). The list is continuously updated and is available on the cystathionine β-synthase website at www.uchsc.edu/sm/cbs.

Out of nearly 400 mutations investigated so far, 100 pathogenic mutations have been described. The vast majority of these mutations are missense mutations, resulting in defective cystathionine β-synthase activity. Two mutations, the G919A (Gly307Ser) and T833C (Ile278Thr) in exon 8 are undoubtedly the most frequently found mutations. Each

account for 25% of the all the mutations found (Kraus *et al.*, 1999). Both, however appear at a higher frequency in certain populations, the T833C (Ile278Thr) is present in more than 50% of affected alleles in the Netherlands (Kluijtmans *et al.*, 1998) and the G919A (Gly307Ser) is present at 71% in affected alleles in Ireland. It also appears frequently in families of Celtic origin, indicating that the G919A (Gly307Ser) might originate from Scandinavia. The other ±98 mutations are all private mutations and only appear in one or two alleles worldwide.

Although extensive work has been done to detect mutations in the cystathionine β-synthase gene, most of the patients investigated are Caucasians from Europe. Little is known about mutations of the cystathionine β-synthase gene in individuals from African origin. Of all the alleles investigated, only four alleles are from African origin of which two are from South Africa (Gordon *et al.*, 1997; Kluijtmans *et al.*, 1995).

5.3.1 Problems with amplification of the cystathionine β-synthase gene

When family 1 was investigated for defects in the cystathionine β-synthase gene, only the cDNA sequence was available. Primers for the amplification of the cystathionine β-synthase cDNA, were designed, using the Genbank sequence (CβS 3233.D, unpublished, 1992) available at the time. Several attempts at amplifying the coding region of the cystathionine β-synthase gene were unsuccessful. Optimizing the $MgCl_2$ concentration and using the nested approach to the PCR still resulted in no PCR product. Nested PCR greatly increase the specificity of PCR as two different sets of primers are used in successive amplifications. A housekeeping gene, such as hypoxanthine-guanine phosphoribosyltransferase could be amplified, indicating that the cDNA extraction had been successful. Cystathionine β-synthase is poorly expressed in lymphoblasts when compared to liver however it is also expressed in the cultures as shown by the enzyme assays.

Comparison between the sequence on Genbank (CBS 3233.D, unpublished, 1992) and the published sequence of Kraus (1993) revealed numerous discrepancies. Matching sites for only two of the four amplification primers could be found in different positions to the sites on the initial sequence on Genbank. Amplification with primers designed according to the published sequence (Kraus 1993 and Kraus *et al.*, 1998) resulted in a 2062 base pair fragment after the first PCR and a 1985 base pair fragment after the second nested PCR. Both these reactions did not yield sufficient DNA for sequencing, although visible on a 1.5% agarose gel. The third set of nested primer reactions, with four

primers, which halved the 1985 bp fragment, resulted in two fragments of 1263 base pairs and 1269 base pairs respectively, representing the 5' and the 3' ends of the coding region.

Initial attempts to manually sequence these two PCR fragments with internal primers were unsuccessful due to background noise and several unreadable stretches on the autorads. When PCR products are sequenced both alleles are screened simultaneously, resulting in point mutations to show up as double bands (one band being the normal band and the other band being the mutant band), which makes it difficult to distinguish the true mutation when the background noise is high. The intensity of each band varies considerably, making it difficult to distinguish double bands from background noise. We overcame this problem by cloning the two amplified halves of the coding region. This procedure allowed us to sequence one allele at a time. The 5' and 3' amplified fragments were ligated into a PGEMT vector, and used to transform $RbCl_2$ competent DH5α cells. This method produced 8 clones from which readable manual sequences were obtained.

5.3.2 Mutations in the cystathionine β-synthase gene of families with homocystinuria

5.3.2.1 Family 1

Cystathionine β-synthase mutation of the paternal allele

The G to A transition at nucleotide 919 in the cystathionine β-synthase gene was detected in five out of eight clones from the index case. This is the most commonly found mutation in the cystathionine β-synthase coding sequence in populations of Celtic origin and is associated with vitamin B_6 non- responsiveness *in vivo* and complete loss of enzyme activity (Hu *et al.*, 1993). This G to A changes a highly conserved neutral polar glycine to a neutral polar serine at amino acid 307. The glycine had been conserved from spinach cysteine synthase to human cystathionine β-synthase (see fig. 69). Amino acid 307 falls in the catalytic region (active core) of the human cystathionine β-synthase that stretches from amino acid residue 37 to 413 (Janosik *et al.*, 2001). The cofactor, pyridoxal 5'-phosphate (PLP) binds in this region. Neutral polar amino acids may bind with non-protein groups. The neutral polar glycine therefore may interact with PLP, whereas the neutral polar serine may not have the same affinity for PLP. Thus rendering the enzyme less active. Recent studies of the protein by Janosik *et al.* (2001), suggested that the C-terminal domain, which covers amino acid residues 414 to 551, has an auto inhibitory effect on the enzyme. Their theory is that the cystathionine β-synthase C terminal domain may auto inhibit the enzyme by partial occlusion of the catalytic site and that

S-adenosylmethionine binding, induces the displacement of this domain which then result in an increase in cystathionine β-synthase activity. They further argue that mutations in the catalytic region might interact with the C terminal domain, resulting in occlusion of the catalytic site. The G919A (Gly307Ser) mutation could therefore be the cause of such inhibition and therefore be the cause of the almost completely deficient CβS enzyme activity detected in the heterozygote father carrying the G919A (Gly307Ser).

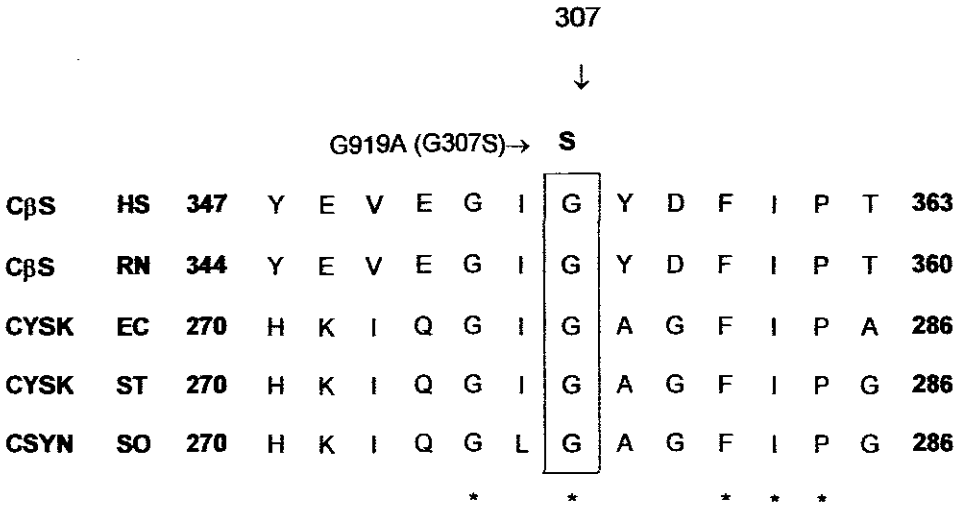


Figure 69. Evolutionary conservation of the cystathionine β-synthase sequence and human mutations showing the G919A (Gly307Ser) mutation. CβS HS denotes human cystathionine β-synthase , CβS RN denotes Rat CβS, cysk EC denotes *E. coli* *O*-acetylserine lyase, CSYSK ST, *Salmonella typhimurium* O-acetylserine lyase, CSYN SO denotes spinach cysteine synthase. Amino acids in each sequence are numbered on each side. Asteriks signify absolute conservation of amino acid residues. The down arrow (↓) signifies the Gly to Ser amino acid change at amino acid 307 (Kraus, 1994).

Restriction enzyme analysis for the G919A (Gly307Ser) mutation

The G919A (Gly307Ser) mutation creates an *Alu* I restriction enzyme (RE) digest site, which has been used by previous investigators to confirm the presence of this mutation. The *Alu* I restriction enzyme however digests the wild type cystathionine β-synthase PCR fragment four times and the mutant fragment, five times. We designed restriction enzyme analysis, using restriction endonuclease *Pvu* II to simplify visualization of the electrophoretic gel. Exon 8 of the cystathionine β-synthase gene was amplified with a forward primer used by Sperandeo *et al.* (1995) and a newly designed mismatch reverse primer, designed specifically to create a *Pvu* II site when the G919A (Gly307Ser) mutation is present. The primer had a C instead of the complementary T to the A at nucleotide 923. This base pair change resulted in the amplification of a 146 base pair fragment that

contained the *Pvu* II restriction site of CAGCT. The fragment was digested into two fragments of 21 and 125 base pairs respectively, when the mutation was present. The wild type was not digested. This method of screening for the G919A mutation was simpler to read than the *Alu* I method. The G919A (Gly307Ser) mutation in the index case was confirmed in genomic DNA. Restriction enzyme analysis of genomic DNA of the extended family showed that the sister, father and a paternal uncle also were heterozygous for this mutation.

Cystathionine β-synthase mutation of the maternal allele

The maternal side of the family was also tested for the G919A (Gly307Ser) mutation and as expected the mother and all the maternal aunts and uncles were normal for the G919A (Gly307Ser) mutation. Manual sequencing the remaining three of the eight cDNA clones of the cystathionine β-synthase gene of the index case of family 1 lead to the identification of a novel missense C to T mutation at nucleotide 1058 in exon 10. Automated sequencing of the cDNA confirmed this mutation. This base pair alteration changed an encoded neutral polar threonine to a non-polar hydrophobic methionine at amino acid 353 (Thr353Met). In the cystathionine β-synthase gene this amino acid has been conserved as an alanine from rat cystathionine β-synthase, *E. coli* O-acetylserine lyase, *Salmonella typhimurium* O-acetylserine lyase and spinach cysteine synthase. However in humans it is replaced with a threonine (Kraus, 1994). This nucleotide also sits in a highly conserved region with several of the adjacent amino acids conserved from spinach to humans (fig. 70).

The tendency of hydrophobic amino acids to react with one another may result in a conformational change of the secondary structure of the enzyme when the C1058T (Thr353Met) mutation is present. This change in structure could lead to a loss of affinity of the enzyme for its co-factor PLP. In addition, the change from a neutral polar amino acid to a non-polar hydrophobic amino acid could also result in loss of affinity for PLP. The C1058T (Thr353Met) site closely interacts with PLP as determined by the program, Protein Explorer. (fig. 72). Sequencing of the genomic DNA of the sister, mother, father, aunts and uncles showed both sister and mother and two maternal aunts and uncles to be heterozygous for the (Thr353Met) mutation, while as expected the father was normal for the C1058T (Thr353Met) mutation. The complete cDNA was sequenced and no other mutations were seen

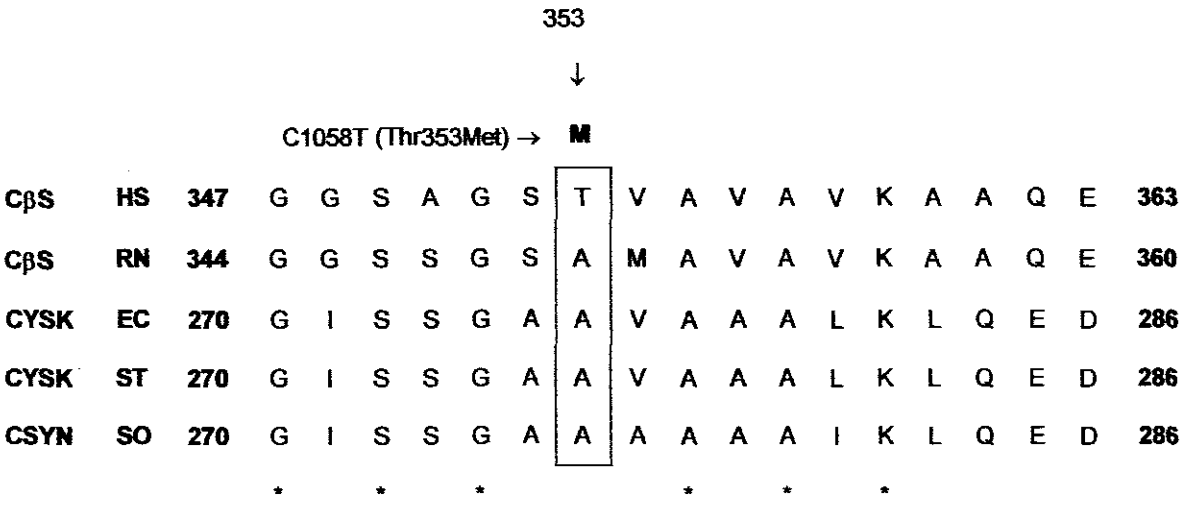


Figure 70. Evolutionary conservation of cystathionine β-synthase sequence and human mutations showing the C1058T (Thr353Met) mutation. CβS HS denotes human CβS, CβS RN denotes Rat CβS, cysk EC denotes *E. coli* O-acetylserine lyase, CSYSK ST, *Salmonella typhimurium* O- acetylserine lyase, CSYN SO denotes spinach cysteine synthase. Amino acids in each sequence are numbered on each side. Asteriks signify absolute conservation of amino acid residues. The down arrow (↓) signifies the Thr→Met amino acid change at amino acid 353 (Kraus, 1994).

5.3.2.2 Family 2

Amplification of the coding region of the cystathionine β-synthase gene for index case F1036 was performed as for family 1. Automated sequencing was now more readily available and the amplified fragments of the coding region of the cystathionine β-synthase gene of index case F1036 was therefore sequenced on a automated sequencer by the ThermoSequenase dye terminator cycle sequencing kit with an ABI 373 DNA sequencer, in the Core facility at The University of Cape Town (UCT). A G to a T transition at nucleotide 1126 in exon 10 was detected on one allele and the second most commonly found T to a C transition at nucleotide 833 in exon 8 on the other allele. The G1126T (Asp376Thr) mutation has not been found before. The entire cDNA was sequenced and no other mutations were detected.

Cystathionine β-synthase mutation of the paternal allele

The G1126T (Asp376Thr) mutation was found on one allele of the father. No other mutation was found on the other allele of the father. The G to T transition at nucleotide 1126 results in an amino acid change of a negatively charged aspartate to a neutral polar threonine at amino acid 376 (Asp376Thr). The aspartate has been conserved for CβS HS

(human CβS), CβS RN, (Rat CβS) as shown in fig. 71. This mutation is also in the highly conserved catalytic region of the cystathionine β-synthase gene. This change might result in conformational change of the secondary structure. The G1126T (Asp376Thr) site interacts closely with PLP as shown in the crystal structure of a cystathionine β-synthase dimer as determined by the program Protein Explorer (fig. 72).

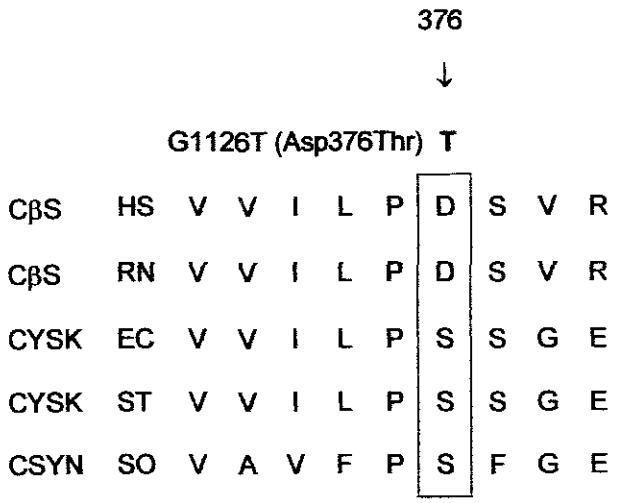


Figure 71. Evolutionary conservation of cystathionine β-synthase (CβS) sequence and human mutations showing the G1126T (Asp376Thr) mutation. CβS HS denotes human cystathionine β-synthase, CβS RN denotes Rat cystathionine β-synthase, cysk EC denotes *E. coli O*-acetylserine lyase, CSYSK ST, *Salmonella typhimurium* O- acetylserine lyase, CSYN SO denotes spinach cysteine synthase. Amino acids in each sequence are numbered on each side. Asteriks signify absolute conservation of amino acid residues. The down arrow (↓) signifies the Aspartate to Threonine amino acid change at amino acid residue 376 (Kraus, 1994).

Cystathionine β-synthase mutation of the maternal allele

The T833C (Ile278Thr) mutation was detected on one allele of the mother and no other mutation was found on the other allele. The T833C (Ile278Thr) mutation that changes a neutral hydrophobic isoleucine to a neutral polar threonine at amino acid 278, is the second most frequently found mutation in patients with especially pyrodoxine responsive homocystinuria (Kraus *et al.*, 1999). This amino acid change could result in the loss of the ability of hydrophobic amino acids to bind with one another, resulting in a conformational change of the secondary structure. The mother has a normal total plasma homocysteine value of 7.5μmol/L so cystathionine β-synthase activity was either not affected or her vitamin B_6 levels were sufficient to counteract the effect of the mutation. Hu *et al.* (1993) and Shih *et al.* (1995) found this found T833C (Ile278Thr) to be associated with pyridoxine responsiveness, especially in the homozygous state when no other mutation is involved.

Kozich and Kraus (1992), showed that cystathionine β-synthase subunits, made by clones containing the T833C (Ile278Thr) mutation were unstable. They suggested that the substitution of a hydrophobic isoleucine by a more hydrophilic threonine may effect either the cystathionine β-synthase conformation or interaction of the subunits and may result in an unstable tetramer.

Cystathionine β-synthase mutation of the siblings

The sister and one brother of the index case had normal total plasma homocysteine and carried no mutation on the cystathionine β-synthase gene. Two other brothers carried the G1126T (Asp376Thr) and T833C (Ile278Thr) mutations respectively. The brother with the G1126T (Asp376Thr) mutation had a fasting total plasma homocysteine of 11.9μmol/L and the brother with the T833C (Ile278Thr) mutation had a fasting total plasma homocysteine of 26.7μmol/L. No clinical history was available for these two brothers. Both these brothers have not been tested subsequently for total plasma homocysteine and it was not known whether the brother with the fasting total plasma homocysteine of 11.9μmol/L had started vitamin treatment or not.

5.3.2.3 Family 3

Cystathionine β-synthase mutation of family 3

Automated sequencing of cDNA of the 3rd index case with a total plasma homocysteine value of 226μmol/L and severe clinical symptoms, showed the T833C (Ile278Thr) mutation on both alleles. This agrees with the finding of Kluijtmans *et al.* (1998), who reported severe disease in four individuals, homozygous for the I278T mutation. Both parents were obligate heterozygotes for the T833C mutation and carried no mutation on the other allele. The mother (F1085) had a normal total plasma homocysteine of 11.6μmol/L. In this case it would appear that the T833C mutation on one allele only, does not cause defective homocysteine metabolism. Shih *et al.* (1995), reported no disease in an individual carrying the T833C Ile278Thr mutation on one allele and a G139R mutation on another allele.

5.3.3 Vitamin treatment

5.3.3.1 Vitamin treatment of family 2

The index case responded positively to vitamin treatment. Total plasma homocysteine levels were lowered from 208.4μmol/L to 65.3μmol/L 12 months after treatment and stayed constant till 27 months after treatment (chapter 4, family 2, fig. 37, page 87). This result agrees with vitamin treatment results found in patients with the pyridoxine responsive T833C (Ile278Thr) mutation (Shih *et al.*, 1995). It would appear that the G1126T (Asp376Thr) mutation did not modify the pyridoxine responsive phenotype of the T833C (Ile278Thr) mutation. The G1126T (Asp376Thr) mutation might also illicit vitamin responsiveness as vitamin treatment in the father who carried the G1126T (Asp376Thr) lowered his fasting total plasma homocysteine from 29.6μmol/L to 19.9μmol/L (chapter 4, family 2, fig. 37, page 87). This slight decrease in total plasma homocysteine could have been the result of interference from other medication the father might be taking as described by Slavik *et al.* (1982).

5.3.3.2 Vitamin treatment of family 3

The index case responded positively to vitamin treatment. Total plasma homocysteine levels were lowered from 225.2μmol/L to 13.7μmol/L after 6 months of vitamin treatment (see table 8, page 90).

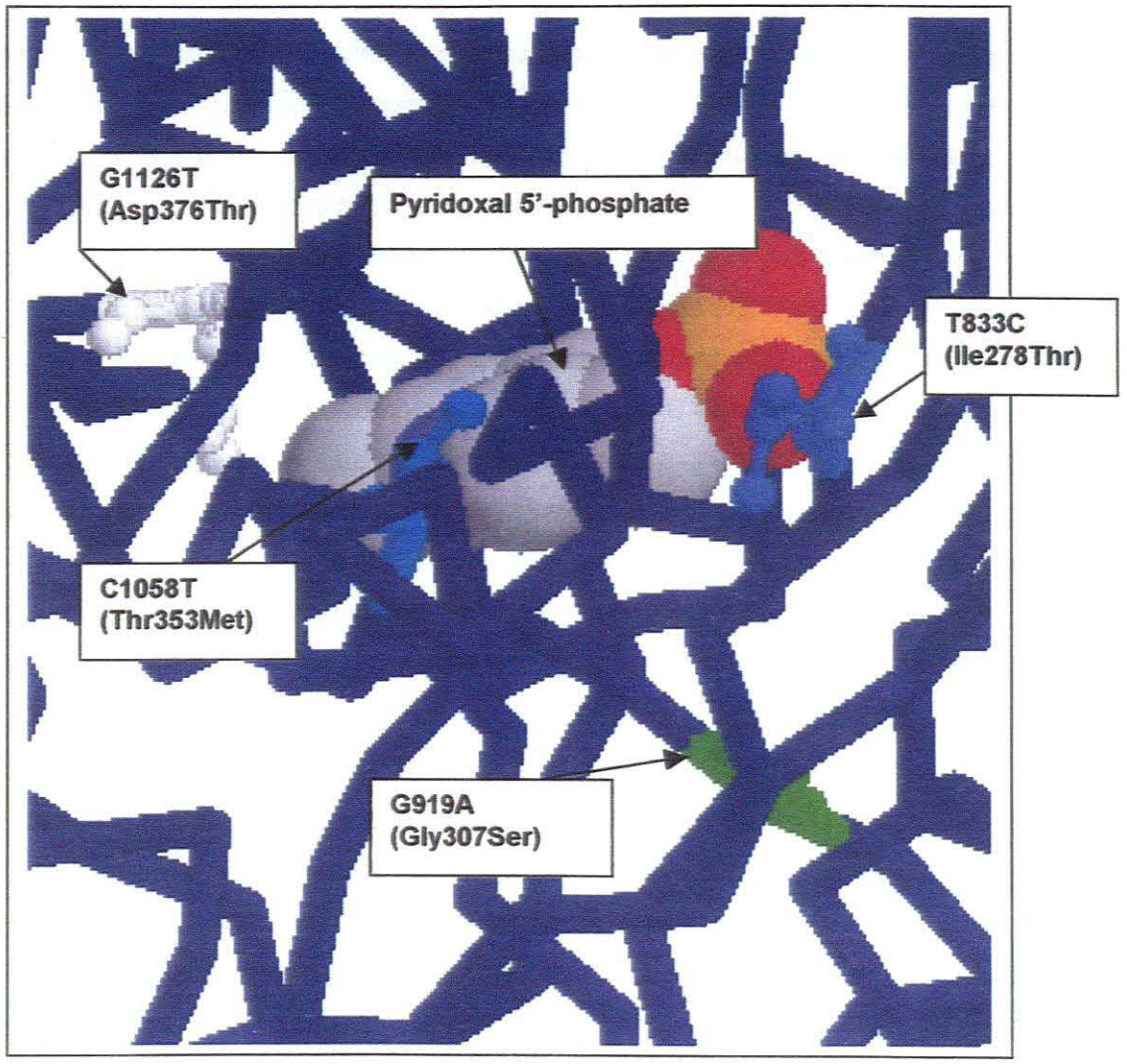


Figure 72. The positions of the four mutations found in the cystathionine β-synthase gene of the three homocystinuric families investigated for this study (Protein Explorer).

5.3.4 Molecular investigation of methylenetetrahydrofolate reductase

The enzyme methylenetetrahydrofolate reductase regulates distribution of folate species to either DNA synthesis or homocysteine remethylation. The commonly found C677T polymorphism that results in an amino acid change from an alanine to a valine, renders the enzyme thermolabile. Both the alanine and valine are neutral non-polar amino acids. The combination of low folate status and the TT genotype has been shown to affect homocysteine homeostasis. Restriction enzyme analysis with *Hinf* I (Frosst *et al.*, 1995) showed that family 1 did not carry this polymorphism and were genotyped CC. All the members of family 2 were heterozygous carrying the CT genotype. The index case of family three and his mother also carried the CT genotype while the father was CC. Nine out of 26 alleles (34%) carried the C677T polymorphism. The TT genotype was not found in any of the families. This frequency of mutant T alleles compared well with the frequency of 34.3% in the affected population versus 33.8% in controls as found by Brattström *et al.* (1998). The presence of this mutation in our patients with homocystinuria again emphasizes the importance of the maintenance of folate status in these patients. A healthy folate status in homocystinuric patients with cystathionine β-synthase defects could increase remethylation and consequently decrease homocysteine. These patients were not screened for the 1298 A to C mutation, as it has been shown by previous investigators not to affect homocysteine homeostasis. Dekou *et al.* (2001), reported no significant effect of this polymorphism on total plasma homocysteine, folate or vitamin B_{12} levels. They also did not detect any evidence of interaction between the A1298C and C677T genotypes. Two of the index cases that carried the T833C mutation and whom responded well to folate and vitamin treatment also carried the C677T mutation. In these cases the presence of the thermolabile variant of the methylenetetrahydrofolate reductase gene could have contributed to the increase in plasma homocysteine.

5.3.5 Summary of the three families

In the three families reported here four different mutations were found of which two were new private mutations. The one C1058T (Thr353Met) mutation has since been reported in two other families of Australian and African American origin (Dawson *et al.*, 1997). The C1058T (Thr353Met) mutation in its heterozygous state does not seem to have an effect on total plasma homocysteine values, because no free homocystine was detected in the mother of family 1 who carried this mutation on one allele and no mutation on the other allele. Her cystathionine β-synthase activity however was in the lower half of the reference range (Kluijtmans *et al.*, 1996). Her normal free homocystine could have been the result of adequate vitamin status. In combination with the G919A (Gly307Ser) mutation it however elicits an excessive increase in total plasma homocysteine values and clinical symptoms found in patients with homocystinuria.

The G1126T (Asp376Thr) mutation has not been reported by other investigators. No other mutations were found in the father's cDNA. This mutation appears in the highly conserved region of the cystathionine β-synthase gene and had quite an effect on fasting total plasma homocysteine in the father (29.8μmol/L) of family 2 but not in his younger son (11.9μmol/L). The lower fasting total plasma homocysteine in the son could have been due to the younger age or adequate vitamin status. Vitamin treatment in the father did lower his total plasma homocysteine to 19.9μmol/L, indicating that the G1126T (Asp376Thr) mutation might illicit vitamin responsiveness in the cystathionine β-synthase enzyme. Drugs could also be the cause of the higher total plasma homocysteine in the father as certain drugs do inhibit methyltetrahydrofolate formation. Methyltetrahydrofolate is the product formed after reduction of methylenetetrahydrofolate by methylenetetrahydrofolate reductase. The G919A (Gly307Ser) and T833C (Ile278Thr) have been found worldwide and are the most common mutations found in the cystathionine β-synthase gene. The two other missense mutations however of which only one has been detected in an African American and Australian, could be the start of a representative picture of mutations found in the homocystinuric and/or hyperhomocysteinaemic patient population of the Western Cape. More alleles will have to be screened to confirm this finding. The studies, so far, has mainly been performed on Caucasians from Europe and very few African alleles have been investigated.

All three the investigated families were from the local Coloured population. The European influence in this South African population is reflected by the presence of the G919A (Gly307Ser) and T833C (Ile278Thr) mutations in the homocystinuria patients of the local

population. Clinical symptoms in our patients were the same as that found in European homocystinuric patients.

The presence of private mutations is a problem when screening for disease carrying mutations in the cystathionine β-synthase gene. The result of this phenomenon is that the whole cystathionine β-synthase gene has to be screened for mutations. This can be done by exon by exon screening or cDNA screening which will require mRNA extraction and reverse transcription to cDNA. It would be of help if another more rapid screening method is recognized that might link a polymorphic marker to homocystinuria or hyperhomocysteinaemia and ultimately to the several vascular diseases caused by both homocystinuria and hyperhomocysteinaemia.

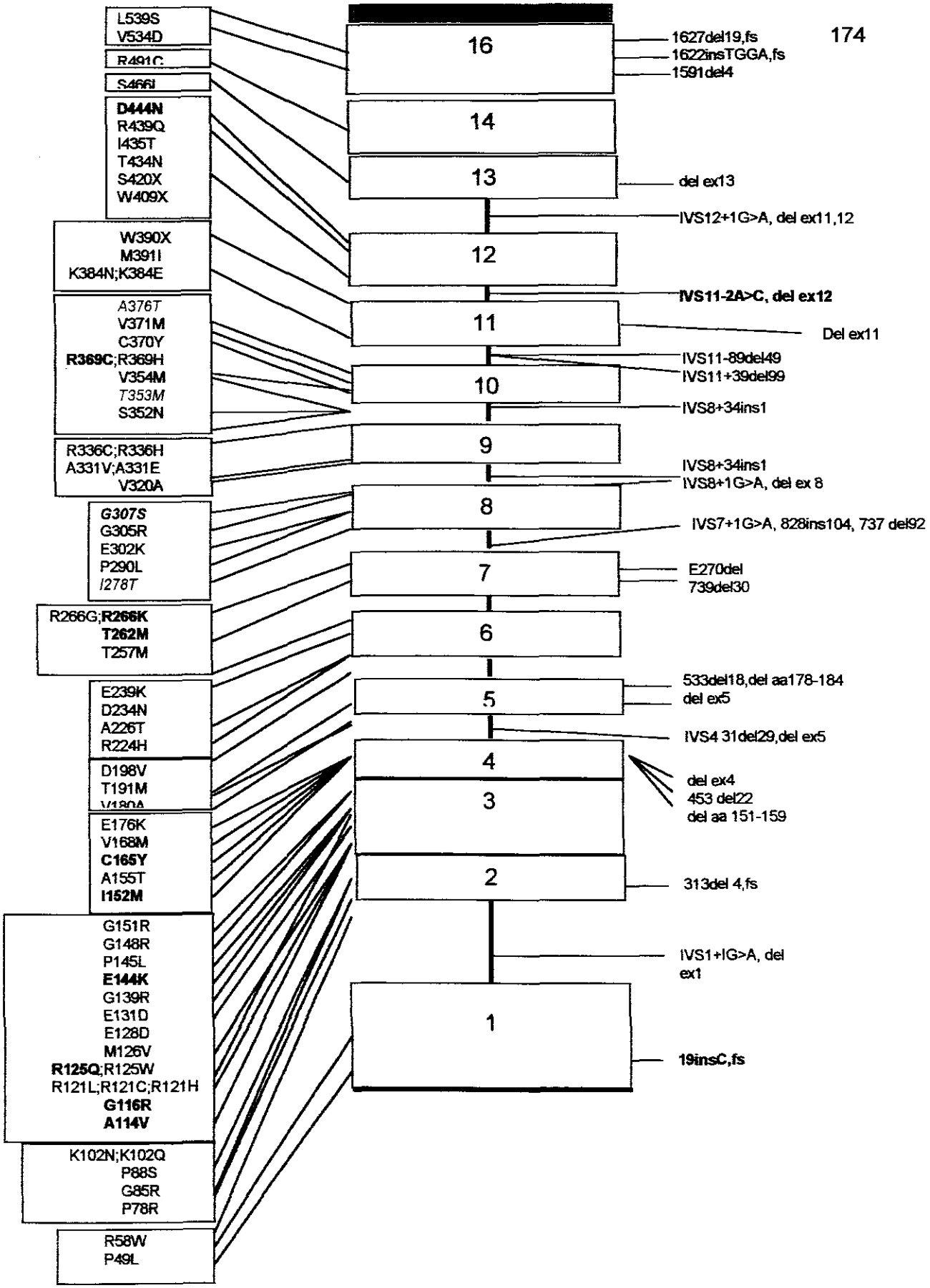


Figure 73. Location of human cystathionine β-synthase mutations. The exons in the coding region are drawn to scale; the introns are not. Shaded areas, denotes parts of 5'- and 3'-untranslated regions of cystathionine β-synthase mRNA found in exons 1 and 16, respectively. The mutations shown on the left are missense and nonsense; the mutations on the right are deletions, insertions, and splicing aberrations. The mutations shown in bold were detected in three or more alleles (Kraus *et al.*, 1999). The four missense mutations detected for this study are shown in blue italics.

5.4 Hyperhomocysteinaemia

Vascular disease is on the increase in Western societies. The Western Cape region of South Africa is no exception, the incidence and cause of vascular disease in the Western Cape however, is still unknown. Numerous studies have now indicated that hyperhomocysteinaemia is associated with several vascular disease states.

Large numbers of patients with vascular disease are admitted annually to the two academic hospitals in the Western Cape and clinicians are increasingly requesting plasma homocysteine levels. There was therefore clearly a need to establish methodology for the identification of hyperhomocysteinaemia in patients with vascular disease.

5.4.1 Total plasma homocysteine measurement

Initially the Inherited Metabolic Disease laboratory at Groote Schuur Hospital and The University of Cape Town was measuring free homocystine in both urine and plasma on a Beckman amino acid analyser by an in-house method. The methodology was modified in order to measure total plasma homocysteine. All the homocyst(e)ine moieties, were reduced by the method of Brattström *et al.* (1984), with slight modification, and total plasma homocysteine after reduction by dithiothreitol (DTT) was measured on the amino acid analyser. The chromatographic run time was shortened, from 100 minutes to 39 minutes because homocysteine eluted earlier than homocystine. The method for total plasma homocysteine proved to be reliable and sufficiently sensitive for the detection of the small increases in total plasma homocysteine, usually found in patients with hyperhomocysteinaemia. Both homocystine and homocysteine standards were linear up to 200μmol/L.

Free homocystine measurement was not adequate to detect hyperhomocysteinaemia because of the slight increase found in low circulating free homocystine levels in patients with hyperhomocysteinaemia. Offering free homocystine as a routine diagnostic test also present other problems. Free homocystine rapidly binds with albumin, when blood is left standing, so plasma has to be separated and deproteinised immediately for accurate measurement. This is usually not possible, even if blood specimens are sent from the ward to the laboratory, within the same hospital. Sample handling measures was less stringent for total plasma homocysteine measurement, however care still needed to be taken to separate the plasma from the red blood cells within an hour, after which plasma was frozen until processed. These precautions were enough to ensure accurate total plasma homocysteine during this study.

5.4.2 Oral methionine

Oral methionine was used during this study, in order to emphasize the small differences that exist between total plasma homocysteine in patients with hyperhomocysteinaemia and healthy individuals. Measuring free and total plasma homocyst(e)ine before and at two hourly intervals post oral methionine in four individuals from the abruptio placentae study, resulted in similar profiles in which peak homocyst(e)ine was reached at 6 hours after oral methionine. Subsequent measurements of homocyst(e)ine were done after an overnight fast and 6 hour post oral methionine.

Oral methionine was originally used to detect heterozygosity for cystathionine β-synthase deficiency by investigators, such as Brenton and Cusworth (1966). Presently it is used to stress all homocysteine metabolising pathways. Studies have shown the need for methionine load as fasting homocysteine levels alone have failed to identify 40% of subjects with methionine intolerance (Bostom *et al.*, 1995). Measurement of free homocystine and total plasma homocysteine after an overnight fast and at two hourly intervals after oral methionine, in four patients from the abruptio placentae study, gave similar profiles. The free homocystine reflected the total plasma homocysteine with peak homocyst(e)ine reached at 6 hours post oral methionine. These results confirmed results from other studies (Boers *et al.*, 1985). Fasting total plasma homocysteine and 6 hour post oral methionine total plasma homocysteine is now routinely offered to clinicians at Groote Schuur Hospital and other hospitals in the region.

Several researchers have now found an association between hyperhomocysteinaemia and abnormalities during pregnancy. Two of these conditions namely, abruptio placentae and pre-eclampsia were investigated for an association with hyperhomocysteinaemia.

5.4.3 Abruptio Placentae

5.4.3.1 Abruptio placentae and homocysteine

Fasting and 6 hour post oral methionine total plasma homocysteine were measured 24 to 48 hours post partum in 21 patients with abruptio placentae and 19 matched controls. The patients with abruptio placentae showed a significant increase ($p < 0.05$) in total plasma homocysteine, both after an overnight fast and 6 hours post oral methionine, when compared to matched control subjects. Fasting and 6 hour post oral methionine, plasma methionine and cysteine, except for 6 hour cysteine ($p = 0.042$) showed no significant difference between patients and control subjects. No valuable additional information was obtained by measurement of plasma methionine and cysteine.

Vollset *et al.*, (2000), found an association between total plasma homocysteine greater than 15μmol/L and abruptio placentae. A study done in the Inherited Metabolic Disease Unit at The University of Cape Town, also found total plasma homocysteine to be significantly higher in patients with abruptio placentae compared to total plasma homocysteine in a matched control group (Owen *et al.*, 1997). A weakness in Vollset's study was that they measured total plasma homocysteine, 10 years after the adverse pregnancies. They suggested that results needed to be confirmed in a study design where total plasma homocysteine is measured before or during early pregnancy because of the folate-independent reduction in total plasma homocysteine occurring during pregnancy (Andersson *et al.*, 1992; Bonnette *et al.*, 1998). This lowering effect during pregnancy should not be of concern if a proper matched control group is investigated simultaneously.

5.4.3.2 Abruptio placentae and vitamin status

No significant differences between abruptio placentae patients and matched control subjects for vitamin B_6, B_{12}, red cell folate and serum folate were detected. Only one subject with post oral methionine hyperhomocysteinaemia (53.1μmol/L) had a low serum folate of 1.3ng/ml (2.9 – 18.7ng/ml). Women from the abruptio placentae study group came from the less affluent Coloured community. Their diet probably consisted of less fresh foods and more processed foods, lacking the necessary vitamins. However, the reason for adequate vitamin status in these women could be due to the vitamin supplementation administered to pregnant women on their first visit to maternity clinics in the Western Cape.

Several studies have shown that plasma concentrations of folate, vitamin B_{12} and vitamin B_6 are inversely associated with total plasma homocysteine concentration (Ubbink *et al.*, 1993; Selhub *et al.*, 1993). Vitamin status has been shown to have an effect on total plasma homocysteine in both patient groups and apparently healthy individuals and numerous investigations have now demonstrated that vitamin supplementation lowers total plasma homocysteine in certain patients from several disease groups (Ubbink *et al.*, 1994; Rasmussen *et al.*, 1996). This short-term vitamin treatment was not sufficient to lower total plasma homocysteine in some of the subjects from this study. Randomized trials have shown that folate supplementation started pre-conceptionally could reduce adverse events during pregnancy (Czeizel and Dudas, 1992).

5.4.3.3 Abruptio placentae and cystathionine β-synthase

Heterozygosity for a defect in the cystathionine β-synthase enzyme was initially thought to be the major cause of hyperhomocysteinaemia in patients with vascular disease, when the patients with abruptio placentae were investigated. The cystathionine β-synthase enzyme activities in these patients, were measured to see if deficient cystathionine β-synthase activity was the cause of the elevated plasma total plasma homocysteine. The method for measuring cystathionine β-synthase activity in fibroblast cultures was adapted for the measurement of enzyme activity in lymphoblast cultures. Culturing lymphoblasts is less labour intensive than tissue culture of fibroblasts. Skin biopsies are also invasive procedures that are best avoided if possible, especially for routine diagnostic testing.

No association was found between low cystathionine β-synthase activity and abruptio placentae. Mean cystathionine β-synthase activity in abruptio placentae patients was 3.9 ±4.8ng/mg protein/hour compared to 5.8 ±7.4 ng/mg protein/hour in the matched control group. Several of the subjects, both in the control and abruptio placentae groups had *enzyme activity approaching levels reported in heterozygotes for cystathionine* β-synthase. The mean activity in the abruptio placentae group was about 70% of the control group, but the two groups were not significantly different owing to the large range of values. Kozich *et al.* (1995) found normal catalytically active cystathionine β-synthase enzyme when expressed in an *E. coli* system, but decreased amounts of the protein were present in cultured cells. Therefore the low enzyme activity (values below the 95th percentile) detected in the study in both affected patients and controls, indicated that enzyme activity in cultured lymphoblasts may not always be a reliable representation of *in vivo* enzyme activity.

No significant correlation existed between low enzyme activity and high total plasma homocysteine in patients with abruptio placentae. Although high total plasma homocysteine correlated with low enzyme activity in some subjects. Sartorio *et al.*, (1986) also did not find any correlation between low cystathionine β-synthase activity and high homocysteine. Inconclusive results from cystathionine β-synthase enzyme analysis called *for genetic investigation of the enzymes along the methyl cycle.*

The first family with homocystinuria had been investigated for defects in the cystathionine β-synthase gene and the common G919A (Gly307Ser) mutation was found in the index case, his father and one paternal uncle. Although the whole cystathionine β-synthase genomic sequence was not yet available, the sequence of exon 8 was available. Screening exon 8 of all the subjects from the abruptio study for the frequent, G919A mutation, with *Pvu* II restriction enzyme analysis, designed for this study, was quick and

easy. This mutation was not detected in any of the subjects from the abruptio placentae study.

Genetic studies have found the G919A (Gly307Ser) mutation to account for 32 to 70% of total homocystinuric alleles investigated (Kraus *et al.,* 1999). Previous genetic studies of the cystathionine β-synthase gene have however mainly been performed in Caucasians from European ancestry. Subjects from the abruptio placentae study group come from the local Coloured community and might therefore not be representative of the European Caucasian population. Dilley *et al.* (2001), found no mutation for the G919A (Gly307Ser) in cases and controls when he investigated African Americans with myocardial infarction.

Although the G919A (Gly307Ser) mutation is commonly found in homocystinuric alleles of European origin, previous studies have failed to implicate a mutation of the cystathionine β-synthase gene as a cause of mild hyperhomocysteinaemia in this population (Kluijtmans *et al.*, 1996; Folsom *et al.*, 1998). It was therefore decided not to screen subjects from our other studies for mutations in the cystathionine β-synthase gene.

5.4.3.4 Abruptio placentae and the methylenetetrahydrofolate reductase

Kang *et al.* (1991) first demonstrated thermolability of the methylenetetrahydrofolate reductase enzyme as a cause of hyperhomocysteinaemia. Several studies have now indicated that folate deficiency, hyperhomocysteinaemia and homozygosity for the methylenetetrahydrofolate reductase thermolabile variant (C677T) are possible risk factors for placentae mediated diseases, such as pre eclampsia, spontaneous abortion and placentae abruption (Ray *et al.,* 1999).

Presence of the methylenetetrahydrofolate reductase C677T thermolabile variant could have been a contributory factor to the hyperhomocysteinaemia found in the affected patients from the abruptio placentae study. All the subjects from the abruptio placentae study was screened for the C677T polymorphism using restriction enzyme methodology with restriction enzyme *Hinf* 1 (Frosst *et al.*, 1995). The CT genotype was present in the affected abruptio placentae patients at a frequency of 19%, which was similar to a frequency of 21% detected in the matched control group. Brattström *et al.* (1998) also found that the CT genotype frequencies were identical between cardiovascular patients and control subjects. Homozygous (TT) genotypes were not detected in either the abruptio placentae patient or control groups. This finding is not reflected by a TT genotype frequency of 12% found in the general Caucasian population (Brattström *et al.*, 1998) but it is consistent with the finding of Dilley *et al.* (2001) who found a low TT genotype frequency of ±2.0% in African Americans. The ethnic origin of our abruptio

placentae population who are from the Coloured community in the Western Cape could explain the absence of the TT genotype. Total plasma homocysteine was similar in patients carrying the CC and CT genotypes of this study. This finding is not surprising as previous studies have only found the TT genotype to be associated with increased total plasma homocysteine.

5.4.4 Pre-eclampsia

Pre-eclampsia is one of the most frequent complications of pregnancy (Raijmakers *et al.*, 2001). Vascular endothelial pathology is thought to play an important role in pre-eclampsia (Roberts *et al.*, 1989). A correlation between hyperhomocysteinaemia and pre-eclampsia (Rajkovic *et al.*, 1997) and hyperhomocysteinaemia and endothelial damage (Celermajer, 1993; Bellamy, 1999) has been reported. Total plasma homocysteine measurement before and after oral methionine, made it possible to determine whether hyperhomocysteinaemia during pregnancy was associated with pre-eclampsia in the local African and Coloured population of the Western Cape.

5.4.4.1 Pre-eclampsia and total plasma homocysteine

Hyperhomocysteinaemia was significantly more prevalent in the pre-eclamptic pregnant women than in the healthy pregnant women ($p < 0.001$). This finding is consistent with that described by Raijmakers *et al.* (2001). No difference in total plasma homocysteine was found between African and Coloured women. Oral methionine rather than screening by fasting levels alone, allowed the identification of an extra five affected women. This finding agrees with previous studies in patients with arterial disease and venous thromboembolism that generally found 50% of patients with abnormal post oral methionine total plasma homocysteine to have fasting total plasma homocysteine within the reference range (van der Griend *et al.*, 1998).

5.4.4.2 Pre-eclampsia and methylenetetrahydrofolate reductase

A study by Lachmeijer *et al.* (2001) have shown the TT methylenetetrahydrofolate reductase thermolabile genotype to be associated with hyperhomocysteinaemia in Dutch women with pre-eclampsia. The TT genotype was not detected in these women. Only the CT and CC genotypes were detected in both women with pre-eclampsia and control women. The pre-eclamptic study group consisted of African and Coloured women from the local population in the Western Cape. The CC (93%; 90.6%) and CT (6.8%; 9.4%) genotype frequencies were the same in both pre-eclamptic and control women

respectively. The TT genotype was not present in pre-eclamptic women or control women. This finding again is consistent with previous studies that found the TT genotype to be present at much lower frequencies in African Americans than in Caucasians (Dilley *et al.*, 2001). The total plasma homocysteine was similar in the CC and CT genotypes and therefore no association existed between high total plasma homocysteine and any specific C677T methylenetetrahydrofolate reductase genotype in the affected group of the pre-eclampsia study. The allele frequency for the C to T substitution was 4% amongst all subjects of this study group, which is more than 50% lower than the allele frequency of 10% found in Caucasians, as reported by Botto and Yang (2000). Results obtained during this study, show that hyperhomocysteinaemia is prevalent amongst both African and Coloured pregnant women with pre-eclampsia.

5.4.5 Peripheral vascular disease

Since methodology to measure total plasma homocysteine has been established in the Inherited Metabolic Disease Unit at Groote Schuur Hospital, clinicians have regularly been investigating patients with peripheral vascular disease (PVD) for hyperhomocysteinaemia. Total plasma homocysteine detected in these patients was compared with the generally accepted reference range for total plasma homocysteine (5 – 15μmol/L), (Ueland *et al.*, 1993) as a matched control group was not available at the time. The peripheral vascular disease patients mainly came from the African and Coloured community in the Western Cape.

5.4.5.1 Peripheral vascular disease and total plasma homocysteine

Total plasma homocysteine was measured in patients with peripheral vascular disease before and after oral methionine. 17% had abnormal fasting total plasma homocysteine compared to 24% who had abnormal post oral methionine total plasma homocysteine. This finding is consistent with results obtained by Loncar *et al.*, 2001, who found one-third of all patients with peripheral vascular disease in his study population (men and women) to have hyperhomocysteinaemia and Dilley, 2001 who found abnormal homocysteine metabolism in patients with peripheral vascular disease. Nine patients who had fasting total plasma homocysteine within the reference range had abnormal post methionine total plasma homocysteine, indicating the necessity for the oral methionine test when hyperhomocysteinaemia is investigated.

Although previous studies (Loncar *et al.*, 2001) have shown differences in total plasma homocysteine values between men and women of different age groups, especially

between the ages of 30 and 50 years, no significant difference in total plasma homocysteine between men and women of the peripheral vascular disease study group was detected for this study.

5.4.5.2 Peripheral vascular disease and vitamin treatment

Total plasma homocysteine in fourteen hyperhomocysteinaemic patients from the peripheral vascular disease study showed that vitamin treatment lowered the mean fasting total plasma homocysteine in this group from 14.2 ±6.0μmol/L to 7.6 ±1.8μmol/L ($p < 0.01$) and the mean post oral methionine value from 57.1 ±15.7μmol/L to 29.2 ±4.7μmol/L ($p < 0.01$). Hyperhomocysteinaemic patients showed marked decreases in total plasma homocysteine (to within the reference range) after 6 weeks of folate treatment. This finding is consistent with results from studies by other investigators (Ubbink, 1997). Ubbink however reported that homocysteine levels, maintained during treatment, was not maintained when vitamin treatment stopped, suggesting that long-term supplementation may be required in some individuals with hyperhomocysteinaemia. The peripheral vascular study group mainly comprised patients from the less affluent Coloured and African communities in the Western Cape and their diets, which may be low in fresh foods, may be the result of impaired folate status.

5.4.5.3 Peripheral vascular disease and methylenetetrahydrofolate reductase

The peripheral vascular disease patients and a group of patients, matched for ethnicity, who attended the hospital for minor ailments and had no sign of vascular disease were screened for the methylenetetrahydrofolate reductase thermolabile variant. No difference in CC and CT methylenetetrahydrofolate reductase thermolabile genotype frequencies (22.5%; 18.6%) (71.8%; 81.0%) between peripheral vascular disease patients and control patients were observed. Four patients (5.6%) compared to one (0.4%) from the control group, carried the TT genotype for methylenetetrahydrofolate reductase. A significant association however could not be established because of the small population sizes. The T allele frequency was 33.6 % in the patients compared to only 12% in the control group. This was also higher than the allele frequency of 10% found in the Caucasian population by previous investigators. Total plasma homocysteine was similar in the CC and CT methylenetetrahydrofolate reductase thermolabile genotypes and therefore no association existed between high total plasma homocysteine and heterozygosity for the C677T methylenetetrahydrofolate reductase genotype.

5.4.6 Summary of hyperhomocysteinaemia in the local populations of the Western Cape

Hyperhomocysteinaemia is prevalent amongst local patients with disease states like abruptio placentae, pre-eclampsia and peripheral vascular disease. Oral methionine was necessary to identify several hyperhomocysteinaemic patients who had normal fasting total plasma homocysteine. Cystathionine β-synthase activity in patients was similar to cystathionine β-synthase activity found in controls, indicating that hyperhomocysteinaemia in these patients might not have been caused by defective cystathionine β-synthase activity. Vitamin status of abruptio placentae patients appeared not to be deficient, but this finding might not be representative of their folate status at the time of conception. The methylenetetrahydrofolate reductase thermolabile variant was not associated with elevated total plasma homocysteine in the abruptio placentae and pre-eclampsia studies. No TT genotypes were found amongst both study groups, consisting mainly of Coloured and African women. A possible association between methylenetetrahydrofolate reductase genotype and total plasma homocysteine was observed for the peripheral vascular disease patients. Vitamin treatment, in hyperhomocysteinaemic peripheral vascular disease patients, lowered total plasma homocysteine effectively after six weeks treatment. The lack of substantial evidence that hyperhomocysteinaemia in the three study groups is due to either reduced folate, vitamin B_{12}, heterozygosity for cystathionine β-synthase or homozygosity for the thermolabile variant of methylenetetrahydrofolate reductase, might support the recent speculation that mildly elevated homocysteine in patients with vascular disease is a consequence rather than a cause (Brattström and Wilcken, 2000). Brattström and Wilcken argue that the impairment of renal function due to vascular disease, might be the cause of elevated total plasma homocysteine, due to a deterioration in plasma clearance of homocysteine

5.5 Ethnic diversity for the methylenetetrahydrofolate reductase genotype

In order to determine the prevalence of the methylenetetrahydrofolate reductase thermolabile variant in the local population, the three study groups were combined and separated into the three local ethnic groups namely, Caucasian, African and Coloured. Screening the three ethnic groups of the local population of the Western Cape for the C677T mutation, allele frequencies of the 17% for the T allele was found in the Caucasians, 12.5% in the Coloured and a low 0.2% in the African population, indicating that the thermolabile variant is also related to ethnicity in the Western Cape. The TT genotype was not present in the African population. This is consistent with the finding of Botto and Yang (2000) and Dilley *et al.* (2001) who indicated that the prevalence of the C677T thermolabile variant of the methylenetetrahydrofolate reductase gene is related to ethnicity. Ubbink *et al.* (1999) also did not find the TT genotype amongst African women.

5.6 An association between peripheral vascular disease, hypertension and variation in the fibrillin-1 and elastin genes

We have shown that hyperhomocysteinaemia is more prevalent in patients with abruptio placentae, pre-eclampsia and peripheral vascular disease. Deficient cystathionine β-synthase or the presence of the thermolabile variant of the methylenetetrahydrofolate reductase gene did not seem to be the cause of the hyperhomocysteinaemia in the above mentioned disease groups in the local population of the Western Cape.

5.6.1 Genotype frequencies

5.6.1.1 Fibrillin-1 f5 locus

Defects in the fibrillin-1 and elastin genes have been implicated in Marfan syndrome and Williams syndrome respectively (Lee, 1991; Urban *et al.*, 1996). Previous investigators have also investigated whether polymorphic markers at specific loci in these genes co-segregated with a specific disease state. Lee (1991) used a penta repeat sequence to demonstrate linkage of the fibrillin-1 gene to Marfan syndrome. Patients with both these two disease states have vascular abnormalities. Powell *et al.* (1996) suggested that changes in the elastic properties of the aging aorta could influence arterial pulse pressure. He further speculated that a variation in the fibrillin-1 gene might be a marker to link the fibrillin-1 gene to these changes in the elastic properties of arteries. Powell *et al.* (1996) genotyped patients with abdominal aneurysm and a control group for the $(TAAAA)_n$ repeat at the fibrillin-1 f5 locus in the fibrillin-1 gene in order to establish if an association existed between the fibrillin-1 genotype and abdominal aortic aneurysm. Powell used the methodology of Lee (1991), to amplify the region in the fibrillin-1 gene where the penta nucleotide repeats occur. Powell recognized four different alleles of the sizes 181, 171, 166 and 161 base pairs and assigned the numbers 1, 2, 3 and 4 to them respectively, calling the smallest allele, allele 4. These alleles gave rise to three common genotypes, namely genotypes 2-2, 2-3 and 2-4. The more rare genotypes were genotypes 1-1, 1-2, 3-3, 3-4 and 4-4.

Patients with hypertension and peripheral vascular disease were typed for markers at two loci in the fibrillin-1 gene and one locus in the elastin gene. If Powell used the primers that Lee used, allele sizes of 22 base pairs less than the allele sizes found in this study should have been amplified as shown in fig. 74. However the difference between Powel's three smaller alleles and the alleles found during this study is 7 base pairs, but it may also be noted that Powell did not detect an allele between 181 and 171, whereas alleles amplified

during this study, ranged from 183 to 168 base pairs with a difference of five base pairs between each one.

(a)

gaaagataag taaaagtagcg atgaaaacaa aagtccagag tactagagtg ttttagggaga gatgaaat taaaataaaa taacataaca taacataaca taaaataaag $(TAAAA)_n$ aagaacttac caacacaaaa tagcctatcg ggagttgaat ggtagccagg gttgcaggca cactgatact tccctatgag g

(b)

gaaagataag taaaagtagcg atgaaaacaa aagtccagag tactagagtg ttttagggaga gatgaaat taaaataaaa taacataaca taacataaca taaaataaag $(TAAAA)_n$ aagaacttac caacacaaaa tagcctatcg ggagttgaat ggtagccagg gttgcaggca cactgatact tccctatgag g

Figure 74. DNA sequence flanking the fibrillin-1 f5 microsatellite polymorphism in the fibrillin-1 gene. The red letters indicate the location of the sense and antisense primers respectively, used to amplify the fragment of DNA that carries the TAAAA microsatellite in this study (a) and in Powell's study (b).

It would seem therefore that Powell may have incorrectly scored his allele sizes and that allele 161 of this study is equivalent to Powell;s allele 168, allele 166 is equivalent to Powell's allele173 etc (see table 46). An allele of 171 base pairs was the most commonly found allele according to Powell. This was also his second largest allele. This study however showed allele 178 [$(TAAAA)_6$ penta nucleotide repeat insert)] to be the most prevalent allele. Allele 178 was the second largest allele detected for this study.

Table 46. Comparison of our allele sizes with that of Powell's.

No	Ours	Powell
1	183	181
2	178	171
3	173	166
4	168	161

Powell *et al.* (1996) did not include the "rare" alleles for chi^2 (distributions) calculations. For this study chi^2 (distributions), were calculated with the rare alleles included and excluded (-rare). Different *p* values were obtained in each case. Powell *et al.* (1996) found the 2-2 fibrillin-1 f5 genotype to be the most prevalent genotype in both healthy subjects and patients with abdominal aortic aneurysm. For this study a significant association between fibrillin-1 f5 genotype and systolic blood pressure in hypertensive

patients with a *p* value of 0.003 was found when the rare alleles were excluded from the statistical analysis. When these rare alleles were included, no significant difference existed. Only a trend towards an association was observed for patients with high pulse pressures when rare alleles were excluded (*p* value of 0.085).

In healthy subjects, Powell showed a significant association between patients and fibrillin-1 genotype and high pulse pressures and only a trend towards an association for systolic blood pressures. During this study no association between fibrillin-1 f5 genotype and high systolic blood pressures or high pulse pressures and normotensive subjects were observed. Powell observed a significant difference in mean systolic blood pressure and mean pulse pressure for the different fibrillin-1 f5 genotypes in abdominal aortic aneurysm patients but not in healthy subjects. Although no significant differences were observed, mean systolic blood pressures and mean pulse pressures were higher in patients carrying the 2-3 genotype. The median systolic blood pressure was 178mm Hg in patients with hypertension, which was higher than the 155mm Hg found by Powell in patients with abdominal aortic aneurysm (table 47).

Median systolic blood pressure and pulse pressure in disease and reference groups of this study and Powell's study (1996)

	HPT (us)	AAA (Powell)
Median systolic blood pressure (mm Hg)	178	155
Median pulse pressure (mm Hg)	69	62
	Normotensive	**Healthy middle aged men**
Median systolic blood pressure (mm Hg)	130	131
Median pulse pressure (mm Hg)	50	50

Table 47. A comparison between median systolic blood pressure and median pulse pressure in the hypertension study group and normotensive control group and the study group with abdominal aortic aneurysm (AAA) and healthy middle age men.

Analysis of the control group showed no difference in pulse or systolic blood pressure between the genotypes. Powell found the distribution about the median pulse pressure to be skewed in his control group, which was not observed during this study. An association between the 2-3 fibrillin-1 f5 genotype and high systolic blood pressure amongst normotensives was found during this study when the rare alleles were excluded from statistical analysis. An unexpected association between the 2-3 genotype and pulse pressure below 50mm Hg amongst normotensives may not be a representative result

because of the small population size of 7. No association between fibrillin-1 f5 genotype and the disease states, hypertension and peripheral vascular disease was found as shown in table 28, chapter 4, page 127.

It appears if Powell may have incorrectly scored allele sizes for the fibrillin-1 f5 microsatellite. Powell did not include the rare genotypes in statistical analysis and different results were obtained when these less frequently observed genotypes were included in the statistical analysis for this study. The 2-2 genotype was the most prevalent genotype. Like Powell, an association between genotype (2-3) and systolic blood pressure and pulse pressure in the hypertension group was found for this study. In healthy subjects, Powell found an association between pulse pressure and the 2-3 genotype and only a trend towards an association when systolic blood pressure was observed. The association observed between fibrillin-1 f5 genotype and systolic blood pressure and pulse pressure during this study might have been not representative due to the small population size. Powell found that both mean systolic and pulse pressures were significantly higher in the 2-3 genotype. Although no significance was demonstrated for this study, mean systolic blood pressure and mean pulse pressures were higher in patients with the 2-3 genotype. Fibrillin-1 f5 genotypes distribution were the same in the two disease groups (HPT and PVD) and the non-hypertensive control group whereas Powell found an association between 2-3 genotype and patients with abdominal aortic aneurysm and popliteal aneurysm.

To conclude, similar results to that of Powell et al. (1996) were obtained for this study. Different *p* values were obtained if rare genotypes were included in the statistical analysis. Powell did not include these and they also might have scored the allele sizes incorrectly.

5.6.1.2 Fibrillin-1 f1 locus

A wide variety of genotypes (±30) were detected at the fibrillin-1 f1 microsatellite. The majority of hypertensive patients and normotensive subjects carried the rare genotypes, rendering this microsatellite unsuitable for statistical analysis.

5.6.1.3 Elastin locus

No significant difference in systolic blood pressure was observed between the different elastin genotypes, although mean systolic blood pressure appeared higher in genotypes 4-7 and 6-7. The 6-7 genotype was more prevalent in the peripheral vascular group at a frequency of 14.3% compared to 8.3% in the hypertensive group and 6.3% in the normotensive group.

5.6.2 Allelic comparisons

Both R_{st} and G_{st} values showed that no significant statistical difference existed between the peripheral vascular disease, hypertensive, and normotensive control groups, when statistical analysis of the allele frequencies for all loci was performed on the groups from diverse racial origin before they were separated into the three different local race groups. Genotype analysis confirmed this result.

A trend towards a significant difference in allele distribution at the fibrillin-1 loci between Caucasians and Coloureds, Caucasians and Africans, was observed. R_{st} and G_{st} values however did not always agree. The allele frequency pattern at locus fibrillin-1 f1 for the Caucasians were different to that of allele patterns for the Coloureds and Africans with the 144 allele being present at a frequency of 40% compared to 20% in the Africans and Coloureds.

Results were different when race groups were separated before statistical analysis was performed on allele frequencies. When allele frequencies were compared between the disease groups for the Caucasians only, a trend towards a significant difference was observed at the fibrillin-1 f5 locus. (f5; G_{st} for PVD vs Control = 0.0773; PVD vs HPT = 0.0653).

5.6.3 Ethnic groups

A trend towards a significant association between genotype and disease group was observed in the Caucasian group between elastin genotype and peripheral vascular disease ($p = 0.068$). An association between the fibrillin-1 f5 genotypes and the Coloured peripheral vascular disease group existed when the rare genotypes were included for the analysis. No association was found between fibrillin-1 f5 and/or elastin genotype and disease states like peripheral vascular disease and hypertension in the African group.

In conclusion no convincing statistical evidence for an association between either genotype or allele and hypertension (HPT) and peripheral vascular disease (PVD) was found during this study. However the evidence towards a possible association between fibrillin-1 genotypes and hypertension (HPT) and peripheral vascular disease (PVD) is enough in order to warrant further study using a better clinical cohort as study population.

CONCLUSION

This study investigated the cystathionine β-synthase gene of three individuals with homocystinuria, diagnosed by this study after excessive total plasma homocysteine in the homocystinuric range was measured in their blood. Their immediate family members were also investigated for homocystinuria and defects in the cystathionine β-synthase gene. The cystathionine β-synthase gene was amplified and investigated for disease causing mutations by amplifying either cDNA or genomic DNA. cDNA was either cloned and sequenced or sequenced directly. Genomic DNA was sequenced directly. Restriction enzyme analysis especially designed for this study or established by other investigators, was used for the rapid detection of mutations established by sequencing, in the cystathionine β-synthase gene. Four different missense mutations were found in the cystathionine β-synthase gene of individuals with homocystinuria. Two of these mutations were private mutations and two were mutations that commonly are found in Europeans with homocystinuria. These two novel mutations resulted in changes in amino acids present in highly conserved regions of the cystathionine β-synthase gene and were in close proximity to the pyridoxal 5'-phosphate binding site.

For this study methodology to measure cystathionine β-synthase activity was established and conditions under which maximal activity could be obtained were defined. Cystathionine β-synthase activity was impaired in the patients with homocystinuria. No association between defective cystathionine β-synthase and abruptio placentae could be established for this study. The commonly found G919A (Gly307ser) mutation was not present in any of the hyperhomocystinaemic subjects from the three vascular disease groups. It therefore appears that hyperhomocysteinaemia in the subjects from the abruptio placentae, pre-eclampsia and peripheral vascular disease study groups is not caused by defective transsulphuration of methionine. No association between the thermolabile variant of the methylenetetrahydrofolate reductase gene and abruptio placentae and pre-eclampsia could be established. Only patients with peripheral vascular disease were homozygous for the C677T thermolabile variant of the methylenetetrahydrofolate reductase gene. The C677T thermolabile variant of the methylenetetrahydrofolate reductase gene was more prevalent in the Caucasian population than in the Coloured population and was barely present in the African population demonstrating the ethnic diversity of these three population groups. Individuals homozygous for the thermolabile variant had higher total plasma homocysteine than heterozygous individuals.

Measurement of free homocystine was replaced by the measurement of total plasma homocysteine after existing amino acid methodology used in the Inherited Metabolic Disease Unit at Groote Schuur Hospital was adapted to measure total plasma homocysteine in patients and controls of three disease groups namely abruptio placentae, pre-eclampsia and peripheral vascular disease. The oral methionine test for the evaluation of methionine metabolism was introduced in Groote Schuur Hospital and other hospitals in the region of the Western Cape. An association between hyperhomocysteinaemia and vascular disease, namely abruptio placentae, pre-eclampsia and peripheral vascular disease, in patients from the local population of the Western Cape was established.

Folate treatment in both the hyperhomocysteinaemic and some of the homocystinuric patients successfully lowered total plasma homocysteine to within the reference range. As no definite genetic link between the cystathionine β-synthase and methylenetetrahydrofolate reductase genes and the hyperhomocysteinaemia found in patients with and vascular disease could be established for this study a possible link between the fibrillin-1 and elastin genes and vascular disease was investigated. These genes have been implicated in disease states such as Marfan's syndrome and Williams' syndrome. Both these disease states have vascular implications and genetic links between fibrillin-1 (Marfan's syndrome and elastin (Williams' syndrome) and these two disease states have been established. Microsatellite analysis was used to investigate possible associations between the fibrillin-1 and elastin genes and peripheral vascular disease and hypertension. Results from this study was compared with results from a study done by Powell *et al.* (1996). Whereas Powell only used genotype analysis to predict an association between blood pressure and a fibrillin-1 genotype, this study used both genotype and allele frequencies of two loci in the fibrillin-1 gene and one locus in the elastin gene. Allele sizes of the fibrillin-1 f5 microsatellite detected for this study did not agree with allele sizes of the same microsatellite from Powell's study. Although a trend towards an association between the penta repeat microsatellite and high blood pressure in patients with hypertension was found no convincing statistical evidence for an association between allele or genotype frequency and peripheral vascular disease and hypertension was found. A trend towards an association however warrants further study using larger study populations.

In conclusion, homocystinuria found in patients investigated for this study was caused by defects in the cystathionine β-synthase gene. New and previously found mutations were detected in these patients which follows the world wide trend of the regular detection of private disease causing mutations found in homocystinuric patients.

Hyperhomocysteinaemia in the Western Cape was associated with vascular disease states namely abruptio placentae, pre-eclampsia and peripheral vascular disease. A possible association between the thermolabile variant of the methylenetetrahydrofolate reductase gene and hyperhomocysteinaemia in peripheral vascular disease patients was found. Folate treatment was effective in both homocystinuric and hyperhomocysteinaemic patients indicating the possibility of a link between dietary and genetic causes for the hyperhomocysteinaemia and homocystinuria found in patients with vascular disease.

A trend towards an association between fibrillin-1 genotype and high blood pressure found during this study warrants further study in order to establish an alternative method for pre-symptomatic diagnosis of vascular disease.

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APPENDIX 1 REAGENT PREPARATION

TISSUE CULTURE

0.125% trypsin/0.5mM EDTA

Add together,	
Trypsin	125μl
0.5mM EDTA	100ml

PSN

Mix equal volumes of	
Penicillin	30mg/ml
Streptomycin	50mg/ml
Neomycin	25mg/ml

Complete medium

Add together	
Fetal calf serum (FCS)	50ml
DMEM	500ml
PSN	5ml

10% glycerol

Add together	
Glycerol	10ml
Distilled H_2O	100ml

DNA EXTRACTION

Ammonium chloride (NH_4Cl_3)

Dissolve	
NH_4Cl_3	23mg
Distilled H_2O	25ml

Tris-EDTA

Dissolve		**final concentration**
Tris-HCl (mw. 157.6)	157.6mg	10mM (pH 8.0)
EDTA	37.2mg	1mM (pH 8.0)
Distilled H_2O	100ml	

5N NaOH

Dissolve	
NaOH	20g
Distilled H_2O	50ml

Make volume up to 100ml with distilled H_2O

0.05m NaOH

Dilute	
5M NaOH	1ml
Distilled H_2O	99ml

1M Tris/HCL pH 8.0

Dissolve	
Tris (mw. 121.14)	121.14g
Distilled H_2O	800ml

Adjust pH to 8.0 with concentrated HCl

0.5M EDTA

Dissolve	
$Na_2EDTA.2H_2O$	186.1g
Distilled H_2O	400ml

Adjust pH to 8.0 with 10M NaOH (use pellets if necessary).

Make up to 1 litre with distilled H_2O

Lysis buffer

Add together		**final concentration**
Sucrose	109.5g	0.32M
1M Tris-HCl (pH7.6)	10ml	10mM
$MgCl_2$ (1M)	5ml	5mM
Triton X	10ml	1%

Make volume up to a litre with distilled H_2O

Digestion buffer

Dissolve		**final concentration**
NaCl	0.58g	100 mM
1M Tris/HCL (pH 8.0)	1ml	10 mM
0.5M EDTA (pH 8.0)	5ml	25mM
SDS	0.5g	0.5%
Protein K	0.1mg/ml	
Make volume up to 100ml with distilled H_2O		

Saline

Dissolve		**final concentration**
NaCl	0.9g	0.9%
Distilled H_2O	100ml	

0.1mM EDTA

Dissolve		**final concentration**
EDTA (mw.=372.24)	3.72mg	0.1mM
Distilled H_2O	100ml	

4M NaCl

Dissolve		**final concentration**
NaCl	33.37g	4.0M
Distilled H_2O	100ml	

Saline/EDTA

Dissolve	
0.1mM EDTA	50ml
4.0M NaCl	3.7ml
Distilled H_2O	200ml
Adjust pH to 8.0	

10% SDS

Dissolve	
SDS	10.0g
Distilled H_2O	100ml

6M NaCl

Dissolve	
NaCl	35.1g
Distilled H_2O	100ml

Phenol:chloroform:isoamylalcohol (25:24:1)

Reagents

8-Hydroxyquinoline

Liquefied phenol

50mM Tris base (pH 10.5)

50mMTris-HCl (pH 8.0)

Chloroform

Isoamyl alcohol

Procedure

Add 0.5g of 8-hydroxyquinoline to a 2 litre glass beaker containing a stirrer. Gently pour in 500ml liquefied phenol (*the phenol will turn yellow due to the 8-hydroxyquinoline, which is added as an antioxidant*). Add 500ml of 50mM Tris base. Cover the beaker with aluminium foil. Stir (at room temperature) for 10 minutes at low speed with a magnetic stirrer. Let phases separate at room temperature. Gently decant the top (acqueous) phase into a suitable waste container. Remove what cannot be decanted with a 25ml glass pipette and suction bulb. Add 500ml 50mM Tris-HCL (pH 8.0). *The pH of the phenol phase can be checked with pH paper and should be 8.0.* If it is not, steps (3)-(7) should be repeated until this pH is obtained. Add 250ml 50mM Tris-HCL (pH 8.0) or TE buffer (pH 8.0) and store at 4°C in a dark bottle. For use in DNA purification procedure, mix 25 volumes phenol (bottom yellow phase) with 24 volumes chloroform and 1 volume isoamyl alcohol. *Phenol prepared with 8-hydroxyquinoline as an antioxidant can be stored for approximate 2 months.*

10M ammonium acetate

Dissolve	
Ammonium acetate *(anhydrous)*	82.1g
Distilled H_2O	40ml
Make up to 100ml with distilled H_2O	

mRNA EXTRACTION

Diethylpyrocarbonate (DEPC)

Dissolve	
DEPC	200µl
Distilled H_2O	100ml

2M Sodium acetate

Dissolve	
Sodium acetate	16,42g
Distilled H_2O	40ml
Glacial acetic acid	35ml

Adjust to pH 4.0 with glacial acetic acid

Make the final volume up to 100ml with distilled H_2O

H_2O saturated phenol

Dissolve	
Phenol crystals	100g
Distilled H_2O	50ml at 60 to 65°C

Aspirate the upper H_2O phase and store up to I month at 4°C.

Chloroform:isoamylalcohol (49:1)

Add	
Isoamylalcohol	1ml
Chloroform	49ml

Guanidinium isothiocyanate stock solution

Dissolve		***final concentration***
guanidinium isothiocyanate	50g	4M
dH_2O	58.6ml	
0.75M sodium citrate	3.5ml	25mM (pH 7.0)
10% sarkosyl	5.28ml	0.5%

Working guanidinium isothiocyanate solution (solution D)

Add	
Guanidinium isothiocyanate (stock solution)	10ml
Mercapto-ethanol	72µl

Ethidium bromide

Dissolve	
Ethidium bromide	10ng
Distilled H_2O	1ml

Take care, very toxic

Agarose formaldehyde denaturing gel

10x running buffer (100ml		**final concentration**
Add		
MOPS	4.63g	200mM
Sodium acetate	0.41g	50mM
EDTA	0.37g	10mM

Make up to 100ml with distilled H_2O

Sample dye

Dilute	
Glycerol	50ml

Up to 100ml distilled H_2O

Add	
Bromophenol blue	400µl

Gel

Dissolve	
Agarose	500mg
Distilled H_2O	36.6ml
10x running buffer	5ml

Melt agarose and cool to 60°C

Add *concentrated* formaldehyde	8.33ml

Sample preparation

RNA	2.6µl
formamide	5µl
formaldehyde	1.4µl
10x running buffer	1µl

Heat samples to 60°C for 5 minutes and then cool on ice

Add 1µl sample buffer and 1µl ethidium bromide (10ng/ml)

Run gel at 50V overnight

cDNA

Dinucleotide phosphates (dNTP's)

dNTP's are usually bought in 50mg amounts. Add a volume of sterile uncontaminated distilled H_2O directly to manufacturer's container so as to make up to 20mM.

Pour all four 20mM solutions into a sterile plastic container, pH to 7.0 with sterile 1M NaOH. Aliquot appropriate amounts into small containers (microfuge tubes) and store at –70°C.

dTTP- (Roche) 11.404g/L ~20mM. Add 4.38ml sterile dH_2O to 50mg for a 20mM solution.

dGTP- (Roche) 11.024g/L ~20mM. Add 4.54ml sterile dH_2O to 50mg for a 20mM solution.

dCTP- (Roche) 10.222g/L ~20mM. Add 4.89ml sterile dH_2O to 50mg for a 20mM solution.

dATP - (Roche) 11.784g/L ~20mM. Add 4.24ml sterile dH_2O to 50mg for a 20mM solution

PCR

10x Tris/KCL/gelatin buffer (15mMMgCl$_2$)

Add together

100mM Tris	1.0ml
1M $MgCl_2$	1.5ml
500mM KCL	5.0ml
0.1% gelatin	1ml
Distilled H_2O.	2.85ml

AGAROSE GEL ELECTROPHORESIS

50x TAE buffer

Dissolve	
Tris	242g
Distilled H_2O	±500ml
Add	
0.5M EDTA (pH 8.0)	100ml
Glacial acetic acid	57.1ml

Make up to 1L with distilled H^2O.

10x TBE buffer

Dissolve	
Tris	216g
Distilled H_2O	±500ml
Add	
Boric acid	110g
0.5M EDTA (pH 8.0)	80ml

Make up to 2L with distilled H_2O.

CLONING

2x TY broth

Dissolve	
NaCl	10g
Yeast extract	10g
Bacto Tryptone	16g
Distilled H_2O	600ml

Make up to 1L with distilled H_2O

10mM ATP

Dissolve	
ATP	5mg
Distilled H_2O	1ml

Glucose;EDTA;Tris (GTE)		**final concentration**
Glucose	45mg	50mM
0.5M EDTA	100µl	10mM
1M Tris-HCl (pH 8.0)	*125µl*	*25mM*

Make up to 5L with distilled H_2O

0.2N NaOH/1%SDS

Add together

5N NaOH	400µl
SDS	100mg

Make up to 10ml with distilled H_2O.

13% Polyethylene glycol

Dissolve

Polyethylene glycol (8000)	130mg
Distilled H_2O	*1ml*

Potassium acetate

Add

Glacial acetic acid	7ml
5M potassium acetate	15ml
Distilled H_2O	3ml

POLYACRYLAMIDE GEL ELECTROPHORESIS

40% Stock polyacrylamide solution

Dissolve	
Acrylamide	250g (whole bottle)
bis-acrylamide	13.1g
Distilled H_2O.	~350ml (warm)

Add warm distilled H_2O to the acrylamide in its reagent bottle. Stir on magnetic stirrer with heat on very low. When dissolved make up to 657ml and store in brown bottle in fridge. The **powder is very toxic so be very careful**. Wear a mask and gloves and do it all in the fume cupboard. **Avoid weighing the acrylamide**.

6% Working polyacrylamide solution

The following reagents are needed

	80ml	**100ml**	**500ml**
Polyacrylamide stock solution	12ml	15ml	75ml
8M urea	33.6g	42g	210g
10x TBE buffer	8ml	10ml	50ml
Distilled H_2O	~26ml	~33ml	~165ml

Dissolve the urea in distilled H_2O and heat gently until dissolved (heating block or microwave). Add the polyacrylamide and TBE buffer and make to volume. Store in a dark bottle in the fridge. This can be stored up to 2 weeks.

Solutions for developing of autoradiographs

Stop solution

2% acetic acid

Fixer

Dilute commercial fixer 1:3

Developer

Commercial developer should be replaced when turning brown.

HOMOCYSTEINE ANALYSIS

S-2-aminoethyl-L-cysteine (AEC); internal standard (2.5mM)

Dissolve

AEC (mw. 200.7)	20.1mg
Li-S buffer (Beckman)	100ml

2.0mM L-Homocystine

Dissolve

L-Homocystine (mw. 268)	1.07mg
Dilution buffer (Beckman)	2.0ml

pH to 12.0 with 3M LiOH (Beckman) to dissolve, then pH back to 7.0.

2.5mM Norleucine

Dissolve

Norleucine (mw. 131.2)	3.3mg
Dilution buffer (Beckman).	10ml

1M DTT

Dissolve

DTT	15.4mg
Distilled H_2O	100µl

Prepare fresh each time

50% Salicyl sulphonic acid (SSA)

Dissolve

SSA	50g
DistilledH_2O	100ml

CYSTATHIONINE β-SYNTHASE ASSAY

Homocysteine from thiolactone

Dissolve

15.4mg thiolactone in

0.1ml 5M NaOH at 37°C for 5 minutes

Adjust pH to 8.6 with 0.18ml 2M HCl. Add 50µl 0.2M dithiothreitol (DTT) and make up to a final volume of 0.5ml with distilled H_2O.

Sonication buffer (0.05M Tris/HCL pH 8.6)

Dilute

1M Tris-HCL 1 in 20 with

Distilled H_2O

APPENDIX 2

UNIVERSITY OF CAPE TOWN

Department of Chemical Pathology

Consent for participation in a biochemical investigation for a research project / Toestemming tot deelname aan 'n biochemiese ondersoek vir 'n navorsingsprojek

Homocysteinaemia, a risk factor for vascular disease. MRC account number 519219.

For this research study we require that the patient, after eating nothing overnight, be administered, by mouth, a measure of **methionine** (a harmless normal nutrient found in all foods) mixed with orange juice. We will then take two blood samples, one immediately after the methionine drink and one 6 hours later. This will enable us to measure levels of methionine and its digestion products in the blood. These results are not only of direct benefit to the patient (it is a test used routinely in the hospital) in assisting the doctor to prescribe the appropriate treatment in cases of vascular (blood vessel) disease, but will also be used in a research project which examines the value of this test in various types of vascular disease.

If for any reason you do not wish to participate in this study, you have every right to refuse and this will not affect the standard of treatment and care that you will receive.

I, the undersigned ..... (Print Name), Hospital Number ..... hereby confirm that I understand the nature and purpose of the above procedure and am aware that the results may be used for research purposes. I agree to participate voluntarily in the above investigation.

Homosisteinemie, 'n risiko vir vaskulêre siektes. MNR rekening nommer 519219.

Die pasiënt moet vir hierdie studie, nadat hy/sy oornag gevas het, **metionien** ('n onskadelike voedingstof wat in alle kossoorte voorkom) opgelos in lemoensap inneem. Hierna sal twee bloed monsters getrek word, een direk na die metionien en een 6 uur later. Dit sal ons in staat stel om metionien en sy neweprodukte in die bloed te bepaal. Hierdie resultate sal tot die direkte voordeel van die pasiënt strek en die dokter daartoe in staat stel om die toepaslike behandeling in die geval van vaskulêre siekte voor te skryf. Hierdie toets word daagliks gebruik in die hospitaal. Hierdie uitslae sal ook gebruik word in 'n navorsingsprojek wat die waarde van hierdie toets in die behandeling van verskillende tipes van vaskulêre siektes sal bepaal.

As die pasiënt om enige rede nie aan die projek wil deelneem nie, kan hy/sy ter eniger tyd weier. Dit sal geensins die standard behandeling van die pasiënt beïnvloed nie.

Ek, die ondergetekende.....(drukskrif), Hospitaal Nommer..... bevestig hiermee dat ek die doel en aard van die bogenoemde prosedure verstaan en is bewus daarvan dat die resultate vir navorsingsdoeleindes gebruik mag word. Ek stem in om vrywilliglik deel te neem aan bogenoemde navorsingsprojek.

Signature of patient / Handtekening van pasiënt:.....

Date / Datum:.....

APPENDIX 3

UNIVERSITY OF CAPE TOWN

Department of Chemical Pathology

Consent for participation in a molecular genetics investigation for a research project

We are undertaking a study to determine the incidence and cause of homocystinuria and hyperhomocysteinaemia in the Western Cape. Canditate genes associated with the disease states; homocystinuria, hyperhomocysteinaemia and hypertension will be investigated at the molecular level.

For this research study we require one 5ml EDTA blood sample. The DNA will not be used for any other purpose other than this investigation.

If for any reason you do not wish to participate in this study, you have every right to refuse and this will not affect the standard of treatment and care that you will receive.

I, the undersigned ..... (Print Name), Hospital Number ..... hereby confirm that I understand the nature and purpose of the above procedure and am aware that the results may be used for research purposes. I agree to participate voluntarily in the above investigation.

Signature of patient.....

Date:.....