THE ROLE OF GENETIC FACTORS IN EARLY ONSET CORONARY HEART DISEASE IN THE NATAL INDIAN

by

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For my dear friend, Vinodh Gathiram ('Gats')

DECLARATION

I, DATSHANA PRAKESH NAIDOO, DECLARE THAT THE WHOLE THESIS, UNLESS SPECIFICALLY INDICATED TO THE CONTRARY IN THE TEXT, IS MY OWN ORIGINAL WORK AND HAS NOT BEEN SUBMITTED FOR A DEGREE AT ANY OTHER UNIVERSITY.

D. P. NAIDOO

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ABSTRACT

Objective:

To determine the role of candidate gene polymorphisms in patients who sustained myocardial infarction at a young age and examine their relationship, if any, to risk factors. Since angiotensin II is known to play a pathophysiological role at the myocardial and vascular level, the genes to be studied are those regulating the renin angiotensin system and tissue metabolism.

Design:

The risk factors and genetic profile is described in 117 young Indians with myocardial infarction recruited over a period of thirty months (Dec 1997-Jun 1999). Controls comprised 80 normal subjects with no clinical evidence of coronary heart disease (CHD) and with a normal effort response.

The key features of this study are the selection of young subjects with myocardial infarction, (mean age 43 ± 6.8 years) in whom the possibility of a genetic basis for the disease was felt to be more likely since the confounding effect of age as a risk factor was reduced.

Setting:

Patients recruited 3-12 months after myocardial infarction from Addington Hospital, Durban. This hospital subserves the Indian community in the north of Durban. The majority of patients were from the Phoenix settlement area.

Results:

1 The clinical profile of the young Indian with myocardial infarction is a young man, slightly overweight with a high prevalence of risk factors, particularly smoking and diabetes, coupled with sedentary behaviour and risk-prone dietary patterns characterised by high red meat intake and low fruit and vegetable consumption, resulting in increased BMI and W/H ratios.

2 There were no differences in the patterns of gene polymorphism in the renin-angiotensin system between the study and control groups. This finding extended across all candidate gene loci studied i.e. those involving aldosterone, G-protein, TGF-β and homocysteine metabolism. Serum triglycerides, haemoglobin A1C and urine microalbumin levels were elevated in the probands together with low HDL-C levels ($p = 0.001$).

3 A striking finding of this study was the substantial proportion of patients found to have diabetes mellitus, totalling 47% of the proband group. Of the 53 diabetic patients, (45 males and 8 females) four (3 males, 1 female) had impaired glucose tolerance. Cigarette smoking, a positive family history of hypertension/diabetes and a family history for premature CHD emerged as

important risk predictors for MI.

Conclusion:

This study, the first to report candidate gene polymorphisms in young Indians with coronary heart disease, has shown no obvious association between the genetic loci studied and acute myocardial infarction. Instead a high prevalence of risk factors, particularly smoking and diabetes mellitus, coupled with coronary-prone behavioural patterns was observed.

In the light of these findings, genome-wide screening of unaffected siblings of subjects with early onset CHD cannot be recommended in this population until common polymorphisms can be clearly identified as risk factors. Indeed this study again supports the dire need for early, school level, education in behavioural lifestyle patterns and disease predisposition. The Indian community is a very high-risk group who should be targeted, not for secondary, but for primordial disease prevention measures.

The study does not rule out the role of other candidate gene polymorphisms in the pathogenesis of CHD in these subjects. The high prevalence of diabetes and insulin resistance suggests that studies of genes regulating glucose and lipid metabolism should be pursued. Such candidate genes should include genes for lipoprotein lipase and paraoxonase polymorphisms which may explain the dyslipidaemia patterns in this group.

PREFACE

This study was approved by the Ethics and Postgraduate Committee, University of Natal, Durban. Permission was also obtained from the medical superintendent at King Edward VIII Hospital for use of the echocardiographic and treadmill facilities, as well as from the medical superintendent of Addington Hospital to study their patients.

The experimental work described in this thesis was carried out in the Department of Medicine, University of Natal Medical School from December 1997 to December 2000 under the supervision of Prof Runjan Chetty. Genetic analysis was performed at the Benjamin Franklin University, Free University of Berlin by myself, under the supervision of Dr Arya Sharma.

During its planning and while the work was in progress, the following publications arose from this thesis:

1. Naidoo DP. Do ACE inhibitors have a role in atherosclerosis regression? Specialist Focus Journal 1996; 1(7): 12-19.

2. Naidoo DP. Diabetes and Coronary Heart Disease. Cardiovascular J of SA 2000; 11(3): 193-198.

Presentations at Congresses:

1. Naidoo DP. Large artery compliance in young myocardial infarct survivors. Faculty Research Day, 6 Sept 1999, University of Natal Medical School, Durban.

2. Naidoo DP, Sharma AM, Brand E, Ringel J, Baig J. Candidate gene polymorphisms in premature myocardial infarction. The 1st Congress of the SA Heart Association, 26th-29th November 2000, University of Stellenbosch.

3. Naidoo DP, Sharma AM, Brand E, Ringel J, Baig J. ACE gene polymorphism in premature myocardial infarction. The 1st Congress of the SA Heart Association, 26th-29th November 2000, University of Stellenbosch.

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ABBREVIATIONS

Apob	:	Apolipoprotein b
Apo a	:	apolipoprotein a
bp	:	base pair
a	:	adenine (purine base)
ACE	:	angiotensin-converting enzyme
Ala to val	:	alanine to valine
Alu	:	repetitive DNA sequence, about 300 bp long
c	:	cytosine (pyrimidine base)
g	:	guanine
t	:	thymine
CHD	:	coronary heart disease
FH	:	Familial hypercholesterolaemia
Lp*(a)*	:	lipoprotein a
LDL-C	:	low density lipoprotein cholesterol
MTHFR	:	methylenetetrahydrofolate reductase
PAI-1	:	plasminogen activator inhibitor-1
Hcy	:	homocysteine
TGF-β	:	transforming growth factor beta
Ang II	:	angiotensin II
Agt	:	angiotensinogen
AT_1	:	angiotensin II type I receptor

CYP11B2	:	aldosterone synthase
11β-HSD2	:	11-beta hydroxysteroid dehydrogenase 2
DD	:	pure deletion (homozygotes)
DNA	:	deoxyribonucleic acid
II	:	pure insertion (homozygotes)
ID	:	insertion-deletion (heterozygote)
EDTA	:	ethylenediaminetetra-acetic acid
Taq	:	thermophylic actinomyces aquaticus
BMI	:	body mass index (Kg/m^2)
BSA	:	body surface area (m^2)
DM	:	diabetes mellitus
E/A	:	Doppler e-wave to a-wave ratio
EDD	:	end-diastolic dimension
SWT	:	septal wall thickness
PWT	:	posterior wall thickness
GTT	:	glucose tolerance test
HDL-C	:	high density lipoprotein cholesterol
HbA1C	:	haemoglobin A1C
LVM	:	left ventricular mass
LVMI	:	left ventricular mass index
PWV	:	pulse wave velocity
RWT	:	relative wall thickness

MI	:	myocardial infarction
TC	:	total cholesterol
VLDL	:	very low density lipoprotein
VSMC	:	vascular smooth muscle cell
W/H	:	waist to hip ratio

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CHAPTER I
BACKGROUND

A) INTRODUCTION:

1. Pathogenesis of Coronary Artery Disease:

In recent years great strides have been made in elucidating the pathogenesis of coronary heart disease (CHD). Although the role of cardiovascular risk factors has emerged as central to the development of CHD (Kannel *et al,* 1976) it is now known that atherosclerosis is a disease of multiple aetiology resulting from a complex series of molecular and cellular changes that begin in the intima of the arterial wall. Furthermore, it is now recognised that the function and activity of cellular components within the plaque are a more important determinant of acute events (unstable angina, myocardial infarction) than the degree of stenosis of the arterial lesion (St Clair, 1997). Thus, while risk factors undoubtedly contribute to disease development, other trigger factors at a cellular level, precipitate acute coronary events in patients with atherosclerotic coronary disease.

Furthermore, differences in the prevalence of the established atherogenic cardiovascular risk factors such as hypertension, smoking and hypercholesterolaemia do not fully account for the variations in geographical prevalence, the severity of CHD, the age at presentation or ethnic predisposition to disease development (Whitty *et al,* 1999, Heller *et al*, 1984).

2. Epidemiology in South Asians

Of all ethnic groups people of Indian origin have been shown to have one of the highest mortality rates for CHD (Balarajan, 1991). CHD has now become a major

public health problem in South African Indians (Seedat *et al,* 1990, 1993). Local studies show that there is an alarming increase in the younger age group (20-39 years), affecting men more than women (Sewdarsen *et al,* 1990, 1991). Risk factors for CHD have been well documented in the South African Indian (Seedat *et al,* 1990, Sewdarsen *et al,* 1991). Smoking, diabetes and a family history have been identified as important risk factors in this community (Seedat *et al,* 1990). A prevalence rate of 15,3% for CHD has been linked to the presence of at least one major risk factor in half the number of patients. In addition, hypertension was present in 19% of a house-to-house survey of 1000 Indians and in another study almost half of 620 male survivors of myocardial infarction had abnormal cholesterol levels > 6.5 mmol/l (Sewdarsen *et al,* 1991). Smoking was the commonest risk factor (79%) in young males.

The rate of premature CHD has been documented to be up to 3 times higher in Indians when compared with subjects of similar age in the Western world (Lowry *et al,* 1991). Epidemiological observations suggest that environmental factors play a significant role in the pathogenesis of premature CHD in Indians (Rajadurai *et al,* 1992). Urbanisation as well as the westward migration of Indians appear to be associated with increased prevalence of CHD in Indians (Klatsky *et al,* 1993, Enas *et al*, 1992). A variety of environmental factors in the Western world might play a potential role in increasing the risk of CHD among the migrant Indians. It has been demonstrated that Indians living in the Western world have an increased incidence of obesity, diabetes, hypertension and emotional stress (McKeigue *et al,* 1991). Although the precise reasons for these metabolic abnormalities are not entirely clear it has been suggested that dietary habits (generally rich in calories/carbohydrates) in association with a lack of physical activity might be responsible for some of these findings.

Recent studies have demonstrated that serum HDL cholesterol levels are generally low and serum triglyceride levels are high among Indians with premature CHD

(Krishnaswami *et al,* 1989). There is evidence that these patients have insulin resistance which is manifest by the presence of hyperinsulinaemia (Seedat *et al,* 1993, Mckeigue *et al,* 1993, Bhatnager *et al,* 1995) glucose intolerance, and truncal obesity. In addition, some studies have suggested that increased levels of lipoprotein *(a)* [Lp*(a)*] might also play a role in premature CHD (Scanu, 1992, Lowry *et al*, 1991). A direct link between the metabolic abnormalities, the lipid levels and the increased risk of premature CHD in Indians, has not been established; and whether these abnormalities are genetically modulated has also not been shown.

3. Pathogenesis of Premature Coronary Atherosclerosis:

The hypothesis that atherosclerosis is a 'response to injury' is now generally accepted and can be summarised as follows:

a) Many factors damage and activate the endothelium of the vessel wall. These are the major risk factors for atherogenesis i.e. hyperlipidaemia, hypertension, diabetes, smoking and ischaemia.

b) Endothelial dysfunction leads to increased permeability so that lipids (low density lipoprotein particles) and circulating cells (monocytes and lymphocytes) enter the subendothelial space and form the initial characteristic lesion of atherosclerosis, the fatty streak.

c) As cell numbers increase together with lipid accumulation there is increased endothelial disruption leading to thrombogenic surfaces to which platelets adhere.

d) The cellular elements within the plaque can themselves release growth modulatory factors leading to proliferation of smooth muscle cells and

fibroblasts (fibrous plaque) and finally to the complex advanced lesion - the atherosclerotic plaque. Ross (1993) has described atherosclerosis as an 'inflammatory fibroproliferative process'. It is thought that lesions with large amounts of lipid are particularly unstable and liable to rupture leading to thrombosis and vessel occlusion, causing ischaemia and manifesting as a clinical event, the acute coronary syndrome (ACS) (Fuster *et al*, 1999). These syndromes (ACS) include unstable angina, non-q wave myocardial infarction, ST-segment elevation, myocardial infarction and sudden death.

Unstable angina and non-q wave MI generally result from rupture of atherosclerotic plaque in the coronary arteries. The term, acute coronary syndrome, is therefore a misnomer, when one considers that the process is a chronic syndrome with exacerbations leading to the formation of occlusive thrombus on minor as well as on critically stenotic lesions. Such episodes are temporary or clinically silent but incorporation of thrombus contributes to the development of a complex plaque. In q wave myocardial infarction larger plaque fissures result in the formation of a persistent thrombus. Abrupt cessation of blood flow for more than an hour results in transmural recrosis (Fuster *et al*, 1999). Myocardial infarction therefore usually results from thrombotic events triggered by rupture of advanced atherosclerotic lesions. The composition of a plaque influences the likelihood of rupture, as fatty lesions are more likely to rupture than more fibrous lesions. Rupture frequently occurs at edges of these lesion, which are fatty in nature and contain foam cells, suggesting that some of the same factors that contribute to early atherosclerosis may also promote rupture (Fuster *et al*, 1990). Thus, both local and systematic thrombogenic factors may influence the degree and duration of thrombus. In addition to the degree of plaque disruption and the severity of stenosis at the site of injury, smoking, level of cholesterol, Lp(*a*), fibrinogen, PAI-I, factors VII and von Willebrand factor contribute to vasoconstriction and

thrombotic complications (Koenig, 1998).

Although the process of atherosclerosis is the same, little is known about the pathogenesis in patients who develop atherosclerosis and premature CHD at a young age (less than 55 years). Evaluation of major coronary risk factors in Indian patients undergoing coronary arteriography has shown that more than one third of patients with CHD have few or no major risk factors (Kaul *et al*, 1986). There is a relatively low prevalence of traditional coronary risk factors such as high cholesterol levels, cigarette smoking and hypertension in patients with premature CHD (Krishnaswami *et al*, 1989, Enas *et al*, 1992, Klatsky *et al*, 1993, Stampfer *et al*, 1991). A review of the family history in South Africans with CHD has indeed suggested that there may be a strong genetic element in risk predisposition (Seedat *et al*, 1996). In contrast, HLA typing has shown no evidence of genetic susceptibility in this ethnic group (Sewdarsen *et al*, 1987). Whether factors other than environmental and/or metabolic abnormalities predispose to early onset of atherosclerosis and to what extent genetic factors interact with these stimuli are not known.

Among genes likely to influence the composition of atherosclerotic plaques are those involved in matrix production, lipid metabolism, as well as the coagulation and fibrinolytic pathways. Lesion stability may also be influenced by haemorrhage from small vessels that grow into the lesions from the media and factors contributing to coagulation influence thrombus formation. Variations of haemostatic factors, such as fibrinogen, PAI-I and factors XIII have been associated with CHD (Koenig, 1998). In the past few years, evidence has emerged that angiotensin II plays a significant role in atherogenesis and interacts with these pathways at various levels in the pathological process ranging from the influence of risk factors to haemodynamic changes and alterations in tissue metabolism and endothelial function (Fig 1). (see 1.2) Early onset of disease with such a multifactorial

aetiology is suggestive of a strong genetic component that may become unmasked in the presence of appropriate environmental stimuli. Thus, the documented high incidence of early CHD in the migrant Indian population might indicate a greater degree of genetic predisposition among them as compared to Europeans. The genetic distance between these two ethnic groups makes the presence of differential disease-allele frequencies plausible, supporting the postulate that genetic differences contribute to the observed differences in disease patterns among Indians and Europeans.

B) EPIDEMIOLOGICAL EVIDENCE OF HERITABILITY OF CARDIOVASCULAR DISEASE

1. The Framingham Heart Study:

In the Framingham Heart Study, death due to CHD in parents was associated with a 30% increase in the risk of coronary artery disease in the offspring (Myers *et al,* 1990). This effect was stronger for early onset of coronary artery disease with age adjusted relative risks of 1.5 for early (occurring at under 60 years of age) and 1.2 for late (in patients older than 60 years) coronary artery disease. No evidence was found for a significant interaction between any of the known risk factors and family history of CHD. In examining persons with a low risk for coronary artery disease as suggested by their risk factor profile (normotensive, non-smoking, non-obese individuals), more than two-thirds of those who experienced coronary artery disease were found to have a positive family history. These findings suggest that primary genetic factors may play a significant role in the pathogenesis of CHD.

2. Segregation studies:

The observation of a familial aggregation of a number of cardiovascular diseases, such as CHD (Schildkraut *et al,* 1989), hypertension (de Faire *et al,* 1975) and left ventricular hypertrophy (Beilen *et al,* 1990) lends support to the role of primary genetic factors in the pathogenesis of cardiovascular disease. A "positive family history" may be the result of shared genes: studies among monozygotic and dizygotic twins and adaptation studies comparing incidence among natural versus adopted siblings of parent-child pairs have consistently shown a greater degree of disease concordance among more closely genetically related individuals, thus distinguishing the effects of genetic and

environmental variables, and supporting the role of hereditary factors (de Faire *et al,* 1975, Feinleib *et al*, 1977). In addition, a number of segregation studies in a large number of families have yielded additional evidence for the importance of genetic predisposition in the occurrence of cardiovascular disease.

C) GENETIC STUDIES OF HERITABILITY

Coronary heart disease shows strong family aggregation, especially when it presents at an early age and when many relatives are affected. Several cardiovascular sequelae including left ventricular hypertrophy, are potentially shared by family members of affected individuals (Schunkert *et al,* 1994). Because familial clustering of risk factors is insufficient to explain the high risk of disease in a sibling of an affected parent, genetic factors are strongly suspected to play a significant role in susceptibility to premature CHD.

1.1 Monogenic Disorders:

As important as the established risk factors are for predicting disease, only a fraction of those subjects who have one or more of these risk factors will actually develop a cardiovascular event; even some with no known factors will experience an event. Epidemiological studies suggest that CHD may have an environmental as well as a genetic basis, which in the latter instance does not follow simple Mendelian patterns of inheritance. Very little is known thus far about the actual genes, or their allelic variants that contribute to the pathogenesis of CHD. In only a small proportion of cases do rare monogenic disorders account for the expression of disease, e.g. familial hypercholesterolaemia (Brown *et al,* 1986).

Although the dyslipidaemias form a well known risk factor for CHD, only a small percentage of CHD patients have recognised mutations in lipoproteins or

their related genes. In the South African Afrikaner, familial hypercholesterolaemia (FH) is highly prevalent and has been diagnosed to be due largely to the LDL receptor gene defect (Kotze *et al,* 1991, Rubinsztein, 1994). Mutations in the LDL-C receptor gene produce familial hypercholesterolaemia; an autosomal codominant disorder characterised by raised plasma LDL cholesterol (LDL-C), tendon xanthomas and premature CHD.

CpG hotspot mutations at the LDL receptor locus have been described in South African Indians (Kotze *et al*, 1997) but the majority of premature CHD sufferers do not have this clinical picture, suggesting some other mode of a genetically determined predisposition to atherosclerosis, probably acting at a cellular level in the intima of the vessel wall.

1.2 Renin-Angiotensin System:

1.2.1 Role of the Renin-angiotensin System *(RAS)* in the Pathogenesis of Atherosclerosis:

Since hypertension is a major risk factor for CHD, (Kannel *et al*, 1976) attention has been focussed on the *RAS* and its major component angiotensin II, a potent vasoconstrictor and mediator of vascular events, in the pathogenesis of CHD. The activity of the *RAS* depends on the availability of angiotensin, renin, angiotensin converting enzyme *(ACE)*, and *Ang II* receptor activity. Angiotensin converting enzyme generates the vasoactive *Ang II* and inactivates bradykinin. It is widely distributed in endothelial and epithelial cells. Each of these components of the *RAS* may play a contributory role in disease pathogenesis.

Alderman *et al* (1991) followed up 1717 subjects with mild-moderate hypertension for 8,3 years and provided the best epidemiological evidence for the link between the *RAS* and the risk of subsequent myocardial infarction.

They showed that the risk of MI was increased over 5 fold among subjects with high- compared to those with low-renin profiles, and this effect was independent of other cardiovascular risk factors. This relationship between plasma renin and risk for myocardial infarction has not been shown in normotensive subjects (Meade *et al*, 1993).

The *RAS* may contribute to the process of atherogenesis via several mechanisms. *Angiotensin II*, an octapeptide, represents the biologically active product of the *RAS*. It is generated by precursors, which are cleared by enzymes. Besides its involvement in blood pressure regulation, *Ang II* acts as a growth factor and contributes to vascular remodelling in most vascular organs, including the heart itself. There is experimental evidence that local *Ang II* formation is increased in atherosclerotic lesions, and that chymase is primarily responsible for this increase (Ihara *et al,* 1999). Angiotensin has also been shown to play an important role in regulating the production of plasminogen activator inhibitor-1 (PAI-1) (Vaughan *et al*, 1995, van Leeuwen *et al,* 1994). This biological role of angiotensin suggests that regulatory changes in the *RAS* may also influence the development of thrombosis in subjects with coronary atherosclerosis.

There is also increased expression of *ACE* in atherosclerotic lesions, leading to local production of *Ang II* and ultimately stimulation of vascular superoxide production (Diet *et al,* 1996). These findings suggest a pathogenetic role for the *RAS* in the early stages of atherosclerosis. This hypothesis is supported by the fact that *ACE* inhibition has been shown to promote regression and even prevention of atherosclerosis (Becker *et al*, 1991). Recently, Mancini *et al* (1996) showed that patients with coronary artery disease treated with an *ACE* inhibitor developed markedly improved coronary vasomotor function. This study, the Trial on Reversing Endothelial Dysfunction *(TREND)* demonstrated that quinapril 40mg/daily given for six months improved acetylcholine - provoked vasoconstriction in patients with CHD. There is now substantial data

from experimental and clinical trials to support the role for *ACE* inhibition retarding atherosclerosis at a tissue level (Naidoo, 1996). There is also evidence of a link between the *RAS* and lipid levels in vascular biology. Hypercholesterolaemia is associated with AT_1 receptor upregulation and increased vascular production of superoxide, secondary to activation of vascular NADH oxidase. This results in attenuation of endothelium-dependent coronary vasodilatation which is demonstrable early in the atherosclerotic process (Zeiher *et al,* 1994). AT_1 receptor blockade has been shown to normalise the activity of NADH oxidase, reduce plaque area and macrophage infiltration and in parallel improve endothelial function (Warnholtz *et al,* 1999).

These data suggest that: the *RAS* may be playing an important role in both the initiation and acceleration of the atherosclerotic process.

1.2.2 Gene Polymorphism and the *RAS*:

Allelic association studies have suggested that polymorphism in genes involved in lipid metabolism, coagulative and fibrinolytic pathways, as well as the renin-angiotensin system may be associated with coronary atherosclerosis. The role of polymorphisms in genes that regulate the *RAS* in the pathogenesis of CHD has recently come under scrutiny (Tiret *et al* 1995, Beohar *et al*, 1995, Arbustini *et al* 1995, Ohishi *et al,* 1993). Although the genetic basis of CHD is still unfolding, many studies have suggested several putative genes (Table 1). A widely studied example is the angiotensin converting enzyme (*ACE*) gene on chromosome 17.

1.2.2.1 *ACE* Gene Polymorphism:

Recently an insertion/deletion polymorphism has been described which is based on the presence (insertion: I) or absence (deletion: D) of a 287-base pair

alu-repeat DNA domain, resulting in three genotypes: homozygous *(DD)* heterozygous *(ID)* and homozygous *(II)*. This polymorphism is codominantly associated with the plasma *ACE* activity, with the *DD* genotype having about double the value of the *II* genotypes (Rigat *et al,* 1990). Both circulating (Rigat *et al,* 1990) and local cardiac (Danser *et al,* 1995) activity of *ACE* are higher in the presence of the deletion polymorphism, which might increase the conversion of *Ang I* to the highly active *Ang II*. Local *ACE* activity could thus promote the development and progression of atherosclerotic plaque in the vessel wall. *ACE* could be involved in the pathogenesis of atherosclerosis by several biological mechanisms. In addition to activating *Ang I* (resulting in decreased tissue perfusion), *Ang II* is involved in vascular smooth muscle cell growth (Daemen *et al,* 1991) and the stimulation of plasmogen-activator inhibitor type I (Ridker *et al,* 1993).

Since Rigat *et al* (1992) identified an insertion/deletion *(I/D)* polymorphism within intron 16 of the *ACE* gene, several studies have reported an association between the *D* allele and an increased risk for coronary artery disease, myocardial infarction, (Cambien *et al*, 1992) restenosis after angioplasty (Kaski, 1994 Hamon *et al,* 1998), ischaemic and dilated cardiomyopathy (Raynolds *et al,* 1993) as well as left ventricular hypertrophy (Iwai, 1994, Schunkert *et al,* 1994).

Cambien *et al* (1992) was the first to report the association of the *D* allele for an increased risk of MI, especially in a low risk group defined by body mass index and serum apo B levels. It is possible that the effect of the *ACE DD* genotype is strongest when no other causative factors are present. In a large case-controlled study Etude Cas-Temoins sur L`Infarctus du Myocarde (ECTIM) an increased risk of MI and an increased frequency of parental MI was demonstrated in subjects carrying the D allele. In this study the relationship between the *ACE DD* genotype and MI was much stronger in patients *at low risk* of MI than high risk subjects (Tiret *et al,* 1995). (refer to

4.2.2).

1.2.2.2 A*CE* Gene Polymorphism and Premature Atherosclerosis:

Variations in the *ACE* gene have also been associated with early changes of carotid atherosclerosis (Kauma *et al,* 1996). The gene effect, however, may be masked by stronger effects of environmental factors such as smoking. The *D* allele has been shown to be a risk factor for MI (Cambien *et al,* 1992, Mattu *et al,* 1995), even in patients with non-insulin dependent diabetes mellitus (Ruiz *et al,* 1994). Angiotensin converting enzyme gene polymorphism has also been shown to be associated with a parental history of MI (Tiret *et al,* 1993, Badenhop *et al,* 1995) and may explain the premature development of CHD and MI in low risk patients (Gardemann *et al,* 1995, Evans *et al,* 1994, Ludwig *et al*, 1995, Takahashi *et al,* 1995). The underlying mechanism maybe related to impaired endothelial function since increased coronary vasomotor tone has been demonstrated in patients with CHD who possess the D allele (Prasad *et al*, 2000).

1.2.3 Angiotensinogen (*Agt*) Gene:

1.2.3.1 *Agt* M235T Polymorphism:

Recent studies suggest that the *M235T* variant of the *Agt* gene is associated with the development of hypertension in Caucasians. This mutation consists of a nucleotide transition in exon 2 leading to an exchange of methioine for threonine in the *M235* position, and is linked to a family history and an early onset of hypertension (Winkelmann *et al,* 1996). It was found to be more frequent in hypertensive patients in studies in Utah and in Paris, (Jeunemaitre *et al*, 1992) and correlated with arterial wall thickness (Castellano *et al,* 1995) measurements. It has been estimated that mutations at the *Agt* locus might be a

predisposing factor in 3-6% of hypertensive individuals younger than 60 years of age (Jeunemaitre *et al*, 1992). Although a large European study has not shown any evidence of linkage in 630 affected sibpairs, we decided to evaluate polymorphisms at the *Agt* locus because frequency of the *Agt T235* allele varies strongly according to ethnic groups. It is more frequent in the Asian population (about 0.75) than in the White population (about 0.40) (Niu *et al*, 1998) and is associated with early onset of familial hypertension (Schimidt *et al*, 1995). Furthermore, Shunkert *et al*, (1997) found that the *T235* allele was associated with higher systolic and diastolic blood pressure. In a meta-anlysis involving 5493 patients Kunz *et al*, (1997) showed that the *Agt T235* allele was significantly associated with hypertension, mainly in studies with a positive family history of hypertension and that the contribution of the *T235* allele may also be dependent on interacting risk factors such as gender, BMI, and menopause. The *Agt M235T* polymorphism has also been shown to be associated with CHD, both independently (Katsuya *et al,* 1995) and also synergistically with the *ACE* insertion-deletion *(I/D)* polymorphism (Kamitani *et al,* 1995).

1.2.4 Core Promoter Element of *Agt* Gene:

In 1997, Inoue *et al* identified a common variant in the proximal promoter of the *Agt* gene: the presence of adenine, instead of guanine *(G-6A)* 6 bp upstream from the initiation site of transcription. The *G→A* substitution located at position *–6* base region upstream of the initial transcription site is of the same frequency and in almost complete linkage disequilibrium with the *T235* allele (Jeunemaitre *et al*, 1997). After an extensive investigation Jeunemaitre *et al* (1997) concluded that, with the exception of *G-6A*, no other known biallelic polymorphism is associated with hypertension. Also, he showed that the *G* and *A* alleles are synonymous with the *M235T M* and *T* alleles respectively.

Since tests of promoter activity showed that *G-6A* nucleotide substitution affects the basal transcription rate of the gene and is in very tight linkage disequilibrium with *M235T*, it marks the original form (in evolutionary terms) of the gene. Both polymorphisms (*M235T* and the *G-6A*) are associated with an increased plasma angiotensinogen level, (Jeunemaitre *et al*, 1992) which could result in an increased formation rate of *Ang II*, especially in tissues where these proteins are rate limiting for *Ang II* generation. Since variants in the *Agt* gene have been associated with an increased risk of CHD (Katsuya *et al*, 1995, Ishigami *et al*, 1995, Kamitani *et al*, 1995) we investigated the *G-6A* polymorphism in the core promoter region of the *Agt* gene in patients with premature MI.

1.2.5 Angiotensin II Type I Receptor *(AT_1R)* Polymorphism:

Angiotensin II is the biological active peptide of the *RAS* that produces vasoconstriction, release of aldosterone and catecholamines as well as the development of cardiac hypertrophy. Most of the actions of *Ang II* are mediated through the angiotensin II subtype I receptors *(AT_1)* (Goodfriend *et al*, 1996).

This receptor is the main effector of the *RAS* and mediates the growth-promoting effect of *Ang II* in humans (Goodfriend *et al,* 1996).

It is now known that several pathways exist for the production of *Ang II* in addition to the effects of *ACE* on *Ang I*. Since the main effector of the *RAS* is the *AT_1* receptor, which also mediates the growth promoting effect of *Ang II* in humans, variations in the gene for the *AT_1* receptor may account for a variable response to *Ang II* stimulation and its possible interaction with risk factors. Little is known about the effect of major risk factors such as hypercholesterolaemia on *RAS* gene expression. The interaction between

lipoproteins and the *RAS* does have some pathogenetic significance and may explain why ACE inhibitors attenuate hypercholesterolaemia – induced atherosclerosis in animal models (Chobanian *et al*, 1990). This may have a genetic basis since hypercholesterolaemia has been shown to enhance AT_1 receptor gene expression and in this way it accentuates the vasoconstrictive effect of *Ang II* (Nickenig *et al*, 1997). More recently macrophages trapped in the vessel wall have been shown to upregulate AT_1 receptors and contribute to increased production of reactive oxygen radicals (Yanagitani *et al*, 1999). The resultant effect of *Ang II* will therefore be instability of the atherosclerotic plaque leading to plaque rupture.

The gene for the human AT_1 receptor has recently been cloned and sequenced (Furuta *et al,* 1992). Several biallelic polymorphisms have been detected in the coding and 3′ untranslated regions of this gene (Bonnardeaux *et al,* 1994). Of these, the *A1166C* gene polymorphism has been shown to be associated with resistant essential hypertension and synergistically increases the effect of *ACE* genotype on the risk of MI (Szombathy *et al,* 1998). A lower frequency of the *C* allele has been reported in patients with CHD and hypertension (Saku *et al,* 1998).

It is possible that AT_1 receptor polymorphism may cause alterations in the activity of this receptor and in this way contribute to a more pronounced potentiation of the effects *Ang II*, explaining the strong cardiovascular risk associated with hypertension (Graves *et al*, 1992). There is also evidence that the AT_1 receptor is a risk marker for arterial stiffness and could modulate the effects of hypertension, aging, and lipids on large arteries (Benetos *et al*, 1995). It has also been associated with decreased aortic wall compliance and could modulate the effect of lipids on large arteries (Benetos *et al*, 1995, Kauma *et al*, 1996). Blockade of this receptor has been shown to correct the endothelial dysfunction of peripheral resistance arteries from hypertensive

patients (Schiffrin *et al*, 1999).

We therefore investigated the role of a common polymorphism *(A1166C)* of the AT_1 receptor gene (A→C transition at position 1166) in our patients. Although this polymorphism is not functional it might be in linkage disequilibrium with an unidentified variant and may reflect a relationship between this variant and the risk of premature MI.

1.2.6 Aldosterone Synthase:

The trophic effects of neurohormones modulate cardiac growth and function. Angiotensin *II* contributes to an increase in LV mass by promoting myocyte growth as well as by stimulating vascular smooth muscle cell growth and proliferation. In addition, increase in myocardial collagen may occur via aldosterone activation (Weber *et al*, 1991). Thus, the combined effect of the renin-angiotensin-aldosterone system is to increase LV mass through several mechanisms. Angiotensin II is a potent fibrogenic factor (Brilla *et al*, 1993). Besides its well-known effect of stimulating aldosterone production from the adrenal cortex, a reciprocal interaction between the hormones has been reported. For instance, an increase in *Ang II* receptor density has been observed in the heart of aldosterone salt-treated rats. (Sun *et al*, 1998) There is also experimental evidence that aldosterone - salt induces cardiac fibrosis through *Ang II* acting on the AT_1 receptor (Roberts *et al*, 1988).

In addition to regulating renal sodium reabsorption and thus intravascular volume, aldosterone may have direct effects on the cardiovascular system. It has been shown to stimulate the growth of cardiac myocytes and accumulation of extracellular matrix proteins (Weber *et al*, 1991). Angiotensin *II* is also an important regulator of noradrenaline release from sympathetic nerve endings and may modulate cardiac sympathetic activity and ischaemic events

(Zimmerman *et al*, 1995). This may explain the salutary effects of the *ACE* inhibitors as well as aldosterone antagonists in reducing event rates in heart failure studies (Lonn *et al*, 1994). These data support a role for all components of the renin-angiotensin system in the pathogenesis of CHD and as trigger factors for cardiovascular complications and event rates.

The genes encoding aldosterone synthase *(CYP11B2)* and 11β-hydroxylase are very similar at the nucleotide level, sharing 95% homology (Fisher *et al*, 1998). Altered activity of these enzymes may be important in the pathogenesis of hypertension and atherosclerosis. Genetic variations within or near the aldosterone synthase gene are associated with aldosterone and 11-deoxycortisol production in males. This may modulate the activity of the *RAS* and thereby contribute to blood pressure regulation. Two biallelic polymorphisms, one in the promoter *(C344T)* and the other in the second intron have been identified in the gene encoding aldosterone synthase, the enzyme catalyzing the steps of aldosterone biosynthesis (White *et al*, 1995).
Preliminary data suggests that genetic variations in *CYP11B2* are associated with variation in baroreflex sensitivity. The *TT* genotype was strongly associated with baroreflex sensitivity as measured by the Valsalva manouevre (Yitalo *et al*, 1998). Amongst the genetic loci studied we therefore included polymorphism in the promoter region of the aldosterone synthase gene which is known to affect the binding of the steroidogenic factor-transcription factor and so influence gene expression. This pathway may not only explain the direct effects of aldosterone on the cardiovascular system, but also support the association between renin levels and MI (Alderman *et al*, 1991).
Aberrations in these genes can lead to changes in arterial pressure and are responsible for a monogenically inherited form of hypertension (Fisher *et al*, 1998). There is evidence that a polymorphism *(C344T)* in the promoter of the *CYP11B2* gene is strongly associated with left ventricular size and decreased

baroreceptor sensitivity in healthy individuals (White *et al*, 1998). This polymorphism may be associated with an increased risk of MI in dyslipidaemic patients (White *et al*, 1998). As altered activity of *CYP11B2* has been proposed as an intermediate phenotype in essential hypertension, we also evaluated gene polymorphism in this gene in our subjects.

1.2.7 11β-Hydroxysteroid Dehydrogenase 2 Polymorphism:

Aldosterone exerts its effects through the binding and activation of the mineralocorticiod receptor (White, 1998). Since cortisol binds to this receptor with the same affinity as aldosterone the enzymatic conversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase 2 *(11BHSD2)* ensures that aldosterone gains access to this receptor.

The gene encoding this enzyme is located on chromosome 16q22. Mutations in the *11BHSD2* gene results in loss of the activity of the encoded enzyme, causing severe hypertension due to mineralocorticoid excess (Stewart *et al*, 1996). Therefore, anomalies in either of the genes encoding the enzymes *11BHSD2* and *CYP11B2* can lead to important changes in arterial pressure and are responsible for several monogenically inherited forms of hypertension (Ulick *et al*, 1979, Mune *et al*, 1995) but these are rare.

A single such polymorphism has been identified in exon 3 of the $11\beta HSD_2$ gene characterised by the GAG to GAA transition at codon 178 (Smolenicka *et al*, 1998, Brand *et al*, 1998). Since common polymorphisms in these genes or their regulatory regions could contribute to genetic variation in the susceptibility to hypertension and its cardiovascular sequelae, we investigated this polymorphism in patients with premature myocardial infarction.

1.2.7.1 Other candidate genes:

The interplay between gene polymorphism and newly identified risk factors for CHD such as hyperinsulinaemia (Despres *et al*, 1996) and abnormalities in apolipoprotein metabolism may also contribute to premature coronary atherosclerosis (Tas *et al*, 1994, van Bockxmeer *et al*, 1992). Therefore, extending the genetic analyses to other candidate genes may explain how insulin and fibrinogen contribute to a procoagulant state, (Robinson *et al*, 1994, Akimova *et al*, 1994) impaired fibrinolysis and altered levels of growth factors and in this way predispose to early onset CHD. Among the candidate genes that have been investigated to date, gene polymorphisms in homocysteine metabolism, *G*-protein and *TGF-β* may provide supportive evidence for a genetic basis to transcriptional changes predisposing to atherosclerosis (Cambien *et al*, 1996).

1.2.8 Homocysteine and CHD:

The sulphur-containing amino acid homocysteine is formed during the metabolism of methionine. Methylenetetrahydrofolate Reductase *(MTHFR)* catalyses the reduction of 5,10-methylenetetrahydrofolate to 5, methylenetetrahydrofolate. This is the predominant circulating form of folate and is a methyl donor for the remethylation of homocysteine to methionine. Reductase activity of *MTHFR* or defects in the *MTHFR* gene itself may lead to raised homocysteine levels.

Homocysteine concentration is elevated in up to 30% of patients with atherosclerosis (Clarke *et al*, 1991). Hyperhomocysteinaemia is known to be an independent risk factor for the development of CHD, (Clarke *et al*, 1991, Murphy-Chutorian *et al*, 1994, Arnesen *et al*, 1995, Robinson, 1994) MI, (Stampfer *et al*, 1992) particularly in young women (Schwartz *et al*, 1997), cerebrovascular disease and venous thrombosis. The serum homocysteine

level is influenced by environmental as well as genetic factors. A high plasma concentration of homocysteine may predispose to atherosclerosis by injuring the vascular endothelium. In a meta-analysis Blom *et al* (1991) estimated that the risk associated with a 5 μmol/L elevation in total plasma homocysteine was equivalent to a 0.5 mmol/L rise in the serum cholesterol. Levels only 12% above the upper limits of normal (15 μmol/L - mild hyperhomocysteinaemia) are associated with a three-fold increase in MI (Nygard *et al*, 1997).

The exact mechanisms by which raised homocysteine levels promote atherosclerosis are not clear but postulates include endothelial damage (Woo *et al*, 1997), platelet dysfunction, (Lentz *et al*, 1991) and LDL-C oxidation (Reinhardt *et al*, 1998). Acute elevation in homocysteine concentration, similar to levels found in MI, is associated with endothelial dysfunction (Chambers *et al*, 1999), and can be prevented by pre-treatment with vitamin C suggesting that the effects are mediated via oxidative stress.

A further report (Tsai *et al*, 1996) has shown that homocysteine inhibits endothelial cell proliferation and stimulates vascular smooth muscle cell proliferation. In addition homocysteine enhances endothelial cell-associated factor V activity (Rodger *et al*, 1986) and inhibits thrombomodulin surface expression (Lentz *et al*, 1991), protein C activation (Rodger *et al*, 1990) and tissue plasminogen activator binding (Hajjar, 1993). These findings suggest that homocysteine may be involved in the thrombotic and atherosclerotic process by promoting prothrombotic activities as well as modulating vascular cell proliferation in the vessel wall.

Homocysteine concentrations are determined to some extent by genetic factors. Genetic aberration in the gene coding for 5,10-methylenetethydrofolate reductase *(MTHFR)* may account for reduced enzyme activity and elevated plasma homocysteine level. (Kang *et al*, 1991). Reduced activity of *MTHFR* and elevated plasma homocysteine levels may be seen in individuals with a

thermolabile variant of *MTHFR*. This phenotype results from a C to T mutation at nucleotide position 677 in the cDNA (Goyette *et al*, 1995). It predicts the replacement of 222 Ala by Val and reduces the basal activity of the enzyme by 50%, leading to decreased thermostability in homozygote individuals (Frosst *et al*, 1995). This mutation is frequent: about 12% of Caucasians are mutant homozygotes. They have lower mean serum folate levels and 25% higher plasma homocysteine concentrations than normal *CC* homozygotes. Up to 17% of patients with CHD are reported to express the thermolabile phenotype.

Since the effects of high plasma homocysteine levels have not been shown to be mediated by the known coronary risk factors (Tonstad, 1997), we hypothesized that the *MTHFR* mutation might be a candidate gene for coronary heart disease, particularly in younger patients with few risk factors.

1.2.9 Transforming Growth Factor Beta *(TGF-β)*:

Excessive proliferation and migration of several vascular cell types (e.g. vascular smooth muscle cells, endothelial cells, myofibroblasts) are important components of the vascular remodelling and the atherosclerotic processes (Ross, 1993, Schwarz *et al*, 1997). In the normal artery cell growth and migration are closely regulated processes: there is a balance of paracrine growth factors, vasoactive factors and circulating hormones that control tissue homeostasis. For instance, endothelial cells produce nitric oxide and other factors that reduce smooth muscle cell proliferation. When the endothelium is damaged, the expression of these factors is altered resulting in enhanced proliferation and/or migration of vascular smooth muscle cells which contribute to neointimal proliferation and the atherosclerotic process.

Transforming growth factor-β is a member of a larger family of growth factors

and cytokines and has a wide spectrum of biological activities (Wahl, 1994) which include regulation of cell growth, differentiation and matrix production (Roberts *et al*, 1988). It is secreted by peripheral blood monocytes, endothelial cells and vascular muscle cells, and induces fibrosis in the kidney, heart and blood vessels (Border *et al*, 1994).

In animal models angiotensin *II* has been shown to act by the induction of proto-oncogenes *efos*, *c-myc* and *c-jun*, and induces expression of several growth factor genes, (Campbell *et al*, 1997, Lonn *et al*, 1994) including the genes encoding for *PDGF* and *TGF-β*. Early activation of these proto-oncogenes followed by sequential activation of growth factor genes ultimately result in vascular smooth muscle cell growth. In addition to these trophic effects *Ang II* has been shown to release an endothelial neutrophil chemoattractant, leading to neutrophil accumulation. All these mechanisms probably contribute to the genesis and progression of the atherosclerotic plaque as well as endothelial function (Bell *et al*, 1990).

At a cellular level the trophic effects of the *Ang II* on smooth muscle cells of blood vessels are therefore determined by the expression of *TGF-β_1* (Gibbon *et al*, 1992, Campbell *et al*, 1997). Elevated plasma levels of *TGF-β_1* have been described in diseases that involve modification of extracellular matrix particularly Type II diabetes mellitus (Pfeiffer *et al*, 1996). Furthermore, several *G*-protein coupled receptor agonists including *Ang II* and α-thrombin are potent mitogens that induce extracellular matrix formation (Weber *et al*, 1994). Data also exists that *TGF-β_1* and *Ang II* regulate the expression of each other (Border *et al*, 1989). Overproduction of *TGF-β_1* mediated in part by *Ang II*, has been linked to LVH and vascular remodelling in hypertension (Villareal *et al*, 1992, Sarzani *et al*, 1989). Recent evidence suggests that *TGF-β_1* is a key inhibitor of atherogenesis and increased plasma levels of *TGF-β_1* are seen in early atherosclerosis but levels are severely depressed in the advanced stages of the disease (Blann *et al*, 1996).

Transforming growth factor-β is therefore a multi-functional cytokine which regulates cell proliferation and is a potent inducer of extracellular matrix production and fibrogenesis. There is experimental evidence that proteoglycan accumulation in atherosclerotic lesions depends on the proximity of platelet derived growth factor *(PDGF)* and *TGF-β* (Evanko *et al*, 1998). Its exact role in human atherosclerosis is unknown but elevated plasma levels have been found in type II diabetes mellitus, correlating with retinopathy, neuropathy and HbA1C levels. Three mutually compatible hypotheses have been put forward to explain the development of atherosclerotic lesions. The 'response to retention' hypothesis emphasizes the importance of atherogenic lipoprotein accumulation in the sub-endothelial region (Williams *et al*, 1995), whereas the oxidation hypothesis (Witztum, 1994) emphasises the role of the oxidative process in the recruitment of macrophages. The 'response to injury' hypothesis proposes vascular smooth muscle cell proliferation as the key event in the formation and progression of the atherosclerotic lesion (Ross, 1993). Transforming growth factor-β_1 could be the missing link that promotes lipid accumulation through increases in proteoglycan synthesis and deposition in the fatty intima. In the human aorta *TGF-β* participates predominantly in the pathogenesis of the lipid-rich atherosclerotic lesion and contributes to specific stages of lesion progression. Studies of human aorta atherosclerotic plaque and restenotic lesions after angioplasty have documented *TGF-β_1* expression by vascular cells within the lesions. Similarly, *TGF-β_1* expression within the vasculature is upregulated in the context of diabetes and hypertension in animal models of vascular disease (Han *et al*, 1995). Transforming growth factor-β_1 in the arterial wall promotes vascular cell differentiation, vascular wall remodelling, arterial lesion growth and regression through apoptotic mechanisms (Schulick *et al*, 1998). It is postulated that vascular structure and lesion formation is determined in part by a balance between cell proliferation

and cell death and by apoptosis (Isner *et al*, 1995, Geng *et al*, 1995, Han *et al*, 1995). Transforming growth factor-β is an inducer of endothelial cell death and it also prevents vascular smooth muscle cell death. All evidence points to it being a proatherogenic cytokine (Bobik *et al*, 1999).

Furthermore *βeta ig-h3* a *TGF-β1*-inducible gene, is over-expressed in ig-h_3 restenotic human vascular lesions (O`Brien *et al*, 1996). Recent evidence suggests that genetic factors may play a role in *TGF-β1* expression, the gene for which is located on chromosome 19q3 and comprises seven exons, of which exon 5, 6 and 7 encode the active *TGF-β1*. Since five polymorphisms have been identified in the TGF-β_1 gene we hypothesized that gene polymorphism might regulate the expression of *TGF-β1* and therefore studied *Thr 263* Ile polymorphism in patients with premature MI. This gene mutation, a *C* to *T* transition at position 76 in exon 5 results in a change from threonine to isoleucine in position 263 *(Thr 263 Ile)* of the propeptide.

1.2.10 *G*-Protein Polymorphism:

In addition to the regulatory effect of *RAS* genes in blood pressure homeostasis and vascular remodelling, genes encoding the components of the transport system that regulate salt and water homeostasis are also potential candidates for influencing blood pressure.

The pH-regulating ion transport system (Na^+/H^+ exchanger [NHE]) which swaps extracellular Na^+ for intracellular H^+ is one such system. Of the five isoforms have been isolated in human tissues one has been associated with hypertension but variations in its transcripts have not been detected (Diez *et al*, 1995).

Since cell lines that display enhanced NHE-1 activity have also shown

increased activity of pertussis toxin-sensitive *G*-proteins, Siffert *et al*, (1998) have located a C → T polymorphism at position 825 of cDNA that encodes the β_3 subunit of the pertussis-toxin-sensitive *G*-protein *($G\beta_3$)*. There is preliminary data to suggest that the response to α_2-adrenergic stimulation in the coronary arteries is genetically determined: patients with *$G\beta_3$ T825* allele had augmented flow reduction compared to *CC* homozygotes (Baumgart *et al*, 1998). The *825T* allele was significantly associated with hypertension. Since hypertension is a major risk factor for CHD we extended our genetic analysis to include *$G\beta_3$* subunit as a candidate gene related to NHE activity and salt and water homeostasis on the basis that enhanced intracellular signal transduction may contribute to premature CHD.

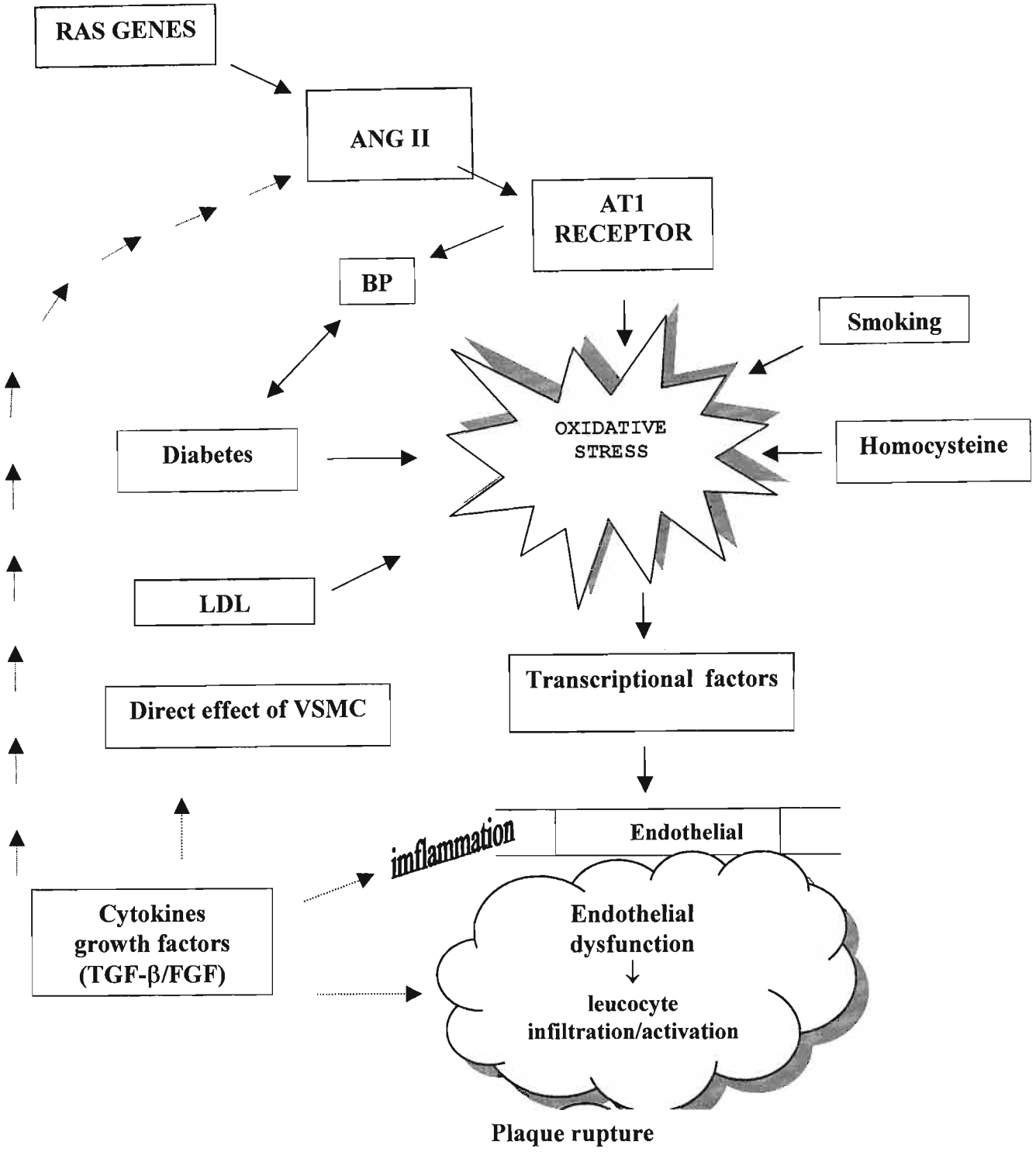

Fig I: **Putative Mechanisms for Angiotensin II in Relation to Risk Factors in Atherogenesis**

In response to angiotensin II monocytes release cytokines and growth factors which cause inflammation of the vessel wall, leading to further release of angiotensin II, as well as a direct effect on vascular smooth muscle cells.

1.3 Arterial Stiffness and Pulse Wave Velocity:

As one ages the arteries become progressively stiffer. This is due to degeneration in the elastic fibres in the media and as a result stress is transferred to the collagenous element in the arterial wall. A further consequence of arterial stiffening is a higher systolic and lower diastolic blood pressure, thereby causing increased afterload and altering coronary perfusion. The principal outcomes of these changes are left ventricular hypertrophy, aggravation of coronary ischaemia and increased fatigue of arterial wall tissues (cyclical stress). Higher systolic blood pressure, pulse pressure and lower diastolic blood pressure and left ventricular blood pressure have been identified as independent risk factors for cardiovascular mortality (Dãrne *et al*, 1989, Levy *et al*, 1990). Increased stiffness of the central arteries not only reflects the presence of hypertension but also risk factors (diabetes, obesity and hyperlipidaemia) that are associated with coronary artery disease and may prove to be a composite measure of risk.

CHD is thought to be associated with a generalised process that begins in the large arteries. Since coronary artery perfusion occurs mainly in diastole, the elastic recoil of the aorta is necessary to produce the energy for the retrograde flow through the coronary arteries. Furthermore, reduced arterial distensibility is responsible for a disproportionate increase in systolic BP, which is an important component of left ventricular stress leading to the development of hypertrophy (Bouthier *et al*, 1985).

A consequence of arterial stiffness is early return of the pressure wave reflection from the periphery leading to summation of the reflected wave with the systolic pressure, generating a higher and later systolic peak. Elevated systolic blood pressure has been shown to correlate with the development of atherosclerosis (Witteman *et al*, 1994). Whether enhanced arterial stiffness is a risk factor contributing to the development of cardiovascular disease has been

debatable (Blacher *et al*, 1999) but several markers do suggest this. As a determinant of pressure load, aortic stiffness correlates with left ventricular mass and wall thickness to radius in essential hypertension (Boutyrie *et al*, 1995). Increasing arterial stiffness occurs with ageing and correlates with the prevalence of atherosclerosis (Wada *et al*, 1994) and end-stage renal disease (London *et al*, 1992). In addition, there is evidence that morphological and structural changes in the aorta may be influenced not only by environmental but also by genetic factors such as AT_1 receptor gene expression (Benetos *et al*, 1995). Therefore, in the absence of risk factors, assessment of arterial compliance in subjects with early onset atherosclerosis may reflect arterial stiffness due to a genetic predisposition in these subjects (refer to 1.2.5).

Several methods have been used to measure compliance/distensibility of large blood vessels (change in vessel volume per mmHg change in pressure) but simultaneous measurements of volume and pressure at a point in the vessel are technically difficult. In recent years it has become increasingly possible to measure arterial stiffness easily and accurately, through determination of pulse wave velocity (PWV). An increase in arterial stiffness increases the speed of aortic pulse travel along the walls of the aorta and the major arteries (increased arterial pulse wave velocity). According to the Moens-Korteweg equation, PWV, which is related to the square-root of the elastic modulus, rises in stiffer arteries. Since the elastic properties of the aorta and central arteries are important determinants of cardiovascular coupling, PWV is clinically relevant to cardiovascular risk.

PWV measurement offers a simple, reproducible, indirect and non-invasive evaluation of regional arterial stiffness. PWV determined from foot-to-foot transit time in the aorta eliminates the influence of arterial wave reflections and closely approximates PWV measurements determined from phase velocity studies (Nichols *et al*, 1998). Correlation of PWV measurements with left ventricular mass have not been studied previously and may indicate early

arterial changes outside the coronary vasculature.

In addition to age and high BP, local hormonal factors may play a role in the modification of the arterial wall, mainly by modifying cell growth. Among these factors *Ang II* plays a critical role, since it induces hypertrophy of vascular smooth muscle cells and increases collagen production by fibroblasts. Most of the actions of *Ang II* are mediated by its effects on the AT_1 receptor. Furthermore, the interaction between lipoprotein and the *RAS* may explain why *ACE* inhibitors attenuate hypercholesterolaemia-induced atherosclerosis in animal models. The identification of polymorphic DNA markers on the AT_1 receptor gene offers a good opportunity to study the interaction of this gene with lipoproteins and its effects on arterial rigidity as measured by PWV. The genetic background may influence large artery function in many ways. The association of genetic and environmental factors may contribute to the development of hypertension, dyslipidaemia, salt-sensitivity and type II diabetes. In addition the genetic make-up of an individual may determine the response of the arterial wall to risk factors such as hypertension, aging, cholesterol and smoking. Identification of such genetic markers may have clinical implications in the detection of high-risk subjects. Polymorphisms of genes coding proteins implicated in cardiovascular regulation are therefore the logical candidates for study.

Thus, in summary, clinical risk factors account only in part for the high propensity to CHD in the Indian population. On their own these risk factors are insufficient to explain the seemingly high prevalence in younger patients, particularly men. Since family history studies suggest that there may be a strong genetic element in risk predisposition and since HLA typing has not shown any evidence of HLA predisposition in these patients, a study of candidate gene polymorphism seems warranted. To date there have been no published studies of gene polymorphisms in Durban Indians, as have recently

been described in the Caucasian population. The Indian population in Natal is an inbred population with a high prevalence of coronary disease. This constitutes a suitable group for analysis of candidate genes by restriction fragment length polymorphism (RFLP).

Patients with CHD comprise a heterogeneous group, ranging from subclinical silent ischaemia to chronic severe, often advanced, yet stable, triple vessel disease. The transition from subclinical cardiovascular disease to overt clinical disease is often precipitated by acute coronary events such as unstable angina or MI. Of all patients with acute coronary syndromes, those with MI form a well-defined, relatively homogeneous group suitable for study. Furthermore the pathogenesis of this condition is now well described, involving thrombotic factors against a background of changing metabolic milieu leading to altered lipid dynamics within unstable atherosclerotic plaques. Since the diagnosis of CHD is clearly established without the need for invasive procedures such as coronary angiography in patients with proven MI and given the high prevalence of CHD in relatively young Indian subjects, we prospectively evaluated a well-defined homogeneous cohort of Indians with early onset MI to determine whether candidate genes regulating the renin-angiotensin system were associated with premature CHD as manifested by MI.

The initial report which implicated the angiotensin converting enzyme gene, was made by Cambien *et al*, (1992) and confirmation of this finding was replicated in many but not all subsequent studies. Therefore, we also focussed on other candidate genes involved in cardiovascular homeostasis as described below (Table I).

D) OBJECTIVES:

The primary objective of this study is to prospectively evaluate the genetic polymorphisms that might be responsible for premature atherosclerosis in Indians who sustained MI at a young age. The focus of this study is on the candidate genes that are components of mediator systems involved in the regulations of vascular tone, cell growth and proliferation (Table I).

The aims can be summarised as follows:-

i) To ascertain the role of candidate gene polymorphisms pertaining to the angiotensin pathway. These include the *ACE* gene, *Agt* gene and the AT_1 receptor gene.

ii) To ascertain the role of candidate gene polymorphisms pertaining to aldosterone metabolism. These are genes for aldosterone synthase *(CYP11B2)* and 11β-hydroxysteroid dehydrogenase 2 *(11β-HSD2)*.

iii) To ascertain the role of gene polymorphisms of the *MTHFR* gene and correlate these with homocysteine levels.

iv) To ascertain the role of polymorphisms in the *G*-protein and $TGF\text{-}\beta_1$ genes.

v) To establish a correlation, if any, between genetic polymorphisms, risk factors, PWV, microalbumin and left ventricular mass.

TABLE I: CANDIDATE GENE POLYMORPHISMS STUDIED

PUTATIVE GENE	CHROMOSOME	LOCUS	DESIGNATION
Angiotensin Converting Enzyme *(ACE)*	17q	Intron 16	I/D
Aldosterone Synthase *(CYP11β2)*	8q22	Promoter	C344T
Angiotensinogen *(Agt)*	1q	Core promoter (5` region, Exon 1)	G6→A
Ang II Type 1 Receptor *(AT_1)*	3q	(5` end of 3` untranslated region)	C1166T
11β-hydroxysteroid dehydrogenase *(11β-HSD)*	16q 22	Exon 3 (Glu^{178}/Glu)	G534A
Transforming Growth Factor *(TGF-β)*	19q 13	Position 76 Exon 5 (Thr263 Ile)	C263T
G-Protein *($GN\beta_3$)*	CDNA	Nucleotide position 825 (β_3 Subunit)	C825T
Methylenetetrahydrofolate Reductase *(MTHFR)*	CDNA	Nucleotide position 677 (Ala^{222}/Val)	C677T

CHAPTER 2
METHODS

2.1 Study Population:

Informed consent was obtained from all subjects *(see Appendix I)*. A detailed clinical questionnaire including family history, patient's history, risk factors, current medical and a physical examination were obtained for each patient. The data were validated with reference to hospital records and dialogued with family members. From all participants, data on medical history, medication and smoking or drinking behavior were obtained by interviews, which included a family history and three-day dietary recall.

The study population was selected from Addington Hospital, Durban. This hospital provides a feeder service to districts north of Durban and most probands came from Phoenix area, which is almost exclusively an Indian littoral district. This population is extremely homogeneous with respect to ethnicity, dietary habits, lifestyles and environmental factors. Only unrelated probands and controls were utilised in the present study. Screening of probands occurred at least 3 months after the index MI.

The diagnosis of MI was confirmed from hospital records on the basis of at least two out of three criteria: typical chest pain, electrocardiographic features showing ST elevation and/or raised cardiac enzymes greater than two times the upper limit of normal.

2.1.1 Controls Subjects:

- The study included 80 unrelated subjects without clinical evidence of CHD. Normalcy was determined by clinical history, examination, electrocardiogram

and a normal effort test. It was originally planned to use the spouse as a control subject. However, since the majority of the probands were male and since the spouse was therefore a much younger female subject, it was decided to select older male subjects as controls. They were randomly selected from Indian volunteers who met the matching criteria of sex, age (± 5yrs.) and time from selection of probands.

Sub-clinical atheroma was not excluded in these subjects since the tests employed i.e. history of angina, abnormal q-waves on the electrocardiogram and a negative stress test, do not exclude sub-clinical atherosclerosis. In order to be absolutely certain that control subjects had no sub-clinical disease, coronary angiography would need to be performed, raising an ethical consideration in performing an invasive procedure in a normal subject. Subjects referred for coronary angiography and shown to have a normal study were not selected because microvascular angina could not be excluded in these patients. Therefore, control subjects who were older (+ 5 years) were selected in order to be more certain of the absence of symptomatic premature coronary heart disease in this group.

Samples from cases and controls were stored for the same duration and were handled together, identically.

The presence of hypertension, diabetes mellitus, hypercholesterolaemia or smoking was determined by history taking, previous medical records, (if any) current medication and by the results of blood tests and examination.

2.2 Procedures:

This included taking an updated medical history and obtaining informed consent. After sampling of fasting venous blood for biochemistry, blood pressure determination, anthropometric measurements, resting electrocardiogram (ECG) and combined echo-doppler ultrasound examination

of the heart were performed, followed by pulse wave velocity determination.

Resting blood pressure (BP) was measured before echocardiography in the right arm with the subject supine for at least 10 minutes and was recorded with a validated automated device (Dinamap) using a cuff of suitable size. The first reading was discarded and the mean of two subsequent readings taken. Hypertension was defined as BP values equal to or above systolic 160mmHg and/or diastolic 95mmHg, or as being present in subjects chronically taking antihypertensive medication.

2.3 Examination and Anthropometric Measurements:

After clinical examination body height was measured in cm with a statometer. Subject's body height and weight were measured in light clothing and without wearing shoes, and the body mass index (BMI) was computed as weight in kilograms divided by height in metres squared (kg/m^2). As body weight measures not only fat, but other components of the body, and BMI is a measure of muscloskeletal mass as well as of body fat, waist and hip measurements were obtained by standard criteria in order to estimate intra-abdominal fat. Obesity was defined as BMI $\geq$ 26 kg/m^2. Waist and hip measurements were taken in cm at the level of the umbilicus and at the level of the anterior superior iliac spine respectively. The data are presented for waist circumference as well since this may provide a better measurement of visceral adiposity (Pouliot *et al,* 1994).

2.4 Venous Blood Sampling:

Informed consent was obtained from probands and controls prior to screening. Subjects were instructed to fast for 12-14 hours and compliance of fasting was determined by interview on the morning of examination. All participants were

examined in the morning and underwent the same procedure.

After a period of 10 minutes of supine rest approximately 20ml antecubital venous blood was collected in serum separator tubes, citrate and EDTA tubes and immediately kept on wet ice. Further processing began within 30 minutes. The plasma was separated by centrifugation at 2500 rpm for 20 minutes and stored at -70^0 for special biochemistry. Serum for lipid analyses was stored at -4^0C and assayed within 24 hours. A 5ml sample of EDTA was lyzed, (sucrose lysis technique – Appendix II); the white cells were then separated and stored as a pellet at -70^0C for subsequent DNA extraction.

Each individual provided a random urine sample for determination of microalbumin level. After the initial blood sample subjects underwent a modified 2-hour glucose tolerance test, with blood samples taken at 1 and 2 hours. Known diabetic patients did not participate in this part of the screening.

2.5 Evaluation of Risk Factors:

2.5.1 Biochemistry:

We evaluated risk factors that were considered standard in the Framingham Study. (Kannel *et al,* 1976). We measured serum cholesterol levels with the use of standard enzymatic methods to determine total and HDL-C cholesterol levels after precipitation of apo-β containing lipoproteins (LDL-C and VLDL) by phosphotungstic acid and magnesium chloride. Subjects with diseases causing secondary hypercholesterolaemia were excluded from the study. We evaluated lipid levels 3-9 months after a MI since total cholesterol and HDL-C levels fall with a rise in triglycerides in the first 24 hours and return to baseline after 10 days (Pfohl *et al,* 1999). Fibrinogen was measured in freshly collected citrated plasma.

Fasting glucose (intra-assay co-efficient of variation 1.9% at 6.7mmol/L), cholesterol, triglyceride and HDL-C were carried out by enzyme tests using the Beckman CX7 autoanalyser. LDL-C was calculated by the Friedwald equation provided total triglycerides did not exceed 4.0 mmol/L (Friedewald *et al,* 1972). Apolipoprotein *(a)* was quantified by radio-immuno assay using antisera from Mercodia (Uppsala, Sweden). HbA1C was estimated by ion exchange chromatography.

2.5.2 Glucose Tolerance Test:

The criteria of the World Health Organisation, based on fasting levels and plasma glucose values measured two hours after a standard oral 75-g glucose load (2-h plasma glucose) were used to diagnose diabetes. Diabetes mellitus was considered to be insulin dependent type I (IDDM) if it was diagnosed before the age of 30 years and treatment with insulin began within one year and continued thereafter. On this basis, no subject in this study was classified as insulin dependent (Type I) diabetes.

2.5.3 Insulin Estimation:

Fasting insulin was used as a measure of insulin resistance based on the observation that fasting insulin level is a good marker of insulin resistance in population studies (Olefsky *et al*, 1973, Laakso *et al,* 1998). In subjects who underwent a glucose tolerance test (GTT) the 2 hour insulin was also measured.

A commercial radio-immunoassay kit was used for measuring plasma immuno-reactive insulin level (Phadeseph - Pharmacia & Upjohn). This assay has 41% cross-reactivity within proinsulin, which is disproportionately low in non-diabetic patients, and less than 0.1% cross-reactivity with C peptide. The detection limit of insulin was 2.5 μU/ml. The intra and inter-assay coefficients

of variation for this test are 5.3 and 7.6%.

2.5.4 Homocysteine Estimation:

Fasting blood samples were drawn and separated within 1 hour. Plasma total homocysteine was measured by high performance liquid chromatography using the modified method of Ubbink *et al* (1996).

2.6 DNA Extraction and Genotyping:

Genomic DNA was extracted from the white cell pellet by the standard method of phenol and chloroform extraction and this was followed by isopropanol precipitation of the DNA (Appendix II).

For each candidate gene an appropriate set of primers, designed to encompass the polymorphic region was used. In each analysis the PCR reaction contained 100 ng DNA template, 0,1 μM of each primer, 1,25 μM of 4DNTPs, 0.5 units of Taq DNA polymerase, and 1,5 mM $MgCl_2$. DNA was amplified for 35 cycles, each cycle comprising denaturation at 94^0 C for 3 minutes, annealing at 60^0C for 40 seconds, extension at 72^0C for 2 minutes. The PCR products were digested where necessary with the appropriate restriction endonuclease and then separated by electrophoresis on 2% agarose gels containing ethidium bromide. After the gels were run and photographed, genotyping was performed by a trained laboratory technologist who was blinded to the subjects' clinical data (Appendix III).

In the case of the *ACE* gene, 4-5% of samples with the *ID* genotype were misclassified as DD when only the flanking primer pair was used. To prevent mistyping of *ID* as *DD* genotype a second PCR, with an insertion-specific primer, was performed in all samples classified as homozygous *DD* in the first PCR (Lindpaintner *et al*, 1995) with 67^0C as the annealing temperature.

2.7 Echocardiographic Measurements:

Two dimensional-guided M-mode echocardiograms were obtained from each subject with a Toshiba SSH 270HG (Toshiba Corporation, Tokyo, Japan) phased-array scanner connected to a 2.5 MHz transducer. Left ventricular internal dimensions were measured at end-diastole and end-systole according to the Penn convention just below the tip of the mitral valve as recommended by the American Society of Echocardiography. (Sahn *et al*, 1978) Only tracings that demonstrated optimal visualisation of the left ventricular interfaces were accepted for analysis. Each recording was also saved on video-tape for subsequent review. Left ventricular mass (LVM) was calculated according to the formula of Devereux *et al* (1977).

$$LVM\ (grams) = (1.04\ [EDD + SWT + PWT]^3 - EDD^3) - 13.6$$

where EDD = internal end-diastolic diameter, SWT + PWT = septal and posterior wall thickness, respectively.

Partition values for the diagnosis of LVH were taken from the Framingham study, with 125 g/m^2 used as the cut-off for both men and women as suggested by Casale *et al,* (1985).

Immediately after M-mode recordings, pulsed Dopper echocardiography was performed. Transmitral flow velocity waveforms were obtained form the apical view in the four-chamber projection with the sample volume placed at the level of the mitral annulus and at the tip of the mitral valve leaflets. The angle between the Doppler beam and the assumed direction of the transmitral flow was less than 10^0 in all subjects. The peaks of the early diastolic velocity wave (E) and late diastolic wave (A) were measured in metres/second and the E/A ratio determined. The whole scanning procedure was recorded on

videotape and a hard copy print of the tracing taken. Imaging was performed blinded to the result of the genetic analysis. To assess reproducibility of the LV mass determination imaging was repeated 7-14 days later in ± 10 subjects. The intra-observer variation was < 10%.

2.8 Pulse Wave Velocity (PWV):

After 15 minutes of rest in the supine position aortic PWV was evaluated transcutaneously with the use of two pressure probes (Ty 306, Fukuda, Tokyo, Japan). This method has been extensively analysed previously (Asmar *et al,* 1995). Briefly, two pressure waves were recorded simultaneously at two sites (at the base of the neck for the common carotid artery and over the right femoral artery). Pulse transit time was determined as the average of 30 consecutive beats in order to cover 5 respiratory cycles. The distance travelled by the pulse wave was measured over the body surface as the distance between the two recording sites. Aortic PWV was calculated as the ratio of distance to transit time. The reproducibility of the measurement for aortic PWV (expressed as percent variation of the mean value) has been found 5,3 ± 3,6% (Asmar *et al*, 1995). The variation coefficient of two consecutive measurements of PWV by the same examiner was previously found to range from 8-9,7%.

2.9 Statistical Analysis:

For all statistical analysis we used the computer software SPSS package for Windows '95 software (Version 9.0). Comparison between groups was done with student's unpaired t-test for continuous variables. Univariate regression analysis was performed according to Pearson. For some parameters log transformation was necessary to obtain a Gaussian distribution which is

necessary for the parametric tests. Multiple regression analysis was performed stepwise on all indicated parameters.

The results for categorical data are expressed as percentages and continuous variables as means ± standard deviation if the distribution was symmetric. Plasma triglycerides, apolipoprotein *(a)*, fibrinogen and urinary protein values were also log-transformed for statistical analysis because of their skewed distribution. Since microalbumin levels were below detection limits in a considerable number of subjects, this parameter had to be analysed by U-test and Spearman's test. Categorical data were analysed by chi-square tests. A p value < 0.05 was considered statistically significant.

The relationship between genotypes and classical risk factors for CHD was assessed by one-way analysis of variance (ANOVA). Statistical significance was again taken at the 5% level. Analysis of covariance (ANCOVA) was used to examine the relationship between genotype, blood pressure (systolic and diastolic) and LVMI. Again, to account for the non-normal distribution of LVMI, the analysis was done using log transformed data.

Alleles and genotype frequencies in cases and control subjects were compared by chi-square (χ^2) tests and by stepwise logistic regression analysis using the SPSS program. Since the genotypes constituted a biallelic marker, comparisons of genotype distributions and allelic frequencies were assessed by t-test with two and one degrees of freedom respectively. Deviation from the Hardy-Weinberg equilibrium was tested by χ^2 test with one degree of freedom. The analysis was also carried out by means of an explorative bivariate logistic regression analysis to assess the independent role of the different factors using MI as the dependent variable and homocysteine, apolipoprotein *(a)*, smoking, BP, lipids, diabetes, family history and genotype as independent variables.

Analysis of genetic polymorphisms are sensitive to the allelic frequencies of the gene in the population studied. To date very few studies have been performed in Asian Indian subjects so that allelic frequencies in these groups

are not known. Furthermore, the few studies to date in Asian Indians have included subjects with a tremendous degree of heterogeneity in the study sample i.e. age range, angina vs MI, geographical area and gender mix (Saha *et al*, 1996, Ramasawmy *et al*, 1996). The number of probands in these studies did not exceed 85 and controls, 108. Furthermore, in the original study reported by Cambien *et al* (1992) the Toulouse sample was not in Hardy-Weinberg equilibrium. This law is relatively robust and deviations from it could be explained by the size of the study sample.

It was therefore decided to include a homogeneous study sample i.e. young age, myocardial infarction, cadastral district, time from index event of at least 50 probands and controls. The number of probands was increased to 100 when the results of glucose tolerance tests revealed a high prevalence of diabetes in this group. Both proband and control groups satisfied Hardy-Weinberg statistical requirements.

CHAPTER 3
RESULTS

3.1 Assessment of Risk Factors:

3.1.1 Demographic Data:

There were 117 probands and 80 controls. In keeping with selection criteria the mean age in the proband group was under 50 years (43,1 ± 6.8). The majority of probands were males (105 males and 12 females). In the control group there were 64 males and 16 females. By selection subjects in the control group were slightly older (46.4 vs 43.1 years). None of the subjects exhibited clinical characteristics of familial hyperlipidaemia i.e. premature arcus, xanthelasma or tendon xanthomas.

The infarct territory in the probands was anterior in 59 (50%), inferior in 48 (41%) and two patients had a lateral and posterior (1) infarct. The remaining eight patients had anterior infarction with inferior extension. Most patients received thrombolytic therapy and during follow up were on aspirin and statin therapy. Three had a very low ejection fraction and were receiving in addition diuretic therapy. A substantial number of patients received ACE inhibitors (n = 33 (28%), β-blockers n = 44 (38%) and twenty-four (20%) were referred for interventional therapy.

Details of the patients and control groups are summarised in Table II. Since gender did not play an important role in our study objectives and because there were so few females data were combined for males and females in further statistical analysis.

3.1.2 Patient Morphometry:

The allometric data revealed significant differences in waist and waist-hip measurements as well as body mass index in the two groups. Probands had higher waist-hip ratios (0.98 vs 0.94) and wider waists (92.2cm vs 87.8cm) than controls ($p < 0.05$).

Table II

DEMOGRAPHIC DATA AND ANTHROPOMETRY			
	PROBANDS n=117	**CONTROLS n=80**	***p***
Age	43.1±6.8	46.4±8.5	.006
Gender M/F	105/12	64/16	
BMI, Kg/m^2	26.4±4.0	25.1±4.0	.015
Waist, cm	92.2±10.8	87.8±9.1	.002
Waist/Hip	0.98 (0.78-1.12)	0.94 (0.78-1.08)	.001
Values are mean ± SD except for waist/hip (median + range)			

3.1.3 Blood Pressure:

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were similar in both groups. Many probands had normal blood pressure but all were taking therapy (cardioprotective ACE inhibitors ± β-blockers) which would have lowered the blood pressure. Measurements were made after an overnight fast so that uniformly trough levels were recorded in all subjects. Subjects were diagnosed as hypertensive if their blood pressure was elevated (> 160/95mmHg) or they had a diagnosis of hypertension and were taking antihypertensive therapy. On this basis 31% of the probands were deemed hypertensive (cf.: controls 5%).

3.1.4 Behavioural Risk Factors (Table II):

3.1.4.1 Smoking:

Table III shows the percentages of subjects in each group who smoked and consumed alcohol. Among the probands only 21 subjects (18%) had never smoked and almost half had not taken alcohol. There was a higher percentage of current smokers among probands, among whom only 10% made attempts to quit the habit completely.

3.1.4.2 Type A Personality:

Measurements of lifestyle were assessed by simple questions regarding drive, motivation and social interaction and revealed that 54% of probands had Type A personality as compared to 16% in the control group ($p < 0.05$).

3.1.4.3 Employment/Education:

Eighty-five probands (73%) had a low level employment and educational level (under standard 9) compared to 50 controls (63%) ($p = ns$).

3.1.4.4 Exercise:

The level of physical activity in all subjects was assessed by questioning them about regular exercise of any sort. Only 26% of probands performed a regular exercise routine i.e. 20 minutes at least twice a week. However, almost half the probands had a sedentary work pattern.

Table III

RISK FACTOR CATEGORIES			
	PROBANDS %	**CONTROLS** %	***p***
Total Cholesterol > 6.2 mmol/l	26	25	0.919 ns
Diabetes	47	21	0.000
Impaired Glucose Tolerance	5	7	
Cigarette Smoking	82	26	0.000
Blood Pressure	31	5	0.000
Family History CHD	80	24	0.000
Family History Premature CHD	48	8	0.000
Family History of Diabetes	44	6	0.000
Family History of Hypertension	41	3	0.000
Overweight, > 25 kg/m^2	62	49	0.058
Waist > 96 cm	36	14	0.001
Waist /Hip > 1.0	30	16	0.026
Type A Behaviour	54	16	0.000
↑ Serum Triglycerides > 2.3 mmol/l	42	15	0.000
↓ Serum HDL-C < 1.0 mmol/l	53	30	0.001
HDL/TC < 20%	56	34	0.002
↑ Alcohol Intake	57	30	0.000
Sedentary Lifestyle	74	54	0.005
Microalbumin > 2.8 mg/mmol creatinine	40	19	0.001

3.1.4.5 Diet:

Of the 117 probands, only 3 patients were vegetarians (cf.: control 5 p = ns). The rest ate meat to a varying degree. The majority changed their eating habits

shortly after the index event (myocardial infarct), only to revert to their former dietary patterns. After a short while many found it difficult to make the lifestyle adjustment required because this meant a change in dietary patterns for the whole family. Only those in the higher income bracket managed to maintain lifestyle changes. Across the group, and except for the vegetarians, diet consisted mainly of curry dishes cooked in sunflower oil. The average meat consumption was similar, approximately three times per week, with only a low intake of fruit and vegetables. Half of the probands and a third of the controls ate fruit and vegetables only once a week.

3.1.5 Metabolic Risk Factors:

3.1.5.1 Diabetes Mellitus:

The proband group had a significantly higher prevalence of diabetes (47%), glucose intolerance (4%: WHO definition) and family history of diabetes (44%). When this became apparent early in the recruitment phase, the number of probands was increased from 50 to 100. All probands had type II diabetes and most were on oral hypoglycaemic agents except for two who were taking insulin. Thirty-two probands and six controls were known diabetics. Diabetes was diagnosed for the first time in 33 subjects (21 probands, 12 controls).

3.1.6 Lipid Profile:

The lipid profile for the two groups showed important differences. Whereas total cholesterol and LDL-C levels, were similar in both groups, significantly elevated triglyceride levels and lower HDL-C levels were present in the probands ($p < 0.05$) (Table IV). The likely explanation was that almost all probands were receiving lipid-lowering therapy in the form of statins. There was however, failure of concomitant attempts at diet-modifying behavioural

changes. In order to assess lipid levels independent of treatment effects it was decided to assay Lp*(a)* on a stored sample (at -70^{0}C) of plasma. Lp*(a)* measured as apolipoprotein *(a)* is essentially unaffected by therapy and correlates well with a genetic predisposition to early onset CHD. This assay did not reveal any striking changes between probands and controls ($p = 0.102$). However, there was a correlation between the apolipoprotein *(a)* level and a family history of premature coronary heart disease.

Table IV

DESCRIPTIVE STATISTICS: BIOCHEMISTRY			
	PROBANDS	**CONTROLS**	***p* (m-w)**
Total Cholesterol	5.4 ± 0.12	5.5 ± 0.13	0.384
LDL-C	3.2 ± 0.10	3.46 ± 0.11	0.074
HDL-C	1.1	1.3	0.000
HDL/TC	0.21	0.24	0.005
Triglycerides	2.8 ± 0.26	1.77 ± 0.18	0.000
Plasma Glucose (0 hr)	7.44 ± 0.33	5.81 ± 0.25	0.000
Plasma Glucose (2 hr)	9.10 ± 0.59	7.5 ± 0.52	0.09
Serum Insulin (0 hr) u/l	19.4 ± 1.85	14.6 ± 1.8	0.799
Serum Insulin (2 hr) u/l	54.9 ± 5.5	52.3 ± 8.7	0.271
Ins/glucose (0 hr)	2.38 ± 0.3	2.4 ± 0.3	0.27
Ins/glucose (2 hr)	8.7 ± 2.2	8.7 ± 2.2	0.93
Haemoglobin A_1C (%)	7.08 ± 0.2	6.21 ± 0.2	0.021
Plasma Fibrinogen (g/l)	3.4	3.2	0.106
Plasma Homocysteine μmmol/l	18.9 ± 1.0	17.53 ± 0.89	0.511
Apolipoprotein (a) μ/l	354.9 ± 25.8	279.0 ± 26.8	0.102
Microalbuminuria mg/mmol creat	7.47 ± 0.97	3.55 ± 0.81	0.000
Values are Mean ± SE			

3.1.7 Summary of Risk Factors:

The distribution of the common risk factors in probands and controls are shown in Table III and reflects common differences in the risk profile of the two groups. Total cholesterol levels were similar in probands and in controls, probably reflecting the higher frequency of lipid-lowering drugs in the former. None of the risk factors shown in Table III differed according to *ACE* genotype

data. (Refer to 3.2)

When the probands were divided according to the presence/absence of diabetes and other risk factors it emerged that almost of all young Indians (113/117) with MI had at least one major risk factor, and that in almost half of them diabetes was a major contributor to risk. When a positive family history was used as an indicator of heritability, 48% of the probands and 8% of the controls had a family history of premature coronary heart disease. A regression analysis was performed with family history of cardiovascular disease (hypertension, diabetes, CHD, stroke). A family history of premature CHD, hypertension and smoking emerged as strong predictors of risk of MI.

3.2 Angiotensin Converting Enzyme Gene Polymorphism:

(Fig 2-7 Appendix)

The *ACE* genotype distributions in probands (χ^2 = 0.03) and controls (χ^2 = 0.69) were in the Hardy-Weinberg equilibrium. The relative allelic frequencies of the *ACE* genotypes in controls did not differ from frequencies in the whole proband group (χ^2 = 1.81), as well as when the probands were subdivided into diabetic and non-diabetic groups (χ^2 = 0.03; p = 0.532) (Tables V & VI).

Table V

GENOTYPE FREQUENCIES IN PROBANDS (n = 117) & CONTROLS (n = 80)							
		HOMO	%	HETERO	%	HOMO m	%
ACE Gene	P	44	**38**	55	**48**	16	**14**
	C	25	**33**	34	**45**	17	**22**
Agt Core Promoter	P	44	**39**	50	**45**	18	**16**
	C	32	**43**	32	**43**	11	**14**
AT_1 Receptor	P	100	**86**	16	**14**	0	**0**
	C	65	**81**	15	**19**	0	**0**
Aldosterone Synthase	P	48	**41**	54	**46**	15	**12**
	C	29	**36**	44	**55**	7	**9**
11β-HSD2	P	115	**98**	1	**1**	1	**1**
	C	76	**97**	1	**1.5**	1	**1.5**
MTHFR	P	94	**80**	21	**18**	2	**2**
	C	64	**81**	14	**18**	1	**1**
TGF-β	P	114	**98**	1	**1**	1	**1**
	C	78	**98**	1	**1**	1	**1**
G-Protein	P	61	**52**	47	**40**	9	**8**
	C	42	**53**	32	**40**	6	7

See Appendix for Hardy-Weinberg Determination and Allelic Frequencies

P = proband
C = control

Table VI

GENOTYPE FREQUENCIES IN NON-DIABETICS							
		HOMO	%	HETERO	%	HOMO m	%
ACE Gene	P	17	**27**	34	**56**	10	**17**
	C	21	**36**	24	**41**	13	**23**
Agt Core Promoter	P	22	**36**	30	**49**	9	**15**
	C	23	**40**	25	**44**	9	**16**
AT_1 Receptor	P	54	**86**	9	**14**	0	**0**
	C	50	**83**	10	**17**	0	**0**
Aldosterone Synthase	P	26	**42**	30	**50**	5	**8**
	C	23	**37**	35	**56**	4	**7**
11β-HSD2	P	60	**98**	1	**2**	0	**0**
	C	57	**98**	1	**2**	0	**0**
MTHFR	P	50	**82**	11	**18**	0	**0**
	C	49	**80**	11	**19**	1	**1**
TGF-β	P	61	**0**	0	**0**	0	**0**
	C	78	**0**	0	**0**	0	**0**
G-Protein	P	6	**10**	26	**43**	29	**47**
	C	4	**7**	27	**45**	29	**48**

PROBANDS VS CONTROLS : Monte Carlo Sig. (2-sided) p = ns
P = Probands
C = Controls

Further analysis to identify a group at low risk of CHD was not performed since almost all subjects had risk factors. None of the recognised risk factors differed according to *ACE* genotype data. Thus, in this Indian cohort of premature MI the *ACE* genotype was not associated with a significantly increased risk of MI. This finding persisted across subgroups without diabetes, as well as in groups with putative genetic risk factors i.e. elevated homocysteine, apolipoprotein *(a)*, strong family history of CHD. When the

clinical and laboratory variables were compared among genotypes in the control group, no significant differences were noted with regard to biochemistry variables.

3.3 Angiotensinogen Core Promoter Polymorphism:

(Fig 8-10 Appendix)

The genotype frequencies in both probands ($\chi^2 = 0.35$) and controls ($\chi^2 = 0.41$) were compatible with Hardy-Weinberg equilibrium. There was no differences in the genotype frequencies between controls and probands ($\chi^2 = 0.22$: p = ns). The allelic frequencies were similar in both groups ($\chi^2 = 0.22$; p = 0.72). The mutant homozygote was present in 18/112 (16%) probands and 11/75 (15%) controls. There were no differences in the risk factor profile of the two groups. The study showed no association between the *Agt G-6A* polymorphism and premature MI in this cohort of patients.

3.4 Angiotensinogen II Type I Receptor Polymorphism:

(Fig 11-13)

The genotype distribution in the proband ($\chi^2 = 0.27$) and control group ($\chi^2 = 0.06$) was compatible with the Hardy-Weinberg equilibrium. There were no differences allelic distribution ($\chi^2 = 0.80$; p = 0.48) between the two groups. The mutant homozygote (*1166C*) was not seen in probands or controls in this study. The frequency of the *A* allele was 93% in probands and 91% in controls.

3.5 Aldosterone Synthase Polymorphism:

(Fig 14-16 Appendix)

The genotype distribution in both case-patients (χ^2 = 0.02) and controls (χ^2 = 4.91) were in Hardy-Weinberg equilibrium. The genotype was TT in 15 cases vs 7 controls, CT in 54 vs 42 and CC in 48 vs 31 (χ^2 = 1.16). The allelic frequencies were similar in both groups (χ^2 = 0.03; p = 0.97).

3.6 11β-Hydroxysteroid Dehydrogenase 2 Polymorphism:

(Fig 18-20 Appendix)

The polymorphism did not exhibit significant deviation from Hardy-Weinberg equilibrium in the proband (χ^2 = 1.31) or control (χ^2 = 1.29) groups. No significant differences in the allelic frequencies (Fisher's exact; p = 0.69) was found between cases and controls. There was only a sample each of the mutated genotype in cases and controls, and one each of the heterozygote genotype.

3.7 Transforming Growth Factor-β Polymorphism:

(Fig 20-22 Appendix)

The genotypes were similar in both groups (χ^2 = 0.14). The polymorphism did not exhibit deviation from the Hardy-Weinberg equilibrium in cases (χ^2 = 1.31) and in controls (χ^2 = 1.30). The allelic frequencies were almost identical in both groups (Fisher's exact; p = 0,69). No individuals homozygous for the rare allele (*TT*) were identified, nor were there any heterozygotes in this sample. These results were confirmed with known heterozygotes identified previously and used as positive controls.

3.8 Methylentetrahydrofolate Reductase Polymorphism:

(Fig 22-26 Appendix)

3.8.1 *MTHFR* Genotype Distribution:

The distribution of the genotypes in both probands ($\chi^2 = 0.02$) and controls ($\chi^2 = 1.26$) was compatible with the Hardy-Weinberg equilibrium. There were no differences in the genotype ($\chi^2 = 0.85$) or allelic ($\chi^2 = 0.18$) frequencies in the two groups. The allelic frequencies were almost identical: C allele 90% vs 89%, T allele 10% vs 11% in probands and controls respectively ($\chi^2 = 0.18$; $p = 0.83$).

Homozygosity for the *MTHFR* polymorphism *Val/val (TT)* genotype was found in one proband (1%) and 2 controls (2.5%). There was therefore no significant difference between patients and controls in the prevalence of the *TT* genotype (p = ns). The prevalence of the T allele in the small number of patients with a strong family history of CHD was higher than in controls but this difference was not significant.

3.8.2 Homocysteine Concentrations:

No substantial differences existed in the homocysteine level among the genotypes. Patients with the *TT* genotype did not have elevated homocysteine levels. The results of this study do not confirm that *MTHFR* genotype is an important determinant of plasma homocysteine levels in this cohort: *TT* genotype carriers have significantly higher levels than carriers of the other genotypes, but this mutation was not common. If homocysteine were indeed a cardiovascular risk factor, especially for premature MI, then one would expect a higher prevalence of the *TT* genotype which would predispose carriers to

accelerated atherosclerosis.

3.9 *G*- Protein Polymorphism:

(Fig 26-28 Appendix)

The genotype frequencies were in Hardy-Weinberg equilibrium in both probands ($\chi^2 = 0.00$) and controls ($\chi^2 = 0.00$). The distribution of the allele ($\chi^2 = 0.00$: $p = 0.09$) and genotype ($\chi^2 = 0.00$) frequencies did not differ significantly between probands and controls.

The *825 T* allele frequencies were 0.93 in probands and 0.91 in controls. The results of this study suggest that it is unlikely that the *GNβ₃ C825T* polymorphism contributes to the risk for premature MI in this cohort.

3.10 Pulse Wave Velocity:

The average PWV was 10.7 ± 0.17 m/sec in probands and 12.2 ± 1.33 m/sec in controls. PWV was higher in the controls but the differences was not significant ($p = 0.393$). There was a significant correlation between PWV and age ($p = 0.001$), pulse pressure ($p = 0.000$), W/H ratio ($p = 0.006$), and a weaker correlation with triglycerides ($p = 0.05$) and HbA1C ($p = 0.045$). Amongst the controls only age, W/H ratio and systolic blood pressure showed correlation with PWV (Table VII). There were significant differences in the echocardiographic parameters in the two groups. In keeping with the previous infarct, ejection fraction was lower and LV diameter bigger amongst the probands (Table VIII). Although this study observed that significant correlation existed between PWV and risk factors particularly BP, diabetic status and waist measurement indices. No significant correlation was shown between PWV and genotype patterns.

Table VII

SPEARMAN'S CORRELATION BETWEEN PULSE WAVE VELOCITY & OTHER RISK FACTORS				
	PROBANDS	***P***	**CONTROLS**	***P***
Diabetes	.298	.001	.232	.038
Age	.569	.000	.054	.634 ns
Blood Pressure	.186	.045	.009	.939 ns
Haemoglobin A_1C	.251	.006	.276	.013
Waist/Hip Ratio	.228	.013	.232	.038
Systolic BP	.379	.000	.061	.589 ns
Diastolic BP	.536	.000	.020	.858 ns
Pulse Pressure	.290	.002	-0.114	.314 ns
Triglycerides	-0.182	.049	-0.851	.542 ns
Microalbuminuria	0.141	0.132 ns	.327	.003

Table VIII

PULSE WAVE VELOCITY AND ECHODOPPLER MEASUREMENTS			
	PROBANDS $\bar{x} \pm SE$	**CONTROLS** $\bar{x} \pm SE$	*p*
PWV, m/sec	10.7 ± 0.17	12.2±1.33	.393
Systolic Blood Pressure	123 ± 2.5	126 ± 2.0	.661
Diastolic Blood Pressure	77 ± 1.2	76 ± 1.2	.421
Pulse Pressure, mmHg	49 ± 1.4	50 ± 1.3	.270
Septum, mm	10.4 ± 0.35	9.0 ± 0.33	.033
LVIDD, mm	53.8 ± 0.65	45.0 ± 1.2	.000
Posterior Wall, mm	6.98 ± 0.48	6.28 ± 0.18	.286
Ejection Fraction	55.0 ± 1.22	64.4 ± 1.08	.000
LV Mass, gm	188±5.83	132±5.9	.000
LV Mass Index, gm/m^2	103±3.23	75±3.7	.000
Doppler E/A	1.42	1.30	.603

CHAPTER 4
DISCUSSION

4. Discussion:

4.1 Assessment of Risk Factors:

Strong familial aggregation of CHD has long been recognised. Statistical regression studies suggest that only 30-50% of the observed increment in familial risk can be attributed to the classical risk factors: raised cholesterol, smoking, hypertension and diabetes, which explain only part of the epidemiological features of atherosclerotic coronary heart disease. Other factors that increase the risk of myocardial infarction alone or in combination with the classical risk factors must therefore be operative. These may involve lipid dynamics within the plaque, antioxidative defences, endothelial factors as well as hereditary determinants that influence atherogenesis. Recent data suggest that these factors may accelerate atherosclerosis in the patient with risk factors, giving rise to premature coronary events.

Evidence for genetically determined predisposition for coronary heart disease is derived in particular from migration studies. A marked susceptibility is observed in regions such as Great Britain, Singapore and South Africa, suggesting that the effect of a changing environment is to unmask the genetic factors. More recently the results of the Bogalusa Heart Study have shown significant clustering of risk factors in both young parents and their offspring (Chen *et al*, 1999). Therefore, the risk factor profile of young Indians with myocardial infarction was determined by interview and assessment of biochemical characteristics in order to define the background risk and compare this with their genetic patterns.

4.1.1 Premature Myocardial Infarction:

Among Western series myocardial infarction in young patients <45 years is thought to be an unusual occurrence. It is quoted that 2-6% of MI occurs in young patients (Jalowiec *et al*, 1989). At least 80% of young patients with MI have typical atherosclerotic coronary heart disease (Warren *et al*, 1979), manifesting most often as non-obstructive or single vessel disease (Zimmerman *et al*, 1995). Multivessel disease appears to be related to the number of risk factors (Wagner *et al*, 1996), particularly diabetes (Hong *et al*, 1994).

Myocardial infarction in the absence of atherosclerotic disease accounts for only 20% of cases in this age group (Choudhury *et al*, 1999). The majority of our patients probably had atherosclerosis as the cause of their disease, for which underlying risk factors were present to a variable extent. None of the patients in our study had clinical evidence to suggest coronary embolism or a systemic vasculitis, nor did they have any overt signs of a hypercoagulable state or history of cocaine drug abuse. Coronary angiography was performed in only those patients who were referred for ongoing pain, so that the number who had rare coronary anomalies, if any, is not known.

Risk factors that account for atherosclerosis in young patients include smoking, hypertension, diabetes, dyslipidaemia, a positive family history and obesity. In addition, hypercoagulable states associated with increased factor VIII activity and reduced fibrinolytic activity as a result of increased PAI-1 levels, use of the oral contraceptive pill and elevated plasma homocysteine levels, have all been reported in patients who sustained MI under the age of 45 years (Choudhury *et al*, 1999).

The baseline characteristics of our study group confirm that young Indian men

and women with MI have significant risk factors, even at a younger age. Among young Indian males with myocardial infarction Sewdarsen *et al* (1987) noted that 96% had at least one major risk factor for coronary heart disease. Smoking was the commonest and present in 79% of patients and serum cholesterol above 6,5 mmol/L was found in 50% of patients. These risk factors were also present in our control group, albeit to a lesser degree. These findings are also in keeping with our survey of medical students which showed that young Indian medical students develop risk factors for CHD at an early age compared with young Black medical students (Morar *et al*, 1998).

This may explain recent trends which suggest an alarming increase in CHD in the South African Indian population (Walker *et al,* 1993) (cf. Framingham data which indicate a decreasing incidence of MI in younger patients).

On the Indian subcontinent the major risk factors (hypertension, diabetes and hypercholesterolaemia) do not play a significant role while insulin resistance appears to be an important risk factor. Abdominal obesity, raised triglycerides and low HDL-C were markers of insulin resistance in this group (Vardan *et al*, 1995, Singh *et al*, 1995). Only 8.2% of patients with CHD had BMI > 27kg/m^2; CHD was present in subjects with BMI 15 kg/m^2 upward (Thomas, 1995), and was related to abdominal obesity.

4.1.2 Behavioural Patterns:

4.1.2.1 Smoking:

Together with alcohol consumption smoking was predominant among the men in our study. It is **the** major risk factor among young Indians with myocardial infarction regardless of their geographical location (Tambyah *et al*, 1996). Findings similar to our study have recently been reported in a case-control study of 200 Indian patients with myocardial infarction in India (Pais *et al*, 1996). The most important predictor of myocardial infarction was current

smoking. Fasting blood glucose and abdominal obesity were also strong predictors of risk but the lipid profile was not associated with myocardial infarction. Smoking contributes to risk via several mechanisms: Hughes *et al* (1998) studied 166 Asian smokers in Singapore and showed that smokers had lower HDL-C and higher triglyceride levels as well as higher plasma fibrinogen and PAI-1 levels contributing to atherosclerosis and thrombosis.

4.1.2.2 Physical Activity and Risk of CHD:

Mortality from CHD has been reported to be inversely related to the level of physical activity and to be reduced in subjects who exercise regularly (Paffenbarger *et al*, 1993). Physical fitness is a determinant of insulin sensitivity (Endre *et al*, 1994) and is a long-term predictor of mortality from cardiovascular disease in healthy middle-aged men (Sandvik *et al*, 1993). A recent study in Japanese men has shown that those who engaged in regular physical activity (>3 days per week) had fewer risk factors than sedentary individuals (Hsieh *et al*, 1998). In our study subjects were classified sedentary if they did not engage in physical activity at least once per week. There was no significant difference in age among the sedentary and physically active groups, but there was a high prevalence of physical inactivity in the proband group, and this was negatively correlated with the HDL-C level.

4.1.3 Dyslipidaemia:

This study confirms previous observations that Indian men and women with MI have significantly lower levels of HDL-C than controls with an approximate 20% difference in HDL-C levels. The expected inverse correlation of BMI and W/H ratio with HDL-C was observed in probands. None of the women were receiving oral contraceptives; so this does not

explain lower HDL-C levels in them. In contrast most men were ex-smokers and had taken alcohol so that part of the explanation for the lower HDL-C may be due to these factors.

Serum levels of high-density lipoprotein or apolipoprotein A-1 (apo A-1) are inversely correlated with the risk of CHD (Gordon *et al*, 1977). Genetic, hormonal and environmental factors determine HDL-C and apo A-I levels within distinct populations. In addition, HDL-C levels differ considerably among different ethnic groups. Obesity and insulin resistance are associated with low HDL-C in many populations, (Reaven *et al*, 1994, Despres *et al*, 1996) particularly in Indians in whom a high prevalence of diabetes exists (Stern, 1995). In addition to insulin sensitivity, the distribution of body fat is an important determinant of HDL-C levels. Intra-abdominal fat is considered to be a chief determinant of metabolic complications associated with cardiovascular risk and is reflected in measures of central obesity. Waist measurement is therefore considered to be a better predictor of CHD than BMI (Depres *et al*, 1990, Larsson *et al*, 1984). Waist/Hip ratio showed a strong inverse correlation with HDL-C in our study (Table IX). The HDL/TC ratio also showed a strong inverse correlation with BMI, W/H ratio and HbA1C levels in both probands and controls.

Table IX

SPEARMAN'S CORRELATION BETWEEN WAIST-HIP RATIO AND OTHER VARIABLES				
	PROBANDS		CONTROLS	
	r	*p*	*r*	*p*
Diabetes	.329	.000	.226	.044
Microalbumin	.281	.002	.092	.416 ns
SBP	.215	.020	.401	.000
DBP	.272	.003	.365	.001
Pulse Pressure	.196	.036	.159	.158 ns
PWV	.228	.013	.232	.038
LV Mass	.323	.000	.240	.032
HDL-C	-0.252	.006	.349	.002
HDL/TC Ratio	-0.290	.002	-0.195	.085 ns

4.1.4 Hyperinsulinaemia and CHD:

Epidemiologic studies have reported an association between insulin levels and cardiovascular events (Haffner *et al*, 1998). Although a recent meta-analysis has shown that hyperinsulinaemia is a weak indicator of cardiovascular risk the relationship appears to be stronger in middle-aged patients (Ruige *et al*, 1998).

A large trial has shown that there is a negative association between insulin sensitivity and carotid intima-media thickness, an indicator of coronary atherosclerosis (Howard *et al*, 1996). Few studies have examined the effect of insulin on arterial stiffness (Salomaa *et al*, 1995, Emoto *et al*, 1998). In the Atherosclerosis Risk in Communities Study (ARIC) the joint effect of hyperglycaemia, hyperinsulinaemia and hypertriglyceridaemia contributed synergistically to an increase in arterial stiffness. In our study we showed

significant correlations between insulin and obesity parameters but no correlation with microalbuminuria (Table X).

Table X

SPEARMAN'S CORRELATION BETWEEN INSULIN, MICROALBUMIN & OTHER VARIABLES					
		PROBANDS		CONTROLS	
		r	*p*	*r*	*p*
Insulin (1 hour)	BMI	.351	.000	.516	0.006
	W	.349	.001	.405	0.036
	W/H	.305	.003	.249	0.211 ns
	Pulse Pressure	.274	.007	.146	0.466 ns
Insulin (2 hour)	BMI	.342	.007	.405	0.076
	W	.309	.015	.072	0.763 ns
	W/H	.280	.028	.025	0.918 ns
Microalbumin	W/H ratio	.092	.416 ns	.281	0.002
	Fibrinogen	.237	.010	.028	0.807 ns
	Homocysteine	.021	.82 ns	.386	0.000
	Smoking	.249	.007	.254	0.023

4.1.5 Ethnicity, Diabetes and CHD:

Type II diabetes carries a strongly increased risk of cardiovascular disease. Since hyperinsulinaemia precedes type II diabetes and is associated with an adverse cardiovascular risk profile, it is thought that insulin resistance might be operative in the pathogenesis of CHD and type II diabetes (Stern, 1996), and may be explained on the basis of endothelial dysfunction which is an early finding in these patients. Insulin resistance, hyperinsulinaemia and impaired glucose tolerance are key components of the metabolic syndrome. Ethnic background appears to be a strong determinant of the relationship between insulin and CHD (Ruige *et al*, 1998). This may explain ethnic differences in the prevalence of diabetes and the propensity to heart disease. South Asian

diabetics have a significantly higher risk of myocardial infarction and death from cardiovascular disease than white diabetics (Mather *et al*, 1998).

Hughes *et al* (1990) reported that the higher mortality for coronary heart disease could not be explained by smoking, blood pressure and serum cholesterol but he felt that low levels of HDL-C and higher rates of diabetes were part of the explanation. He showed that Asian patients with NIDDM had higher mean body mass index, waist/hip ratios and abdominal girth. They also had a higher prevalence of hypertension, lower HDL-C and higher PAI-1 levels, components of the metabolic syndrome that increase the risk of atherosclerosis and thrombosis. However, he found no differences from controls for cigarette smoking and LDL-C, (Hughes *et al*, 1998). These correlations are also borne out in our study (Table XI).

Table XI

SPEARMAN'S CORRELATION BETWEEN DIABETES AND OTHER RISK FACTORS				
	PROBANDS	***p***	**CONTROLS**	***p***
BMI	.433	.000	.254	.023
Waist	.426	.000	.355	.002
W/H Ratio	.329	.000	.072	.524
BP	.193	.037	.161	.153
Pulse pressure	.231	.013	.205	.069
PWV	.298	.001	.232	.038
LV mass	.038	.686	.231	.039
E/A ratio	-0.242	.008	.145	.204
Triglycerides	.235	.002	.160	.155
Microalbuminuria	.276	.003	.355	.001

As a group Indians have a high genetic risk for diabetes. Adult offspring of diabetic parents exhibit hyperinsulinaemia and decreased insulin sensitivity long before the development of glucose intolerance (Ramachandran *et al*, 1998). Similarly, our study of risk factors in medical students revealed that Indian students exhibited metabolic changes including hyperinsulinaemia more frequently than their Black counterparts (Morar *et al*, 1998).

A field survey of South African Indians in Durban, (Seedat *et al*, 1990) showed that at least two thirds of Indian subjects had a major risk factor for coronary heart disease with a high prevalence of insulin resistance. In Britain Mckeigue *et al* (1991) found that the high prevalence of insulin resistance in the South Asian population was associated with coronary heart disease and obesity. Similar results have been shown for Asian Indians in the USA in whom resistance to insulin suppression of free fatty acid levels has been shown to be associated with metabolic risk factors (Laws *et al,* 1994). In an

early report of coronary heart disease in 3 different racial groups in Durban diabetes was detected in 47% of Indians with coronary heart disease (Thandroyen *et al*, 1980). Subsequently, Sewdarsen *et al* (1991) studied 131 Indian type 2 diabetic patients 3-4 months after myocardial infarction. He found that the diabetic subjects were older and more likely to be hypertensive, whilst smoking and a family history of coronary heart disease was common in non-diabetic subjects. Amongst women with myocardial infarction he found that diabetes mellitus was the commonest risk factor, was present in 78% of patients and was associated with a family history of myocardial infarction in first-degree relatives (Sewdarsen *et al*, 1988).

4.1.6 Family History:

A family history of premature CHD in a first degree relative is recognised as an independent risk factor for coronary disease (Myers *et al*, 1990). Epidemiological studies suggest that first degree relatives of coronary patients have a 2.5 to 7 fold increase in risk of death from CHD compared to those without a family history of CHD (Slack *et al*, 1966). In our study, the inherited basis of CHD is likely to be represented in the large group with a strong family history of premature CHD (48%), although the influence of factors such as raised homocysteine and Lp*(a)* levels cannot be excluded.

Whilst clustering of risk factors within families suggests that this may be due to a genetic influence, particularly in those with diabetes, it is apparent from the dietary and behavioural habits that an environmental influence probably contributed to their cardiovascular disease. Most interesting in our study was the fact that there were hardly any subjects who had no identifiable risk factors (n = 4). In most subjects shared environmental influences are likely to have had an important effect. Whilst single gene abnormalities such as the LDL receptor mutation have been linked to CHD later in life, in most cases the development of atherosclerosis is likely to have had a polygenic basis against a

setting of environmental risk factors.

Inherited vascular risk may be mediated by a number of mechanisms. Several major risk factors such as diabetes and hyperlipidaemia are known to be genetically influenced, including levels of newly identified risk factors such as homocysteine, Lp*(a)* and fibrinogen. In addition, endothelial dysfunction has been demonstrated in humans with clinical evidence of atherosclerosis (Vita *et al*, 1990) as well as in young asymptomatic subjects who have established cardiovascular risk factors such as smoking (Celemajer *et al*, 1993) and diabetes (Clarkson *et al*, 1996). It is likely that a variety of genes interact to accelerate damage from these risk factors.

Identification of unaffected siblings at risk therefore becomes an important target in the primary prevention of CHD in this high-risk population. A marker to select those young people with a strong family history of premature CHD who are at risk may lie in determination of genetic patterns, insulin and homocysteine levels, markers of endothelial dysfunction (microalbuminuria) and arterial stiffness (PWV).

4.2 ACE Gene Polymorphism:

The *ACE* genotype had no influence on the development of MI in this cohort of young Indians. Furthermore, no major impact of these polymorphisms on blood pressure, patient morphometry and biochemical parameters were detected.

4.2.1 Studies in Asian Indians:

There are three studies of *ACE* gene polymorphism in Indian patients with coronary heart disease. In Singapore Saha *et al* (1996) studied 155 Chinese and 72 Indians with myocardial infarction and showed no significant

association of the *ACE* gene with CHD or MI in either race group. In addition he showed that the frequency of the *D* allele was significantly lower than that reported in Caucasians.

In another study, Ramasawmy *et al* (1996) compared 85 young Mauritian male MI survivors with 108 control subjects and found no association between *ACE I/D* polymorphism and susceptibility to early onset myocardial infarction. In this study the frequency of the *D* allele was 42% in probands and 43% in control subjects. Although Gardemann *et al*, (1998) has shown that the *D* allele is associated with coronary heart disease in younger subjects (< 61.7 years) no clear association between *ACE* genotype and coronary heart disease has been demonstrated in the Indian population studies.

4.2.2 Meta-analyses:

A meta-analysis of 46 studies, totalling 32,715 subjects has shown that the *D* allele is associated with a 28% increase in plasma *ACE* activity, a 21% increase in the risk of MI and 16% increase in ischaemic heart disease for the *DD* genotype vs. *ID* and *II* genotypes. However, none of these risks were significantly increased when the largest studies were examined separately (odds ratio for MI: 0,9, for IHD: 1,09), suggesting that *ACE* gene polymorphism affects plasma *ACE* activity but not blood pressure and the risk of MI (Agerholm-Larsen, 2000). Given the confounding effects of *ACE*-inhibitor therapy, which almost all probands received, we did not measure renin, aldosterone or *ACE* activity in our patients.

In another meta-analysis of fifteen studies totalling 3394 myocardial infarction cases and 5479 control subjects Samani *et al* (1996) provided pooled estimates of the association between *ACE I/D* polymorphism and the risk of myocardial infarction but the strength of the association was weak. Several important limitations have emerged from this meta-analysis that could explain the

heterogeneity of results among studies. The studies were carried out in highly select groups of cases with less than ideal control subjects. Often cases were collected some time after the MI raising the possibility of selection by survival. Thirdly, using a funnel plot of sample size vs. odds ratio Samani *et al* (1996) showed that a significant trend towards positive associations were reported in the smaller studies, resulting an over-estimation of the true effect of the *D* allele. This reflects the importance of the size of the study needed to prove an association. If the risk of MI with the *DD* genotype is 26% then 1400 probands and a similar number of controls are required to have 80% power to detect a difference between *DD* and *II/ID* genotypes at a probability of 0,05.

Subjects similar to ours have been reported in a Norwegian study by Bohn *et al* (1993) who showed that the differences could be due to chance, undetected selection bias, different gene-environment interactions, or to preferential loss of *DD* individuals in the high-risk cohort.

4.2.3 Low Risk and *DD* Genotype:

There were too few subjects defined to be at lower risk of MI (by low body mass index and low cigarette consumption) and therefore one cannot comment on the association of the *DD* genotypes with MI by this stratification.

Although a few studies (Cambien *et al*, 1992, Ludwig *et al*, 1995, Keavney *et al*, 1995, Gardemann *et al*, 1995) have reported that the risk of MI associated with the *DD* genotype is increased in low risk subjects, a large well-designed study (Tiret *et al*, 1993) did not show this. In another cohort analysis of 388 Italian patients the *D* allele showed the strongest association for atherosclerosis but the distribution of the risk factors among the three genotypes was similar (Arbustini *et al*, 1995).

4.2.4 Mortality and *DD* Genotype:

Cambien *et al* (1992) reported that the *DD* genotype was slightly more prevalent in 610 patients who survived MI than in 733 controls (32% vs 27%), especially in individuals with below average lipids and body mass. In subjects at low risk of MI (apo B < 1, 25 g/l and BMI < 26 kg / m^2) the risk ratio of the *DD* vs. *ID* + *II* was 2,7 ($p < 0,0005$). In our study there was no difference in the frequencies of the genotypes between probands and controls, even when comparing 'low risk' probands to control subjects by this stratification.

The biological role of angiotensin converting enzyme suggests that the *ACE* gene is a candidate for myocardial infarction. The *D* allele has a frequency of 0,53 in Caucasians and is codominantly associated with higher plasma and cellular angiotensin converting enzyme levels. It therefore appears that the increased risk associated with the *DD* genotype may be related to higher intracardiac production of *Ang II* leading to coronary vasoconstriction and hypertrophy. This may also explain the higher prevalence of restenosis after PTCA in subjects with the *DD* genotype (Ohishi *et al*, 1993). In a large case-control study (ECTIM) an increased risk of MI and an increased frequency of parental MI were demonstrated in subjects carrying the *D* allele (Tiret *et al*, 1993). This relationship was stronger in subjects at low risk of myocardial infarction. Further evidence that the *D* allele could increase the risk of death in patients with coronary heart disease comes from the Belfast Monica Project which showed an increase frequency of the *D* allele in autopsy cases of MI (Evans *et al*, 1994).

Perhaps the largest study to date with conflicting findings is the prospective study of US male physicians by Lindpaintner *et al* (1995). In this study 1250 men with CHD were compared to 2340 control subjects according to age and smoking history. The presence of the *D* allele confirmed no appreciable increase in risk of coronary artery disease or 'myocardial infarctions' even

among low-risk subgroups.

In a recent analysis Samani *et al* (1996) prospectively studied 684 patients with MI and followed them up to assess the impact of the genotype on prognosis. He found that the genotype distribution did not influence the short-to- medium term prognosis after MI.

4.2.5 Diabetes Mellitus and *ACE* Genotype:

A field survey in Durban (Seedat *et al*, 1990) has shown that at least two thirds of Indians subjects have a major risk factor for coronary heart disease. Diabetes mellitus was strongly associated with a positive history of coronary heart disease. This finding is interesting since PIMA Indians have a low incidence of coronary heart disease despite a high prevalence of diabetes (Nagi *et al*, 1998)! It is thought that the lower frequency of the *D* allele among PIMA Indians may underlie their low risk of coronary heart disease. In contrast Ruiz *et al* (1994) found that the *DD* genotype is an independent risk factor for coronary heart disease in type II diabetes. The *D* allele has been found be a risk factor for MI in type II diabetes mellitus especially in those subjects judged to be low-risk. Keavney *et al* (1995) studied 173 newly diagnosed Caucasian type II diabetic patients who developed myocardial infarction and showed that the *D* allele was associated with MI in those with low plasma LDL-C or low triglyceride levels. We found no difference in the genotype frequencies in the diabetic subgroups in our study. In an extensive review Kennon *et al* (1999) performed a meta-analysis and showed that the *ACE* genotype had a significant impact on the progression of diabetic nephropathy. Significant odds ratios were shown in individuals with the *DD* genotype for coronary heart disease, myocardial infarction and both diabetic and non-diabetic renal disease in his study.

4.2.6 Limitations to Genotype Studies:

4.2.6.1 Homogeneity of Sample:

Studies of linkage disequilibrium are highly sensitive to selection of a genetically appropriate control sample (Samani *et al*, 1996). Case-control studies from populations with a heterogeneous genetic background can be misleading. For example, in two areas of Finland the prevalence of the *DD* genotype was different in two population-based samples (Perola *et al*, 1995).

In the original study by Cambien *et al* (1992) the Toulouse sample was not in Hardy-Weinberg equilibrium. This law is relatively robust and deviations from it could be explained by selection or by the size of the sample. The number of subjects in our study is small but few studies have been carried out in homogeneous populations including so many subjects (Agerholm-Larsen *et al*, 1997). Coronary heart disease is a multifactorial disease influenced by environmental and genetic factors. The effects of the genotype are determined by gene-gene as well as by gene-environmental interactions. Because of marked geographical differences in prevalence of risk factors gene polymorphisms must be evaluated in the homogeneous samples with the same coronary heart disease risk.

The conflicting results of association studies may also be partially explained by the remarkable differences of the allele frequencies in the populations investigated. For instance, the frequency of the *D* allele and the *DD* genotype is significantly increased in the hypertensive African-American population (Duru *et al*, 1994) compared with normotensives controls.

4.2.6.2 D Allele Dropout:

Studies reporting an association of the *I* allele of *ACE I/D* polymorphism could be biased by a deficit of the *D* allele in older subjects, as a consequence of the lethality of the *D* allele (Evans *et al*, 1994). In the present study those patients who died before admission to hospital or within the first few hours after admission were not included. The mean age of our subjects was under 45 years and we could find no evidence of a '*D* allele-dropout' when we stratified subjects by age. In addition, there was no significant deviation from the Hardy-Weinberg equilibrium for any of the polymorphisms studied. We are confident that the genotyping data are accurate since an insertion-specific primer was used in the analysis and the gels were read independently by two trained observers. Thus these results cannot readily be explained by selective loss of *DD* homozygotes, a finding that has been described previously (Morris *et al*, 1994).

In our study the restriction of the inclusion criteria to young patients with coronary heart disease who had myocardial infarction could have introduced a selection bias. Young patients were chosen to eliminate the confounding effect of age as a risk factor, and also to possibly enrich the genetic determinant in the study group. Several other pathogenetic, including thrombotic, mechanisms could be operative in this group.

4.2.6.3 Size of Sample:

Significant difficulties in the assessment of genetic studies have been the small numbers of subjects studied, (resulting in low discriminating power) and the presence of mixed genders. In the ECTIM study, an odds ratio of 1.3 was observed for those having the *V* allele in the *MTHFR* polymorphism. Assuming a dominant model and an allele frequency of 0.35 in controls, this would imply an allele frequency of 0.37 in cases and to replicate the results of the ECTIM study with 80% power, 950 cases and 950 controls would be required. The fewer number of subjects investigated may explain the lack of

an association of MI with the gene polymorphisms studied. It is therefore possible that larger investigations are needed to draw final conclusions.

Polymorphisms in genes related to cardiovascular homeostasis have often required multiple and large studies (ECTIM) to clearly define attributable genetic risk. The current study is therefore unable to answer this question and anticipates the pooling of data from different regions to achieve numbers affording adequate power. Successful application of this type of case-control study design, however, is dependent on the ability to identify a group of cases and control subjects from the same genetic pool. If allele frequencies of cases and control subjects differ for any reason that is not due to the disease process, such as population stratification, then false positive, or false negative results may occur.

In order to minimise this problem, cases and control subjects were drawn from the same homogeneous population in our study.

4.3 Angiotensin Core Promoter Element:

Previous reports suggest that variants of the angiotensinogen gene were associated with an increased risk of essential hypertension (Jeunemaitre *et al*, 1992, Brown *et al*, 1994, Hegele *et al*, 1994, Kamitani *et al*, 1994, Caulfield *et al*, 1994). Another potential mechanism by which angiotensinogen may influence susceptibility to CHD may be related to its abundant expression in human adipose tissue (Ailhaud, 1997). This may represent a potential link between blood pressure and insulin resistance, especially in obese subjects (Aubert *et al*, 1990).

4.3.1 MI, Hypertension and *Agt* Genotype:

Several gene loci encoding components of the *RAS* have been implicated in

cardiovascular diseases. Whereas there is continuing controversy regarding the role of the *ACE DD* genotype, the angiotensinogen gene has been consistently linked to hypertension and also contributes to the risk of CHD (Katsuya *et al*, 1995).

Tiret *et al* (1995) studied two *Agt* polymorphisms: *T174M* and *M235T* in 630 MI subjects. He found that the *T174M* and the *M235T* genotype distributions did not differ between MI survivors and controls, but subjects with BMI < 26 Kg/m^2 had a higher prevalence of hypertension if they carried the *M174* allele. In a recent meta-analysis, Staessen *et al* (1999) analysed 69 studies totalling 27,906 subjects and showed that the *T* allele was associated with an increased risk of hypertension in Caucasians, but not in Blacks or Asians. The *T* allele was associated with 7-11% rise in circulating angiotensinogen levels but was not associated with atherosclerosis or microvascular complications. In contrast to these findings, Staessen's *et al* (1997) earlier meta-analysis of 145 reports on 49,959 subjects showed that possession of the *D* allele of *ACE* gene polymorphism was associated with an increased risk of atherosclerotic and renal microvascular complications. In comparison with the *II* group the excess risk in *DD* homozygotes was 32% for CHD, 45% for MI, 94% for stroke and 56% for diabetic nephropathy. The corresponding risk in *ID* heterozygotes amounted to 11% for CHD and 13% for MI.

4.3.2 LV Size and Genotype:

Experimental and clinical data show that the *RAS* may influence progressive ventricular dilatation, ventricular function and outcome after MI. In addition to a direct toxic effect on myocardial cells, *Ang II* may induce hypertrophy in non-infarcted areas, activate the sympathetic nervous system, stimulate fibroblast proliferation, increase ventricular afterload and impair diastolic relaxation (Riegger *et al*, 1996). Modulating these mechanisms may explain

the beneficial effects of *ACE* inhibition, started early after MI and accounts for the reduction in morbidity and mortality for major cardiovascular events in clinical trials.

4.4 AT_1 Receptor Polymorphism:

The findings of the present study are at variance with previous reports. We have not shown any relation between the AT_1 receptor genotype and the risk of premature MI. This may be related to the low prevalence of severe hypertension in our patients. The AT_1 C allele has been shown to be more frequent in patients with severe hypertension and myocardial infarction (Tiret et al, 1994). In an allelic interaction study Hingorani et al, (1995) has shown that both SBP and DBP track with the I allele of the ACE gene and the 1166C allele of the AT_1 receptor gene. Although the AT_1 receptor genotype has been associated with hypertension, in cross-sectional studies this polymorphism has not been found to be associated with aortic stiffness.

In a study of all three *RAS* candidate gene polymorphisms, *(ACE I/D, Agt M235T, T174M* and *AT_1R A1166C)*, Riegger *et al (1996)* found that hypertensive patients reporting a parental history of MI before age 60 years had a higher prevalence of the *ACE D* allele. Similar results were obtained in patients reporting a parental history of stroke before age 65 years. The *Agt T235* allele prevalence was higher in male hypertensives and the AT_1 *C1166* allele higher in females.

Other studies of *RAS* gene polymorphisms have shown an interaction between the AT_1 receptor genotype and *ACE DD* genotype on the occurrence of MI. There is also recent evidence of synergism between the *ACE DD* genotype and the AT_1 1166 CC genotype that further increases the risk of ischaemic events after MI (van Geel et al, 1998). In addition the interaction between the AT_1

genotype and the ratio of TC/HDL on aortic stiffness suggests that the AT_1 1166 C allele confers a combined genetic susceptibility to cardiovascular risk in association with clinical risk factors. This does not appear to be so in our patients and probably applies to Caucasians.

4.5 Aldosterone Synthase Polymorphism:

This study does not support a role for *CYP11B2 C344T* polymorphism in this selected cohort of patients with premature myocardial infarction. The results are in keeping with a previous report which showed that genetic variation in the promoter of *CYP11B2* may influence blood pressure regulation but its association with CHD is at best weak (Hautanen *et al*, 1997).

Recently, White *et al* (1998) showed that the *-344 C* allele *CYP11B2* polymorphism is strongly associated with increase in LV size (White *et al*, 1998, Kupari *et al*, 1998) and decreased baroreceptor sensitivity and could be associated with MI in susceptible individuals. Analysis of 2007 participants in the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease Study), however, did not find any significant effects of this polymorphism on serum aldosterone, BP or cardiac size and function (Shunkert *et al*, 1999).

4.6 11β-Hydroxysteroid Dehydrogenase 2 Polymorphism:

Decreased activity of *11β-HSD2*, the enzyme that converts cortisol to cortisone may be associated with salt sensitivity, hypertension and the metabolic syndrome (Sharma *et al*, 2000). Although a recent study found an association of the *11BHSD2 G534A* marker with end-stage renal disease (Smolenicka *et al*, 1998). Brand *et al, (*1998) studied a population of 347 hypertensive sibling pairs and found no association with hypertension in

Caucasians. She concluded that this gene may be relevant in other populations. The frequency of the substitution has been reported to be about 5% (Brand *et al*, 1998). In this study we investigated the prevalence of *11BHSD2 G534A* gene polymorphism in premature MI and found no association since the genotype frequencies were similar in cases and controls. The association was also not significant when comparing subgroups including hypertensive probands.

4.7 Transforming Growth Factor-β Polymorphism:

The analysis reveals that the *Ile 263* polymorphism is infrequent in the Indian population. To test the validity of our technique we used known positive mutant homozygotes as controls from Benjamin Franklin University and were able to reproduce the findings. Interestingly we identified no individuals homozygous for the rare allele of the gene variants, although this was expected with the fewer numbers included in the present study. Given the low frequency of the *Ile 263* variant it is not possible to make any inferences about the genetic control of serum levels of *TGF-β_1* in this cohort. Whether other polymorphisms in the promoter region account for this and so predispose to cardiovascular pathology is not clear.

Although increased serum levels of *TGF-β* have been reported in diabetes (Pfeiffer *et al*, 1996) and atherosclerotic disease (Blann *et al*, 1996) studies of five polymorphisms in the *TGF-β_1* gene have not shown any association with CHD or hypertension (Syrris *et al*, 1998). Further studies have reported not only elevated circulating levels of this cytokine in coronary artery disease (Wong *et al*, 1997, Tashiro *et al*, 1997) but also increased expression in heart muscle after MI (Sharma *et al*, 1999, Fukumoto *et al*, 1998, Sun *et al*, 1998) and on restenotic vascular lesions (O'Brien *et al*, 1996). Furthermore, there is evidence that many cytokines are upregulated in the intima and there is increased responsivity to PDGF and *TGF-β* in medial smooth muscle cells in

diabetic patients. These changes lead to proliferation and migration of smooth muscle cells into the intima and further accelerate the formation of the atherosclerotic lesion (Miyagawa *et al*, 1999).

Seven polymorphisms in the *TGF-β_1* gene have been reported in the ECTIM study (Cambien *et al*, 1996). One of these polymorphisms, the presence of the Arg^{25} allele, was associated with higher blood pressure and a family history of hypertension in the normotensive controls compared with individuals with the Pro^{25} allele. The Arg^{25} allele has also been associated with increased *TGF-β_1*, production and fibrosis.

4.8 Methylenetetrahydrofolate Reductase Polymorphism:

This is the first study to report homocysteine levels in relation to *MTHFR* genotype in young Indian MI survivors. An abstract by Kotze *et al* (1999) has shown that the *MTHFR* gene polymorphisms were more common in Caucasian than in Africans in South Africa but she did not report findings in Indians. Although raised homocysteine levels were common, our results could not confirm that the *TT* genotype in the *MTHFR* gene is a strong predictor of raised plasma homocysteine since the genotype was infrequent in this cohort. The positive results from retrospective studies may reflect a consistent bias resulting from measuring homocysteine after the acute vascular events.

Although the association between homocysteine and atherothrombotic events appears to be independent and dose-related it remains to be established whether it is causal and modifiable. Due to the close coupling between folate metabolism and the methylation of homocysteine to methionine, the plasma level of homocysteine is strongly related to serum and red cell folate (Jacques *et al*, 1996). Because blood levels of folate and B12 are related inversely to homocysteine, nutritional deficiency leads to an increased risk of hyperhomocysteinaemia. Although we did not measure serum folate and B12

levels in our patients their nutritional status appeared satisfactory and we did not suspect this as a cause of raised homocysteine levels in our patients.

The mechanism by which homocysteine may cause vascular damage is unclear. There is experimental evidence that homocysteine promotes atherogenesis by facilitating oxidative arterial injury, so damaging the vascular matrix and enhancing vascular smooth muscle cell proliferation. It may also promote thromboembolic disease by causing oxidative damage to the endothelial lining leading to impaired vasomotion and procoagulant effects (Hankey *et al*, 1999).

4.8.1 Homocysteine and CHD:

An elevated plasma homocysteine is reported to be a risk factor for accelerated CHD. As pointed out earlier, the effects of high plasma homocysteine levels has not been shown to be mediated by the known coronary risk factors (Tonstad, 1997). These findings are in contrast to a study in an Indian population with relatively low cholesterol levels in which raised fasting homocysteine was an independent risk factor for CHD (Chambers *et al*, 1999). In our study there was no correlation between homocysteine levels and myocardial infarction. Our findings are in keeping with Chacko (1998) who studied 56 patients with CHD and found that plasma homocysteine was not a major risk factor in the Indian population.

Recently, a large study of 2453 male Caucasians showed that there was indeed no association between *MTHFR C677T* gene polymorphism and the risk of CHD and myocardial infarction (Gardemann *et al*, 1999). Carriers of the *TT* genotype with high-risk profiles, however, had higher CHD scores than individuals with one allele.

4.8.2 *MTHFR* and Premature CHD:

Tsai *et al*, (1996) studied a patient population of premature CHD, similar to our study and showed that 39% of their patients with CHD had elevated plasma homocysteine. However, although the *TT* genotype was more common (22.8%) in individuals with raised homocysteine (10.7%) the frequency of the *TT* genotype was not increased in patients with premature CHD (10.6%) as compared to controls (14.6%). This finding is agreement with recent publications (Ma *et al*, 1996, van Bockxmeer *et al*, 1997, Brugada *et al*, 1997, Christensen *et al*, 1997, Verhoef *et al*, 1997, Gardemann *et al*, 1998). Thus, data from several studies suggest that the *TT* genotype is not an important risk factor for CHD (Verhoef *et al*, 1997). It appears that plasma homocysteine may be an innocent bystander or marker rather than be playing a causative role in the atherosclerotic process in the vessel wall. Alternatively, the *TT* genotype may be a marker of risk in individuals with low folate levels.

4.8.3 Limitations to the Study:

Whereas some investigators have identified the *TT* genotype as a risk factor for CHD others have failed to identify a link between this genotype and accelerated atherosclerosis. These discrepancies might be due to differences in the study design. This study failed to detect an association of *MTHFR* gene polymorphism with non-fatal myocardial infarction. It should be noted that probands constituted survivors of myocardial infarction and not patients with fatal outcome of this disease. This bias in selection could explain, in part, some of the discrepancy in the findings. It is also not known to what extent other gene variations, also postulated to be associated with CHD, might interfere with the link between the *MTHFR 677T* gene polymorphism and the risk of myocardial infarction. Potential candidates are polymorphisms of the *RAS* genes, as well as genes involved in lipid and glucose metabolism, since

diabetes mellitus was clearly a major contributor to CHD risk in this cohort.

4.9 G-Protein Polymorphism:

This study reveals no association between *GNβ3 (825T)* polymorphism and premature MI. Although Siffert *et al*, (1998) demonstrated a significant association of this polymorphism in hypertensive patients a large scale study in 681 hypertensive and 564 MI subjects failed to show any association with hypertension or MI (Brand *et al*, 1999). In the present study the *825T* allele frequencies were 0.73 in both probands and controls. Because increased NHE-1 activity is associated with LVH and susceptibility to nephropathy in insulin-dependent diabetes, (Siffert *et al*, 1996) we also analysed the diabetic subgroup separately but found no increase in the frequency of *825T* allele here.

There is also new evidence that the G-protein β_3 subunit *825T* allele is associated with high BMI in male Caucasians (Sharma *et al*, 2000). Recently, Naber *et al*, (1998) investigated *GNβ3 C825T* polymorphism in 617 patients undergoing coronary angiography. The *825T* allele was associated with a two-fold increase in the risk of CHD and MI.

4.10 Pulse Wave Velocity:

Although close correlations have been shown between PWV and atherosclerosis in elderly subjects (van Popele *et al*, 1998) no study has looked at PWV in younger patients (< 55 yr), so removing the effects of ageing with established CHD. Nor has any study attempted to correlate PWV with risk factors and gene polymorphism. In this study PWV was measured in all fasting subjects (at trough level for all cardiovascular medications) and correlated with the risk factors, including echocardiographic parameters and

genetic patterns.

4.10.1 PWV and Gene Polymorphisms:

In this study polymorphisms of the *RAS* genes were not related to the development of aortic stiffness. Benetos *et al* (1995) showed no influence of *ACE* gene polymorphism on aortic stiffness but Castellano *et al*, (1995) showed that medial-intimal thickness of the carotid artery is associated with the presence of the *D* allele. Significant differences in aortic stiffness have been found among the AT_1 genotypes: the presence of the *C* allele of the *A1166-C* polymorphism was associated with increased aortic stiffness in hypertensive but not normotensive patients. (Benetos *et al*, 1995). In a study of other *RAS* genes Pojoga *et al* (1998) analysed two polymorphisms of the aldosterone synthase *(CYP11B2)* gene and found that the presence of the –344C allele was associated with elevated levels of plasma aldosterone and PWV in patients with essential hypertension.

4.10.2 Pulse Wave Velocity and Risk Factors:

In keeping with previous studies (Chanudet *et al*, 1989), this study showed that age, blood pressure and diabetes were the main determinants of central PWV (as measured in the aorta). We also found significant correlations between PWV and W/H ratio in keeping with the effect of BMI (Toto-Moukouo *et al*, 1986) and anthropometric measurements (London *et al*, 1995) on PWV.

Surprisingly there was no correlation between PWV and smoking although both parameters were independently correlated with microalbuminuria. Active smoking deteriorates elastic properties of the human aorta (Nakamoto *et al*, 1989). Numerous studies show that smoking causes a significant increase in PWV in both medium-sized and large arteries (Failla *et al*, 1997, Levenson *et*

al, 1987). Our results could be related to the fact that although 80% of probands smoked, the smoking consumption was not quantified and PWV was not correlated with the degree of exposure to nicotine.

4.10.3 PWV and Diabetes:

Studies in Type II diabetes suggest that increased PWV results from an underlying diffuse atherosclerotic process, often not detectable clinically, (Lehmann *et al*, 1992) and related to the duration of diabetes (Tanokuchi, 1995). Significant alterations have been seen even in younger subjects and relate to glycaemic control (Paillole *et al*, 1989, Asmar *et al*, 1995). Significant correlations have also been shown between PWV and the presence of microalbuminuria or proteinuria in diabetic patients, suggesting that PWV may be a reliable index of vasculopathy in these patients (Takegoshi *et al*, 1991) PWV may be used to detect siblings of diabetic patients who are at high risk for CHD (Hopkins *et al*, 1996).

4.10.4 PWV and CHD:

In the early stages of atheroma infiltration of LDL-C into the intima leads to an increase in aortic distensibility. In patients with FH, however, no significant correlation has been found between PWV and serum cholesterol (Avolio *et al*, 1985). With advancing age, the 'atherosis' develops a sclerotic component in the vessel wall leading to a decrease in aortic distensibility (Lehmann *et al*, 1992,1995).

Increased aortic PWV in patients with CHD was first demonstrated by Simonson *et al* (1960) when they showed a mean difference of 1.68 m/s between patients with and without CHD. It has been suggested that aortic/carotid distensibility might be used as an indicator to distinguish between patients with and without CHD (Dart *et al*, 1991, Barenbrock *et al*,

1995). In a study similar to ours, the relationship between arterial compliance and risk factors was examined in a group of newly diagnosed patients with CHD and controls matched for age, sex, smoking status and serum cholesterol levels. The beta stiffness index was significantly higher in the CHD group (Cameron *et al*, 1995). They found that there was no significant correlation shown between compliance and lipid levels, but there was a correlation between PWV and microalbuminuria ($p = 0.003$).

4.10.5 Limitations of the Study:

This study showed strong correlations between PWV and major risk factors reflecting the vasculopathy associated with this parameter. Although PWV is strongly correlated with direct measurements of arterial distensibility and is an excellent surrogate evaluation of arterial stiffness, there are limitations in this technique: the critical factors are the precise measurements of pulse transit time and the length of the vascular segment. The surface vascular length is an approximation that might underestimate the true vascular length, particularly in the elderly with unfolded aortas. Our patients were young, so that this was not a problem and an on-line pulse wave recording by a validated method permitted precise measurement of transit time (Asmar *et al*, 1995). However, our control subjects were slightly older and this may explain the absence of any difference between probands and controls since PWV increases with age. Furthermore, the effects of smoking on PWV need to be re-evaluated taking into account the degree of nicotine exposure.

4.11 Allelic Association Studies:

Until recently, no study has systematically evaluated genetic polymorphisms of all components of the *RAS*. Tiret *et al* (1994) was the first to report a synergistic effect of angiotensin converting enzyme and AT_1 receptor gene

polymorphism on the risk of myocardial infarction. He found a significant interaction between these polymorphisms. The odds ratio for myocardial infarction associated with the angiotensin converting enzyme *DD* genotype was 1.05 without AT_1 receptor C allele, 1.52 in *AC* heterozygotes and 3.95 in *CC* homozygotes (test for trend $p < 0.02$). This interaction was even stronger in low risk (Apo B < 1.25 g/L and BMI < 26 $kg.m^2$) subjects.

Three different genotypes were analyzed in the CORGENE Study (Jeunemaitre *et al*, 1997): *ACE I/D*, *Agt M235T* and AT_1 receptor *A1166C*, but no significant association was observed between these polymorphisms and the MI (n = 156) and non-MI (n = 307) subjects. Only the *Agt 235T* allele was associated with the extent of the coronary lesions or angiographic score. Similarly Biggart *et al* (1998) analysed *ACE*, *Apo E* and *TGF-β* genes in patients with early onset of CHD and found no association.

More recently Gardemann *et al* (1998) re-evaluated the evidence for ACE/AT_1 receptor synergism by studying 2244 Caucasians with angiographically-defined CHD. He found no association for a synergistic effect in patients with MI or coronary heart disease. In another case control study Ludwig *et al* (1997) searched for association between the *ACE I/D* and angiotensinogen *M235T* polymorphisms in two different population samples. He found that within selected groups in the ARIC sample, *ACE D* and *Agt 235T* alleles were associated with CHD and MI and that there was a synergistic interaction between the two alleles. Comparable tests in the Framingham sample, however, failed to support an association of these markers with CHD.

Another study which searched for interaction between the *ACE I/D* and the AT_1 receptor locus was reported by Berge *et al*, (1997) in 235 MI survivors. This study found no such interaction but a higher frequency of *CC* homozygotes was found in males in the low risk group. Thus, only few studies have examined the potential interaction at the *RAS* genetic loci and these have shown that the association is neither strong nor consistent, and probably involves a complex interaction among risk factors and genotypes.

CHAPTER 5

CONCLUSION

5.1 Established Risk Factors and Premature MI:

5.1.1 Obesity as a Risk Factor:

Weight gain increases the risks of type 2 DM, premature atherosclerosis and hypertension. There is a linear association between weight gain and CHD risk: for every 10% increase in weight CHD mortality increases by 17% (Rosengren *et al*, 1999).

In this study we took the cut-off point for increased risk as BMI > 25 kg/m^2 which currently defines overweight in Caucasians. At a recent obesity symposium (Sharma *et al*, 2000) it was pointed out that obesity-associated co-morbidity may increase rapidly in non-Caucasians when BMI exceeds 18 g/m^2. The cut-off point in our Indian population should therefore be lower, probably around 22-23 kg/m^2. Using this level at least 80% of our probands were classified overweight, as opposed to 66% of the control population ($p < 0.05$).

5.1.2 Dyslipidaemia as a Risk Factor:

Hypercholesterolaemia is a major risk factor for the development of CHD. The interaction between lipoproteins and the *RAS* may explain why *ACE* inhibitors attenuate the hypercholesterolaemia-induced atherosclerosis in various animal models (Sugano *et al*, 1996, Chobanian *et al*, 1990). Since *Ang II* exerts its vasoconstrictive and proliferative effects through activation of AT_1 receptors we looked for interaction between hypercholesterolaemia and AT_1 receptor gene polymorphism.

Although there was no correlation between cholesterol levels (TC and LDL-C) and the risk of MI, (probably because patients received treatment), the

atherogenic lipoprotein phenotype was especially prominent in probands and was characterized by low HDL-C and high triglyceride levels.

Epidemiologic data indicate a strong inverse correlation between plasma HDL-C and CHD (Gordon, 1977, Rhoads *et al*, 1976). In fact the most frequent lipid abnormality in patients with CHD before the age of 60 years is a low HDL-C level. In experimental studies HDL-C administration inhibits the development of fatty streaks and induces regression of atherosclerotic lesions in cholesterol-fed rabbits (Badimon *et al*, 1989,1990). Although the generally accepted mechanism for this effect is reversed cholesterol transport (Stein *et al*, 1997) there is now evidence showing that HDL-C can reduce the atherogenicity of LDL-C by inhibiting oxidative modification (Mackness *et al*, 1995). Further evidence of a more direct anti-atherogenic effect of HDL-C are its ability to improve endothelial dysfunction (Cockerill *et al*, 1995) inhibit platelet aggression (Nofer *et al*, 1998) and cholesterol accumulation in foam cells (Viñals *et al*, 1997).

5.1.3 Smoking as a Risk Factor:

In a field survey smoking (> 10 cigarettes per day) was found to be a major risk factor for CHD in South African Indians (Seedat *et al*, 1999). It was present in 40.8% males and 5,7% females. A substantially higher proportion of females (33%) smoked fewer cigarettes (> 1 a day).

Taken together the analysis of major modifiable risk factors, i.e. smoking, hypercholesterolaemia, hypertension and diabetes, showed that 68% of the Indian population had at least one major risk factor for CHD. Hypertension and smoking were the most frequent combination in men, while in women hypertension and diabetes frequently occurred together. It is therefore not surprising that 82% of probands were smokers in our cohort of premature MI.

5.1.4 Insulin as the Intermediate Phenotype:

In order to establish whether a candidate gene is truly associated with the disease the intermediate phenotype i.e. gene product must be shown to be related to the genotype. Identification of a key intermediate phenotype for diabetes, insulin levels, revealed an association with the metabolic syndrome. Better determination of the phenotype in future studies may help in clarifying the association of genotype with disease e.g. urinary aldosterone and cortisol levels in relation to aldosterone synthase and/or *11β-HSD*, and plasma renin and *ACE* levels in relation to the *RAS* genes. This was not performed in this study because the majority of patients were receiving *ACE* inhibitor and beta-blocker therapy.

5.2 Molecular Genetics in the Understanding of CHD:

5.2 .1 Gene Polymorphisms in the Promoter Region:

This study was unable to show that polymorphisms in the *RAS* genes were related to early onset MI in Indians. Since mutations in the coding regions of the candidate genes that we studied were uncommon, attention was turned to polymorphism in the regulatory (promoter) regions of these genes. Such DNA variation may alter gene transcription and is a potential basis for clinically important gene-environment interactions.

Therefore, polymorphisms in the promoter region of the angiotensinogen gene and the aldosterone synthase genes were examined and again no genetic susceptibility to MI was found. Other newly discovered polymorphisms in the promoter regions include genes for the LDL-C receptor, lipoprotein lipase and PAI-1, all of which are associated with MI at a young age. These genetic components may contribute to the dysregulated extracellular matrix metabolism that is so characteristic of atherosclerosis and plaque rupture.

Common polymorphisms occurring in the promoter region of these candidate

genes could account for the substantial differences in the metabolism of triglyceride-rich lipoproteins and the ensuing risk of CHD. There is also evidence that fatty acids control triglyceride metabolism by modulating the transcription of the lipoprotein lipase and *Apo C-III* genes through activation of peroxisome proliferator-activated receptor (PPAR) gamma (Hamsten, 1996).

5.2.2 Genetics and Obesity:

Identification of genetic determinants of complex disorders such as obesity, hypertension and atherosclerosis is a daunting task. In this study we did not specifically examine obesity-related genes. However, it appears that the *RAS* is involved in the development of obesity-related hypertension since there is convincing evidence (Sharma *et al*, 2000) that *Ang II* plays an important role in the development of adipose tissue by virtue of its ability to recruit adipose precursor cells.

5.2.3 The Putative Gene for CHD:

A clinical consequence of genotype-specific regulation of biological mechanisms would be that certain individuals are more prone to atherosclerosis when exposed to environmental risk factors. Thus, knowledge of an individual genotype might be predictive of future atherosclerotic risk. Although unidentified, genetic loci probably underlie the variable susceptibility to environmental risk factors for CHD in our subjects. Individuals identified by genotype may be monitored with special care and given specific advice on lifestyle changes geared to their genotype.

Our study, however, does not show any influence of genotype patterns of the polymorphisms we studied on the risk of premature MI. Since gene pools, lifestyles and gene-environment interactions differ between populations one

cannot assume that a given genetic trait will have similar impact in risk in all populations. Recent reports of molecular genetic analyses using allelic association studies have suggested that genes involved in lipoprotein metabolism and fibrinolytic pathways are also associated with coronary atherosclerosis or MI.

Mild to moderate hypertriglyceridaemia, generally accompanied by low levels of HDL-C was present in a substantial number of case-patients with premature MI. The molecular genetic pathology of this condition is still ill defined. Potential candidate genes include the Apo-A-1, C-III, A-1V cluster and lipoprotein lipase which need to be studied in this high risk group. In contrast Lp*(a)* might be a monogenic risk factor for CHD, accounting for a significant portion of the familial predisposition to CHD that cannot be explained by other established risk factors in low risk groups.

The strong family history of MI and biochemical parameters suggests that the low levels of HDL-C and raised triglycerides may be occurring against a background of another major risk factor. This is likely to be a polymorphic gene that is directly involved in HDL-C metabolism. Examples of such biallelic genes include lipoprotein-lipase, hepatic lipase and cholesterol ester transfer protein, which need to be evaluated in future studies in this population (Nordestgaard *et al*, 1997).

CHAPTER 6
SUMMARY AND CLINICAL IMPLICATIONS

6.1 Summary:

When familial clustering of coronary heart disease was first recognised in the 1950's it was noted to be especially prominent in families of patients with early-onset disease. In most cases the risk associated with a positive family history could not be attributed to familial aggregation of established risk factors. Until recently little was known of the genetic basis of underlying individual susceptibility to coronary heart disease. Since coronary heart disease and essential hypertension often overlap genes involved in blood pressure regulation became logical candidates for studying susceptibility to CHD. To delineate the genetics of this common and multifactorial disease is extremely difficult because a host of genes are likely to be contributory factors. Furthermore, it is likely that clinical disease results from interactions between heritable and environmental factors.

6.1.1 Clinical Characteristics

Established major risk factors for CHD could be identified in the present study sample. These included lipid parameters, diabetes mellitus, cigarette smoking and family history. In addition, coronary prone dietary and sedentary behavioural patterns as well as lower educational status were contributory factors. Our findings are consistent with those of Hughes *et al* (1990) who found that the higher mortality from CHD to be associated with lower HDL-C and higher rates of DM in this group.

Markers of insulin resistance: hyperinsulinaemia, increased waist, W/H ratio, atherogenic lipoprotein phenotype, diabetes and family history suggested that

some degree of heritability could have predisposed to CHD in the setting of risk factors; in this respect genetic variations may have played a significant role in pathogenesis.

6.1.2 Genetic Analysis:

Analysis of eight candidate gene polymorphisms for CHD showed no differences in genotype distribution or allele frequencies in case-patients and normal control subjects. Both case-patients and controls showed no deviation of genotype distribution from the Hardy-Weinberg equilibrium. The genotype frequencies for these eight polymorphisms are different from those described in Western series, emphasizing the importance of choosing samples from ethnically homogeneous populations in studies of common genetic variations (Hamsten, 1996).

There are very few studies that have evaluated potential interaction of the angiotensin converting enzyme gene with other *RAS* gene polymorphisms on the risk of MI. There was also no significant interaction between these alleles and those at any of the other loci studied for the presence of CHD. There was no relationship of risk factors to the candidate genes studied, nor was there a significant association of any of the genetic markers with CHD.

6.1.3 Newer risk factors:

In this study Lp*(a)* and homocysteine levels were elevated in the case-patients but the differences were not significant. Larger cohorts are needed to show that these risk factors are markers of premature atherosclerosis in this population.

6.2 Conclusion:

In conclusion the present study does not support the relevance of the candidate gene loci studied to myocardial infarction in young Indian MI survivors. It is likely that complex diseases such as CHD are usually not controlled by a single disease locus but often involve multiple genetic and/or environmental factors that are subject to specific gene environment and gene-gene interactions.

To date, no study has assessed the cumulative effects of several candidate loci simultaneously. In the present study polymorphism of five candidate genes of the *RAS* was examined. There was no evidence of any statistically significant association between gene cluster polymorphism and MI. There was also no association between these polymorphic sites and lipid levels in this cohort of predominantly male, young adult survivors.

The findings of this study therefore reinforce the major contribution of behavioural and environmental risk factors in the causation of CHD in this ethnic group. The findings do not exclude the role of predisposing genetic factors in this group. Indeed diabetes mellitus was a major, probably heritable, risk factor and therefore studies of adequate power, designed to investigate candidate genes regulating glucose, lipid and tissue metabolism are strongly indicated. Potential candidate genes are lipoprotein lipase, paraoxonase and PPARγ. These gene variations may account for the dyslipidaemia associated with insulin resistance and the predisposition to the atherogenic lipoprotein phenotype so characteristic of this cohort (Aubo *et al*, 2000).

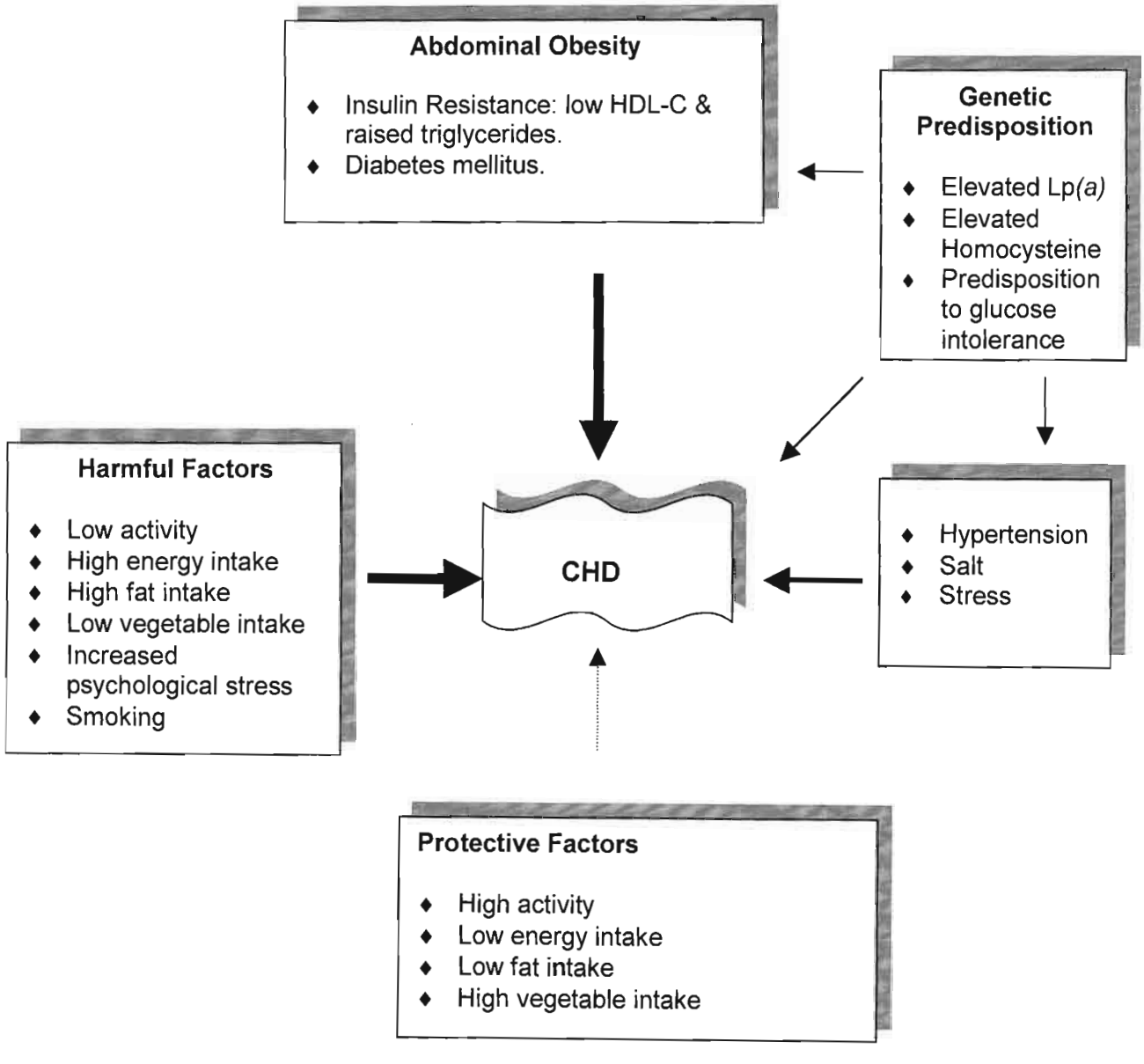

Fig 29: **Role of Genetic Predisposition in Relation to Risk Factors in Atherosclerosis**

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APPENDIX I

Please indicate what you will tell the subjects in simple language and addressed to them. The procedure or treatment which will be applied should be described and reference should be made to possible side effects, discomfort, complications and/or benefits.

It must be made clear to the patient that he/she is free to decline participate or to withdraw at any time without suffering any disadvantage or prejudice.

Index case: You have heart disease due to blockage of blood vessels that supply blood to your heart muscle. It is not exactly known why you have this kind of heart disease at such a young age. We would like to ask you a few questions, study your heart and take a sample of blood from your vein to find a reason for this.

You are free to decline and this will not affect your treatment in the future.

APPENDIX II

DNA Extraction Protocol:

A) DNA Extraction Method for Whole Blood (EDTA)

5-10 ml aliquot

1. Add 35 ml reagent 1 to 5 ml EDTA anticoagulated blood.
2. Rotary mix for 4 minutes, then spin at 3500 rpm for 4 minutes.
3. Discard the supernatant.
4. To the WBC pellet, add 2 ml reagent 2 and vortex to resuspend.
5. Add 500 µl reagent 3 and rotary mix for 15 minutes.
6. Incubate at 65^0 C for 25 minutes, occasionally mixing by hand.
7. Add 2 ml ice cold chloroform and rotary mix for 10 minutes.
8. Spin at 3500 rpm for 5 minutes.
9. Aspirate the supernatant, place into a clean tube, add an equal volume of phenol:chloroform: isoamyl alcohol (pH8.0) and rotary mix for 5 minutes.
10. Spin at 3500 rpm for 5 minutes and aspirate the upper phase to a clean tube. Repeat the P:C:IAA extraction until the interface is clear of protein deposit.
11. Add 5 ml cold absolute ethanol to precipitate the DNA.
12. Pellet the DNA: spin at 4000 rpm for 5 minutes.
13. Wash the pellet with 70% ethanol.
14. Dry the pellet and dissolve in 1 ml 1 x TE buffer (pH8).

(B) REAGENTS:

Reagent 1:

		1L:
Magnesium chloride hexahydrate	(MW 203,3) - 5mM	1.02 g
Sucrose	(MW 342.3) - 320mM	109.54 g
Tris. HCI	(MW 157.56) - 10mM	1.58 g
Triton X- 100		100 ml

- Adjust the pH to 8.0 with 2 M NaOH (MW 40) - 8g in 100ml water.

Reagent 2:

			0.5L:
Tris. HCI	(MW 157.56)	- 400 mM	31.51 g
Sodium Chloride	(MW 58.44)	- 150 mM	4.38 g
EDTA	(MW 372.24)	- 60 mM	11.17 g

- 1% SDS to be added after autoclaving the above solution - ie add 50 ml of a 10% SDS solution. To make 50 ML 10% SDS, add 5g SDS to 40 ml water, heat to 68^0 C and when dissolved, add water to make up to 50 ml.
- Adjust the pH to 8.0 using 2 M NaOH.

Reagent 3:

		10 ml:
Sodium perchlorate	(MW 140.46) - 5 M	7.023 g

APPENDIX III: PCR CONDITIONS:

1. Angiotensinogen Core Promoter (Agt-CP) PCR:

All PCR to detect the Agt-CP mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01. Gene Amp 9600 PCR System 1991) and primers as reported by Jeunemaitre *et al*, (1992). The sense and antisense primers were as follows:

sense: 5`TCACTAAgACTTCCTggAAgA-3`
antisense: 5`AgACCAgAAggAgCTgAGG-3`

Samples were amplified for 35 cycles consisting of denaturation at 94^0C for 45 sec, annealing at 56^0C for 30 sec and extension at 72^0C for 45 sec, followed by a final extension step at 72^0C for 10 min. Because the nucleotide G-6A mutation created a restriction site for Eag I enzyme (New England Biolabs) the resulting amplification product (251 bp) after PCR was therefore digested with Eag I restriction endonuclease for four hours at 37^0C. (New England Biolabs, Schwalbach/Ts, Germany). Following digestion, restriction fragments (176 bp and 75 bp) were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction, Eag I digestion and scoring were repeated.

2. Angiotensin II Type I Receptor (AT_1 R) PCR:

All PCR to detect the AT_1 R mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by Hingorani *et al* (1995). The sense and antisense primers were as follows:

sense: 5`gTAAgCTCATCCACCAAgAAgg-3`
antisense: 5`gCAAgTgTAgCAgCAgTTgC-3`

Samples were amplified for 35 cycles consisting of denaturation at 95^0C for 30 sec, annealing at 60^0C for 30 sec and extension at 72^0C for 60 sec, followed by a final extension step at 72^0C for 10 min. Because nucleotide A/C1166 mutation created a restriction site for Dde I (New England Biolabs), the resulting amplification product after PCR was therefore digested with Dde I restriction endonuclease for two hours at 37^0C (New England Biolabs, Schwalbach/Ts, Germany). Following digestion, restriction fragments were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction Dde I digestion and scoring were repeated.

3. **Angiotensin Converting Enzyme (ACE) PCR:**

All PCR to detect the ACE gene mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by Rigat *et al* (1992). The sense and antisense primers were as follows:

sense: 5`CTggAgACCACTCCCATCCTTTCT-3`
antisense: 5`gATgTggCCATCACATTCgTCAgAT-3`

Samples were amplified for 35 cycles consisting of denaturation at 94^0C for 45 sec, annealing at 60^0C for 45 sec and extension at 72^0C for 60 sec, followed by a final extension step at 72^0C for 10 min. The PCR product is a 190 bp fragment in the absence of the insertion (deletion) and a 490 bp fragment in the presence of the insertion. Following PCR, restriction fragments (190 bp and 490 bp) were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. To increase the specificity of deletion homozygote (DD) genotyping, a second PCR with the annealing temperature at 68^0C and primer pair that recognizes the insertion-specific sequence, was performed in all samples classified as DD homozygotes in the first PCR.

Sense: 5`TgggACCACgCCCgCCACTAC-3`
Antisense: 5`TCgCCAgCCTCCCATgCCCATAA-3`

Only the I allele produces a 355 bp amplicon. The reaction yields no products in samples of the DD genotype. In cases of ambiguity the PCR reaction-specific PCR scoring were repeated.

4. Aldosterone Synthase Promoter (CYP11-β2) PCR:

All PCR to detect the CYP IIβ_2 mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by White *et al* (1998). The sense and antisense primers were as follows:

CYP 304 sense: CAgggCTgAgAggAgTAAAA
CYP 171 antisense: CAgggggTACgTggACATTT

Samples were amplified for 35 cycles consisting of denaturation at 94^{0}C for 45 sec, annealing at 52^{0}C for 45 sec and extension at 72^{0}C for 60 sec, followed by a final extension step at 72^{0}C for 10 min. Because nucleotide C344T mutation created a restriction site for *Hae*III enzyme (New England Biolabs), the resulting amplification product (231bp) after PCR was therefore digested with *Hae*III restriction endonuclease for two hours at 37^{0}C (New England Biolabs, Schwalbach/Ts, Germany). Following digestion, restriction fragments (140bp and 91bp), were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction *Hae*III digestion and scoring were repeated.

5. PCR for 11β-Hydroxysteroid Dehydrogenase 2 (11β-HSD2):

All PCR to detect the 11BHSD2 mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by Smolenicka et al (1998). The sense and antisense primers were as follows:

HSD 540 A sense:	5`ggAAgTTTgCTgCTgggCTgA-3`
HSD 870 B antisense:	5`AgTggggCAgCTCAgCTTTgg-3`

Samples were amplified for 35 cycles consisting of denaturation at 94^0C for 30 sec, annealing at 63^0C for 30 sec and extension at 72^0C for 60 sec, followed by a final extension step at 72^0C for 10 min. Since the nucleotide G534A mutation created a restriction site for Alu I enzyme (New England Biolabs), the resulting amplification product after PCR was therefore digested with Alu I restriction endonuclease for four hours at 37^0C (New England Biolabs, Schwalbach/Ts, Germany). Following digestion, restriction fragments were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction, Alu I digestion and scoring were repeated.

6. PCR for Transforming Growth Factor-β:

All PCR to detect the TGF-β_1 mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by Pociot et al (1998) for a mutagenically separated PCR (MC-PCR) assay. In this MS-PCR three primers were used in a single reaction mix:

Sense (1):	5`ACCgCCCCATTCTgCTTCTCATggCCCT-3`
Sense (2):	5`TggCCACCATTCATggCATgAgTCggCCTT-TCCTgCTTCTCATggACAC-3`
Antisense:	5`AAggCCTCCATC CAg gCT ACA Agg CTC AC-3`

Samples were amplified for 35 cycles consisting of denaturation at 94^0C for 45 sec, annealing at 68^0C for 45 sec and extension at 72^0C for 60 sec, followed by a final extension step at 72^0C for 10 min. The nucleotide *Thr 263* Ile polymorphism does not require a restriction endonuclease. In the presence of *Thr 263* a PCR product of 150 bp was amplified, whereas a 129 bp fragment was amplified in the presence of the Ile 263 variant. Following the PCR, the amplification products (150 bp and 129 bp) were sized fractionated on 2% agarose gels and repeated with Nutrasieve to improve separation of the bands. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction size fractionation on Nutrasieve gel and scoring were repeated.

7. PCR for Methylenetetrahydrofolate Reductase Gene Polymorphism:

All PCR to detect the *MTHFR* mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01. Gene Amp 9600 PCR System 1991) and primers as reported by Frosst *et al*, 1995. The sense and antisense primers were as follows:

sense: 5`TGAAGGAGAAGGTGTATGAGGGA-3`
antisense: 5`AGGACGGTGCGGTGAGAGTG-3`

Samples were amplified for 35 cycles consisting of denaturation at 94 ^{0}C for 15 sec, annealing at 58 ^{0}C for 45 sec, and extension at 72 ^{0}C for 45 sec, followed by a final extension step at 72^{0}C for 10 min. Because the nucleotide *C677T* mutation creates a restriction site for *Hinf I* (New England Biolabs,) the resulting amplification product (246 bp) after PCR was therefore digested with *Hinf I* restriction endonuclease for 20 hours at 37^{0}C (New England Bioloabs, Schwalbach/Ts, Germany). Following digestion, restriction fragments (175 bp and 71 bp) were size fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS + KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction, *Hinf I* digestion and scoring were repeated.

8. **PCR for G-Protein:**

All PCR to detect the *G*-Protein mutation were carried out with 100 ng of genomic DNA as a template, using a DNA thermal cycler (Perkin Elmer Version 2.01 Gene Amp 9600 PCR System 1991) and primers as reported by Siffert *et al* (1998). The sense and antisense primers were as follows:

sense: 5`TgACCCACTTgCCACCCgTgC-3`
antisense: 5`gCAgCAgCCAgggCTggC-3`

Samples were amplified for 35 cycles consisting of denaturation at 94^0C for 45 sec, annealing at 60^0C for 45 sec and extension at 72^0C for 60 sec, followed by a final extension step at 72^0C for 10 min. Because nucleotide C→T mutation created a restriction site for BSEDI (MBI Fermentas) the resulting amplification product (268 bp) after PCR was therefore digested with BSE DI restriction endonuclease for two hours at 60^0C MBI Fermentas. Following digestion, restriction fragments (152 bp and 116 bp) were sized fractionated on 2% agarose gels. Genotype analysis was carried out by two independent investigators (AMS and KS) who were unaware of the clinical data. In cases of ambiguity the PCR reaction, BSEDI digestion and scoring were repeated.

ACE-ID-PCR:
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **120** tubes
Buffer	1.0	**120**	2	**240**	3 min 94^0
MgCI	0.75	**90**	1.5	**180**	45 sec 94^{0}C
DNTP 1.25M	1.0	**120**	1.5	**180**	45 sec 60^{0}C
Taq	0.03	**3.6**	0.03	**3.6**	60 sec 72^{0}C
ACE P 1 (20µM)	0.1	**12**	0.1	**12**	10 min 72^{0}C
ACE P 2 (20µM)	0.1	**12**	0.1	**12**	
DMSO	0.5	**60**	0.7	**84**	
H_2O	6.52	**782.4**	4.07	**488.4**	
DNA-template	0.0		10		
TOTAL	10.0		20		

CODE: 1 = II ; 2 = ID ; 3 = DD.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	2	2	3	2	1	2	2	2	2	0	2
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	3	2	1	2	1	2	1	2	1	0	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	1	1	1	2	1	2	2	2	2	1	1	2
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	2	2	1	2	2	3	2	1	1	2	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	3	1	1	2	0	2	1	2	1	0	2	2
F	7	15	23	34	41	56	87	130	147	167	177	188
	2	2	1	2	2	1	2	2	2	2	2	3
G	8	16	24	35	42	57	94	133	156	168	178	189
	1	3	1	2	2	1	3	1	2	1	0	2
H	9	17	25	36	43	60	93	136	158	169	179	190
	2	2	1	1	2	2	2	1	3	1	1	1

FIG 2:

(See page 140) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-ID-PCR:

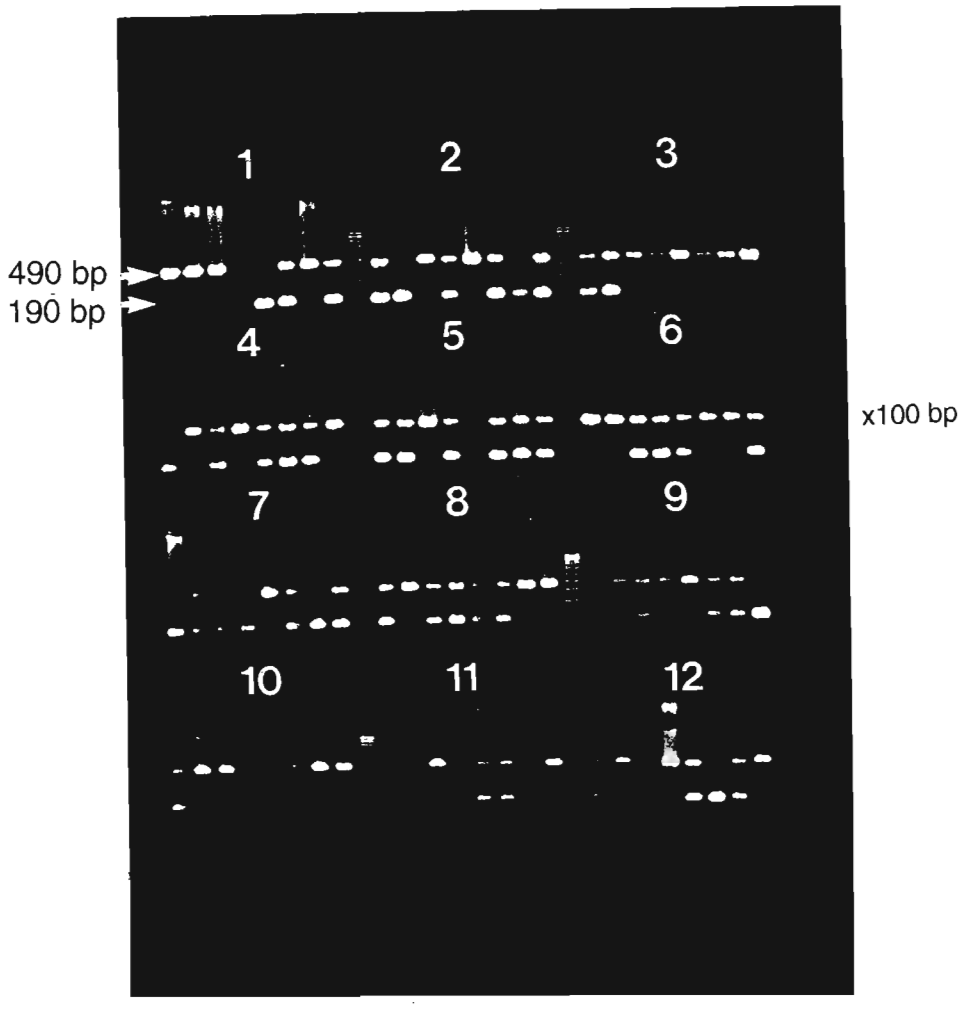

***FIG 2*:ACE-ID-PCR: PROBANDS**

This PCR yielded 2 products: a 190 bp fragment in the absence of the insertion and a 490 bp fragment in the presence of a deletion.

ACE-ID-PCR:
DNA: PROBANDS & REPEATS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For 50 tubes
Buffer	1.0	**44**	2	**75**	3 min 94^0
MgCl	0.75	**33**	1.5	**60**	45 sec 94^0C
DNTP 1.25M	1.0	**44**	1.5	**50**	45 sec 60^0C
Taq	0.03	**1.32**	0.03	**1.5**	60 sec 72^0C
ACE P 1 (20µM)	0.1	**4.4**	0.1	**5**	10 min 72^0C
ACE P 2 (20µM)	0.1	**4.4**	0.1	**5**	
DMSO	0.5	**22**	0.7	**30**	
H_2O	6.52	**286.88**	4.07	**310**	
DNA-template	0.0		10		
TOTAL	10.0		20		

CODE: 1 = II ; 2 = ID ; 3 = DD.

	1	2	3	4	5
A	191 PR 2	H2O 0	223 PR 2	116 PR 2	165 1
B	192 PR 3	203 PR 3	224 PR 3	153 PR 1	H2O 0
C	193 PR 3	204 PR 3	225 PR 1	197 PR 3	172 0
D	195 PR 3	206 PR 2	H2O 0	198 PR 2	174 1
E	196 PR 3	209 PR 1	208 PR 1	6 PR 1	178 1
F	199 PR 1	220 PR 2	148 PR 2	20 1	180 2
G	200 PR 0	221 PR 2	207 PR 1	167 2	188 3
H	202 PR 1	222 PR 1	28 PR 2	168 1	

FIG 3:

(See page 142) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-5). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-ID-PCR:

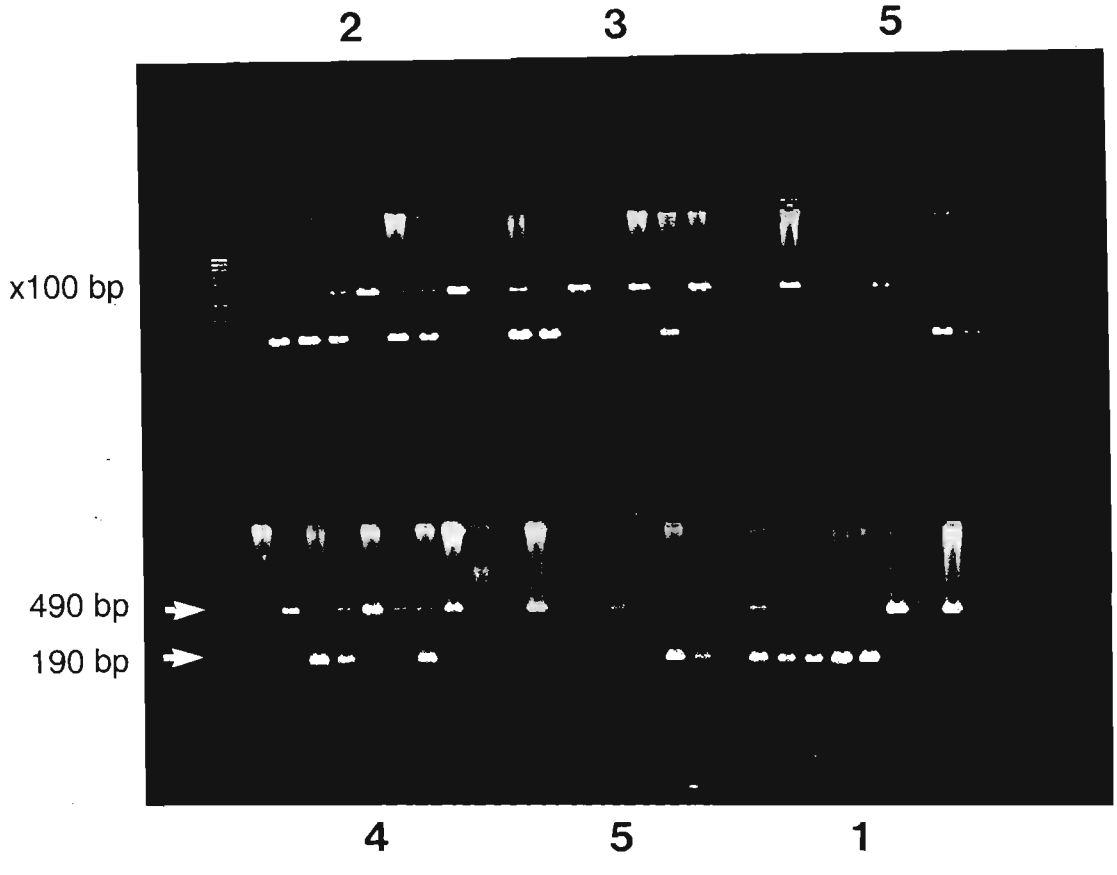

***FIG 3*:ACE-ID-PCR: PROBAND & REPEATS**

This PCR yielded 2 products: a 190 bp fragment in the absence of the insertion and a 490 bp fragment in the presence of a deletion.

ACE-ID-PCR:
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **44** tubes
Buffer	1.0	**44**	2	**66**	3 min 94^0
MgCI	0.75	**33**	1.5	**52.8**	45 sec 94^0C
DNTP 1.25M	1.0	**44**	1.5	**44**	45 sec 60^0C
Taq	0.03	**1.32**	0.03	**1.32**	60 sec 72^0C
ACE P 1 (20μM)	0.1	**4.4**	0.1	**4.4**	10 min 72^0C
ACE P 2 (20μM)	0.1	**4.4**	0.1	**4.4**	
DMSO	0.5	**22**	0.7	**26.4**	
H_2O	6.52	**286.88**	4.07	**272.8**	
DNA-template	0.0		10		
TOTAL	10.0		20		

CODE: 1 = II ; 2 = ID ; 3 = DD.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A	1 1	9 2	17 2	25 3	33 3	40 0	48 1	A012 3	A080 2	A119 1	A161 2
B	2 2	10 1	18 1	26 2	H2O 0	41 1	49 2	A005 2	A098 2	A120 3	A162 2
C	3 2	11 1	19 2	27 1	34 2	42 1	50 2	A019 2	A104 2	A123 3	A166 2
D	4 0	12 1	20 2	28 1	35 1	43 2	H2O 0	A024 3	A106 2	A149 3	A171 1
E	5 1	13 1	21 3	29 3	36 0	44 2	A006 1	B003 2	A108 2	A150 3	A205 0
F	6 2	14 2	22 2	30 2	37 2	45 2	A004 1	B004 3	A109 3	A154 3	A170 3
G	7 1	15 2	23 0	31 2	38 0	46 2	A008 1	B005 1	A110 1	A157 1	A086 2
H	8 3	16 1	24 3	32 2	39 0	47 3	A010 0	A064 2	H2O 0	A160 1	A006 ND

FIG 4:

(See page 144) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-ID-PCR:

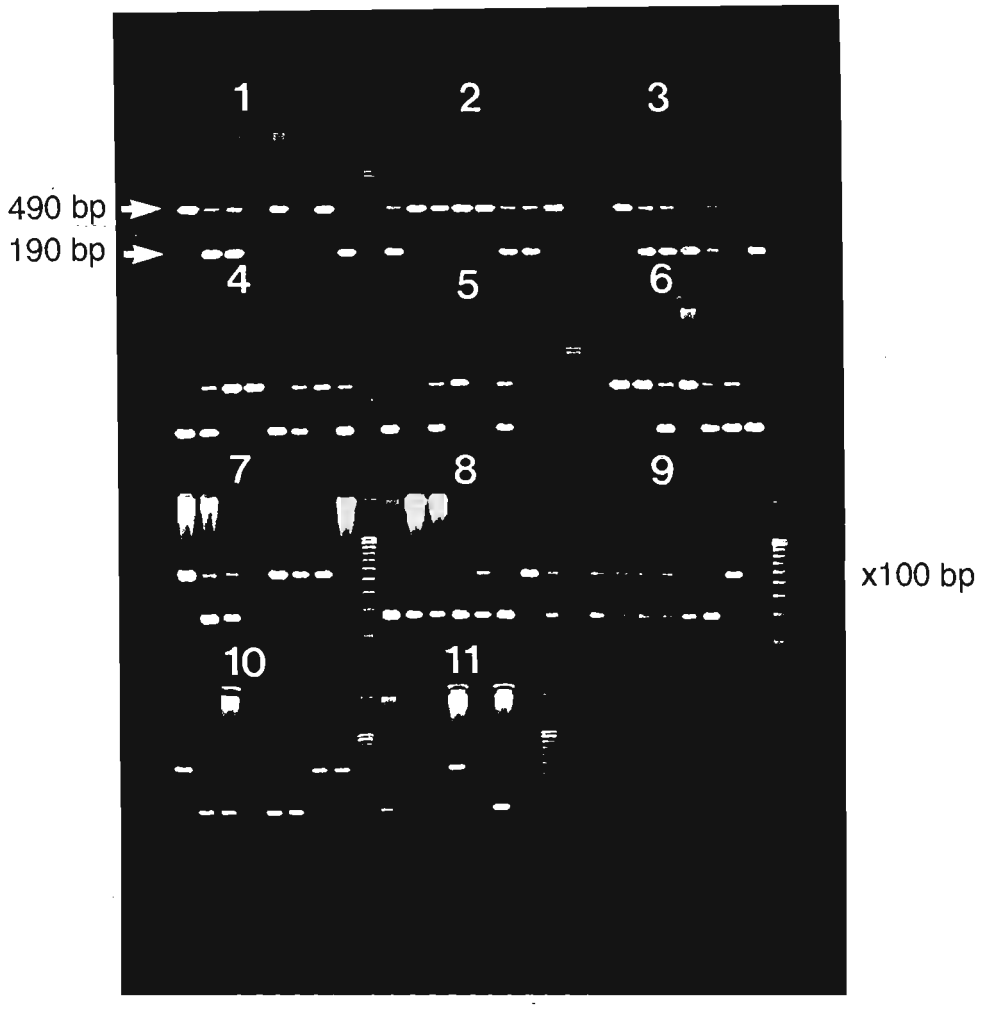

***FIG 4*:ACE-ID-PCR: CONTROLS**

This PCR yielded 2 products: a 190 bp fragment in the absence of the insertion and a 490 bp fragment in the presence of a deletion.

ACE-II-PCR: INSERTION SPECIFIC PRIMER
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	1	**210**	1.3	**273**	3 min 94^0
MgCI	0.75	**157.5**	0.9	**189**	45 sec 94^{0}C
DNTP 1.25M	1	**210**	1.3	**273**	45 sec 68^{0}C
Taq	0.03	**6.3**	0.03	**6.3**	120 sec 72^{0}C
II P 1 (20µM)	0.13	**27.3**	0.15	**31.5**	10 min 72^{0}C
II P 2 (20µM)	0.13	**27.3**	0.15	**31.5**	
H_2O	6.96	**1461.6**	3.96	**831.6**	
DNA-template	0		3		
TOTAL	10		14		

CODE: I = II/ID ; 3 = DD.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	2 I	10 I	18 I	26 3	37 I	44 I	63 I	99 I	137 I	159 I	H20 0	180 I
B	3 I	11 3	19 I	27 I	38 I	46 I	65 I	102 I	140 I	163 I	172 0	181 I
C	4 I	12 I	20 I	30 I	39 I	49 I	77 I	103 I	144 I	164 I	174 I	184 I
D	H2O 0	13 I	21 I	31 I	40 I	54 I	78 3	105 I	145 I	165 I	175 I	186 I
E	5 3	14 I	22 I	33 I	H2O 0	55 I	85 I	111 I	146 I	H2O 0	176 I	187 I
F	7 I	15 I	23 I	34 I	41 I	56 I	87 I	130 I	147 I	167 I	177 I	188 3
G	8 I	16 3	24 I	35 I	42 I	57 I	94 3	133 I	156 I	168 I	178 0	189 I
H	9 I	17 I	25 I	36 I	43 I	60 I	93 I	136 I	158 3	169 I	179 I	190 I

FIG 5:

(See page 146) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-II-PCR:

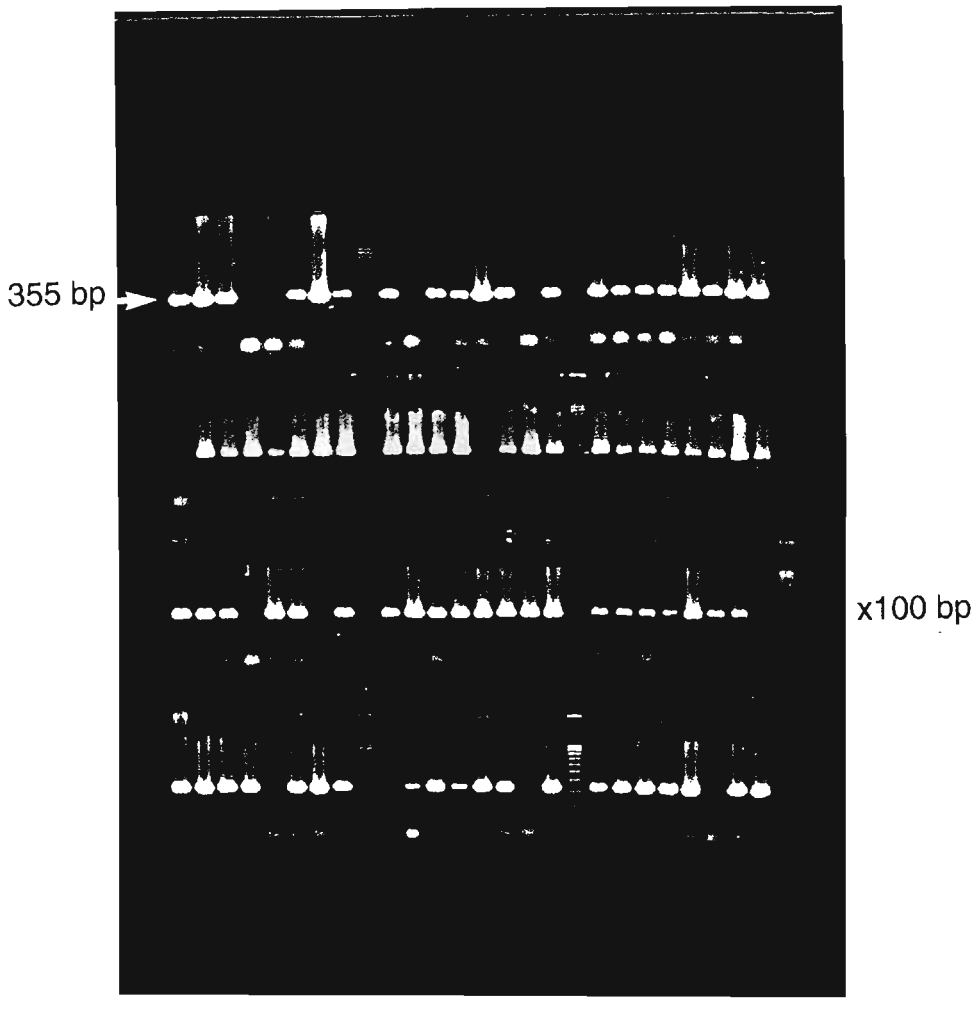

FIG 5: **ACE-II-PCR: PROBANDS**

This PCR used a primer pair that recognises the insertion-specific sequence and identifies ID genotypes that have been misclassified as DD when only the flanking primer pair was used. Only the I allele produces a 355 bp amplicon, while the reaction yields no products in samples of the DD genotype.

ACE-II-PCR: INSERTION SPECIFIC PRIMER
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	1	**210**	1.3	**273**	3 min 94^0
MgCI	0.75	**157.5**	0.9	**189**	45 sec 94^{0}C
DNTP 1.25M	1	**210**	1.3	**273**	45 sec 68^{0}C
Taq	0.03	**6.3**	0.03	**6.3**	120 sec 72^{0}C
II P 1 (20μM)	0.13	**27.3**	0.15	**31.5**	10 min 72^{0}C
II P 2 (20μM)	0.13	**27.3**	0.15	**31.5**	
H_2O	6.96	**1461.6**	3.96	**831.6**	
DNA-template	0		3		
TOTAL	10		14		

CODE: I = II/ID ; 3 = DD.

	1	2	3	4	5	6	7	8	9	10	11
A	1 I	9 I	17 I	25 3	33 3	40 0	48 I	A012 3	A080 I	A119 I	A161 I
B	2 I	10 I	18 I	26 I	H2O 0	41 I	49 I	A005 I	A098 I	A120 3	A162 I
C	3 I	11 I	19 I	27 I	34 I	42 I	50 I	A019 I	A104 I	A123 3	A166 I
D	4 I	12 I	20 I	28 I	35 I	43 I	H2O 0	A024 3	A106 I	A149 3	A171 I
E	5 I	13 I	21 3	29 3	36 I	44 I	A006 I	B003 I	A108 I	A150 3	A205 I
F	6 I	14 I	22 I	30 I	37 I	45 I	A004 I	B004 3	A109 3	A154 3	A170 3
G	7 I	15 I	23 I	31 I	38 0	46 0	A008 I	B005 I	A110 I	A157 I	A086 I
H	8 3	16 I	24 3	32 I	39 0	47 3	A010 I	A064 I	H2O 0	A160 I	6 CO I

FIG 6:

(See page 148) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-II-PCR:

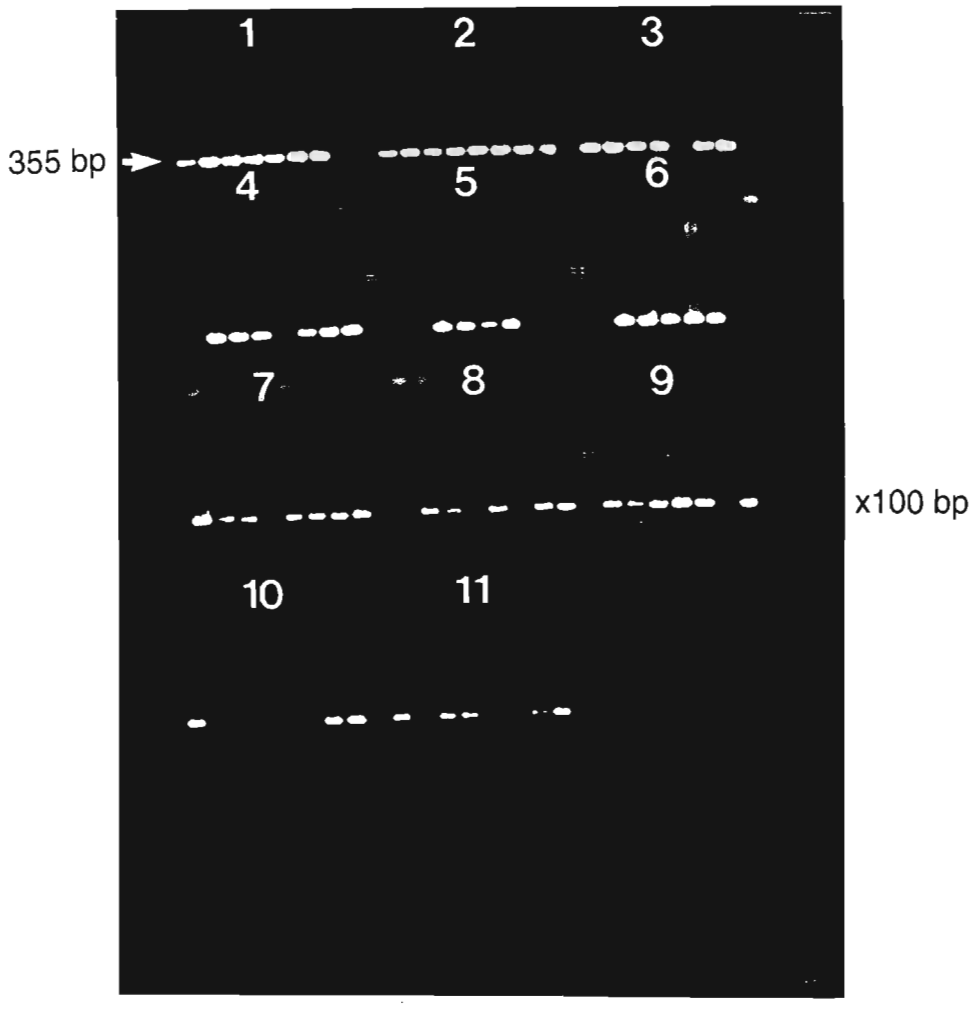

FIG 6: **ACE-II-PCR: CONTROLS**

This PCR used a primer pair that recognises the insertion-specific sequence and identifies ID genotypes that have been misclassified as DD when only the flanking primer pair was used. Only the I allele produces a 355 bp amplicon, while the reaction yields no products in samples of the DD genotype.

ACE-II-PCR: INSERTION SPECIFIC PRIMER
DNA: PROBANDS & REPEATS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	1	**40**	1.3	**52**	3 min 94^0
MgCI	0.75	**30**	0.9	**36**	45 sec 94^{0}C
DNTP 1.25M	1	**40**	1.3	**52**	45 sec 68^{0}C
Taq	0.03	**1.2**	0.03	**1.2**	120 sec 72^{0}C
II P 1 (20µM)	0.13	**5.2**	0.15	**6**	10 min 72^{0}C
II P 2 (20µM)	0.13	**5.2**	0.15	**6**	
H_2O	6.96	**278.4**	3.96	**158.4**	
DNA-template	0		3		
TOTAL	10		14		

CODE: I = II/ID ; 3 = DD.

	1	2	3	4
A	191 PR I	H2O 0	223 PR I	116 PR I
B	192 PR 3	203 PR 3	224 PR 3	153 PR I
C	193 PR 3	204 PR 3	225 PR I	197 PR 3
D	195 PR 3	206 PR I	H2O 0	198 PR I
E	196 PR 3	209 PR I	208 PR I	6 PR I
F	199 PR I	220 PR I	148 PR I	
G	200 PR 0	221 PR I	207 PR I	
H	202 PR I	222 PR I	28 PR I	

FIG 7:

(See page 150) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-4). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

ACE-II-PCR:

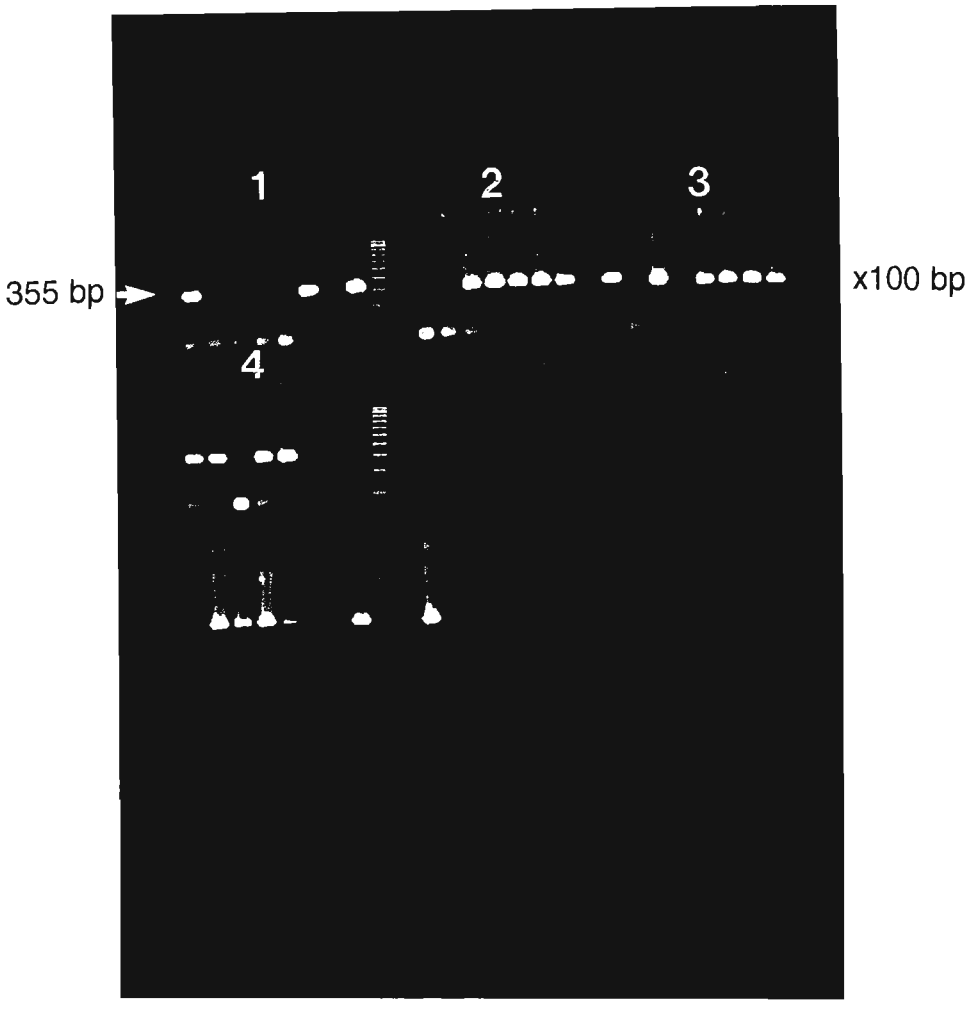

FIG 7: **ACE-II-PCR: PROBANDS & REPEATS**

This PCR used a primer pair that recognises the insertion-specific sequence and identifies ID genotypes that have been misclassified as DD when only the flanking primer pair was used. Only the I allele produces a 355 bp amplicon, while the reaction yields no products in samples of the DD genotype.

AGT-CORE PROMOTER-PCR DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**110**	2.3	**253**	3 min 94^0
MgCI	0.4	**44**	0.75	**82.5**	45 sec 94^{0}C
dNTP 1.25M	1	**110**	1	**110**	30 sec 56
Taq	0.03	**3.3**	0.03	**3.3**	45 sec 72^{0}C
AGT-HAP-1 (10μM)	0.5	**55**	0.5	**55**	10 min 72^{0}C
AGT-HAP-2 (10μM)	0.5	**55**	0.5	**55**	
H_2O	6.57	**722.7**	14.92	**1641.2**	
DNA-template	0		3		
TOTAL	10.0		23		
NEB 3 Buffer	2	**220**		**1 = AA**	
Eag 1 enzyme	0.1	**11.0**		**2 = AG**	37^{0}C forever
H_20	7.9	**869**		**3 = GG**	
DNA-template	10				

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	1	2	2	1	1	3	1	3	2	0	2
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	2	2	2	1	0	1	2	2	3	0	2
C	4	12	20	30	39	49	77	103	144	164	174	184
	2	2	2	1	1	3	1	1	1	0	2	2
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	1	1	3	1	2	1	2	3	2	2	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	3	1	1	2	0	2	3	2	2	0	2	3
F	7	15	23	34	41	56	87	130	147	167	177	188
	1	1	3	1	2	2	1	2	1	3	3	2
G	8	16	24	35	42	57	94	133	156	168	178	189
	2	2	2	2	1	2	2	2	1	2	0	2
H	9	17	25	36	43	60	93	136	158	169	179	190
	3	1	1	1	2	1	3	2	1	1	2	0

FIG 8:

(See page 152) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AGT-CORE PROMOTER PCR:

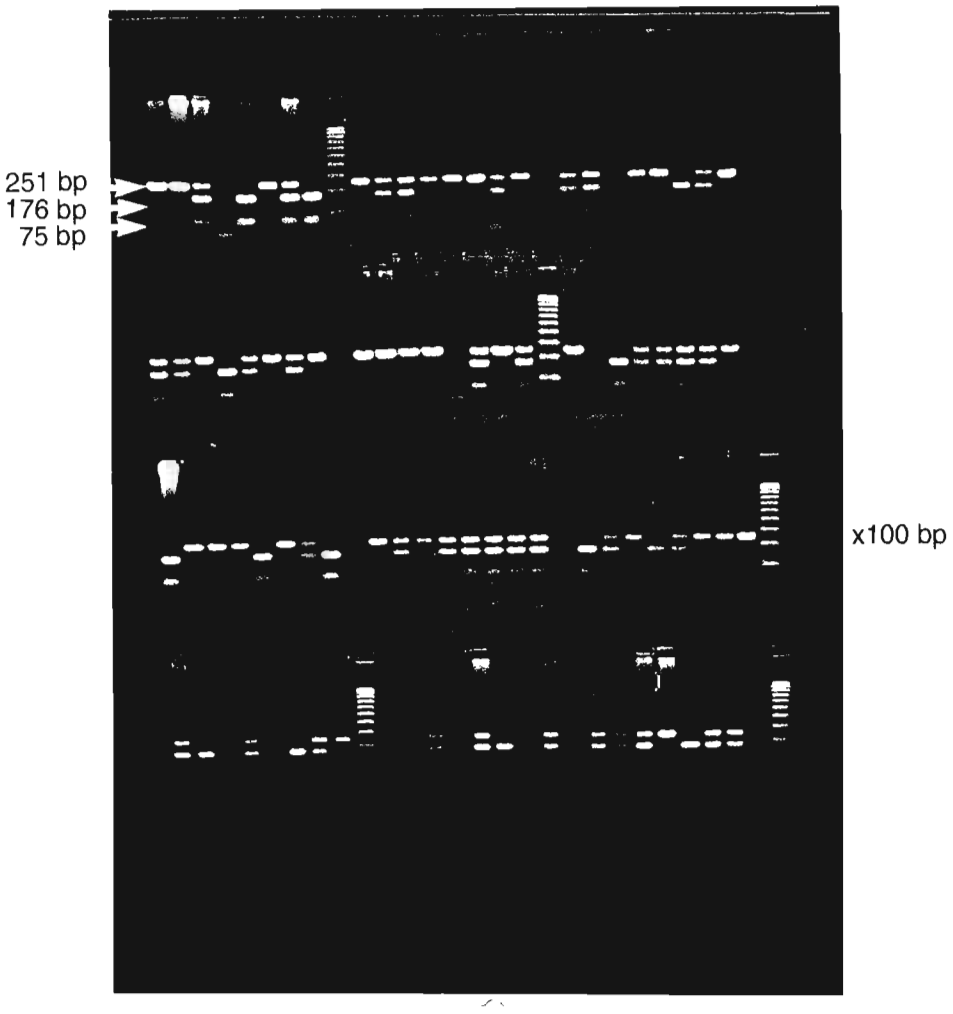

FIG 8: **AGT G6→A CORE PROMOTER PCR: PROBANDS**

After the digestion with Eag 1 enzyme the PCR yielded three products: 75, 176 and 251 bp fragments corresponding to GG, AG and AA genotypes respectively.

AGT-CORE PROMOTER-PCR
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**130**	2.3	**299**	3 min 94^0
MgCI	0.4	**52**	0.75	**97.5**	45 sec 94^{0}C
dNTP 1.25M	1	**130**	1	**130**	30 sec 56
Taq	0.03	**3.9**	0.03	**3.9**	45 sec 72^{0}C
AGT-HAP-1 (10µM)	0.5	**65**	0.5	**65**	10 min 72^{0}C
AGT-HAP-2 (10µM)	0.5	**65**	0.5	**65**	
H_2O	6.57	**854.1**	14.92	**1939.6**	
DNA-template	0		3		
TOTAL	10.0		23		
NEB 3 Buffer	2	**420**		**1 = AA**	
Eag 1 enzyme	0.1	**21**		**2 = AG**	37^{0}C forever
H_20	7.9	**1659**		**3 = GG**	
DNA-template	10				

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
A	1 2	9 2	17 0	25 3	33 1	40 0	48 2	A012 2	A080 2	A119 2	A161 2
B	2 2	10 2	18 1	26 2	H2O 0	41 1	49 3	A005 2	A098 1	A120 1	A162 0
C	3 1	11 2	19 1	27 3	34 2	42 2	50 3	A019 1	A104 2	A123 1	A166 0
D	4 2	12 2	20 2	28 3	35 1	43 2	H2O 0	A024 2	A106 3	A149 0	A171 1
E	5 1	13 2	21 2	29 1	36 0	44 3	A006 3	B003 1	A108 1	A150 0	A205 0
F	6 0	14 1	22 3	30 2	37 3	45 2	A004 2	B004 1	A109 1	A154 2	A170 1
G	7 1	15 1	23 0	31 2	38 0	46 1	A008 1	B005 3	A110 2	A157 2	A086 1
H	8 1	16 1	24 2	32 1	39 0	47 1	A010 1	A064 2	H2O 0	A160 0	A006 1

FIG 9:

(See page 154) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AGT-CORE PROMOTER PCR:

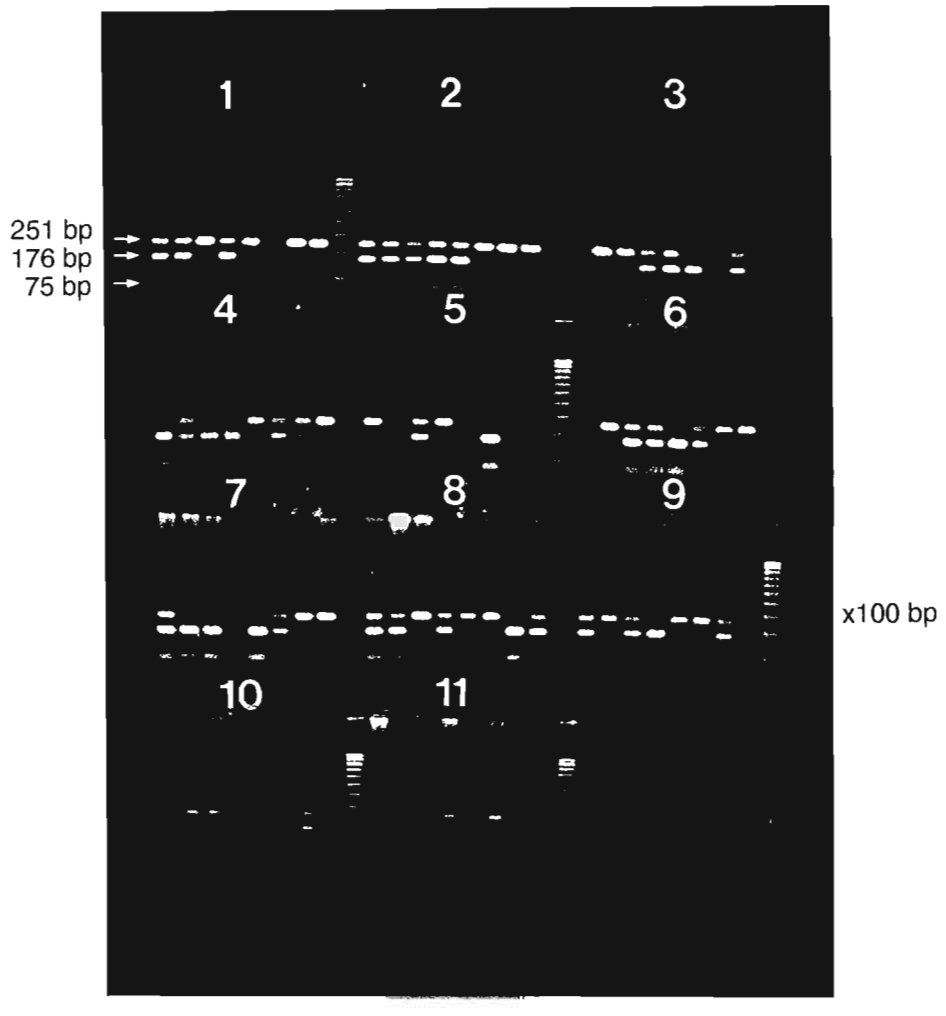

FIG 9: **AGT G6→A CORE PROMOTER PCR: CONTROLS**

After the digestion with Eag 1 enzyme the PCR yielded three products: 75, 176 and 251 bp fragments corresponding to GG, AG and AA genotypes respectively.

AGT-CORE PROMOTER-PCR
DNA: PROBANDS & REPEATS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**130**	2.3	**299**	3 min 94^0
MgCl	0.4	**52**	0.75	**97.5**	45 sec 94^{0}C
dNTP 1.25M	1	**130**	1	**130**	30 sec 56
Taq	0.03	**3.9**	0.03	**3.9**	45 sec 72^{0}C
AGT-HAP-1 (10µM)	0.5	**65**	0.5	**65**	10 min 72^{0}C
AGT-HAP-2 (10µM)	0.5	**65**	0.5	**65**	
H_2O	6.57	**854.1**	14.92	**1939.6**	
DNA-template	0		3		
TOTAL	10.0		23		
NEB 3 Buffer	2	**420**		**1 = AA**	
Eag 1 enzyme	0.1	**21**		**2 = AG**	37^{0}C forever
H_20	7.9	**1659**		**3 = GG**	
DNA-template	10				

	1	2	3	4
A	191 PR 3	H2O 0	223 PR 2	116 PR 0
B	192 PR 3	203 PR 1	224 PR 1	153 PR 2
C	193 PR 0	204 PR 2	225 PR 1	197 PR 1
D	195 PR 2	206 PR 2	H2O 0	198 PR 3
E	196 PR 2	209 PR 1	208 PR 0	6 PR 2
F	199 PR 1	220 PR 1	148 PR 0	
G	200 PR 0	221 PR 1	207 PR 1	
H	202 PR 3	222 PR 1	28 PR 1	

FIG 10:

(See page 156) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-4). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AGT-CORE PROMOTER PCR:

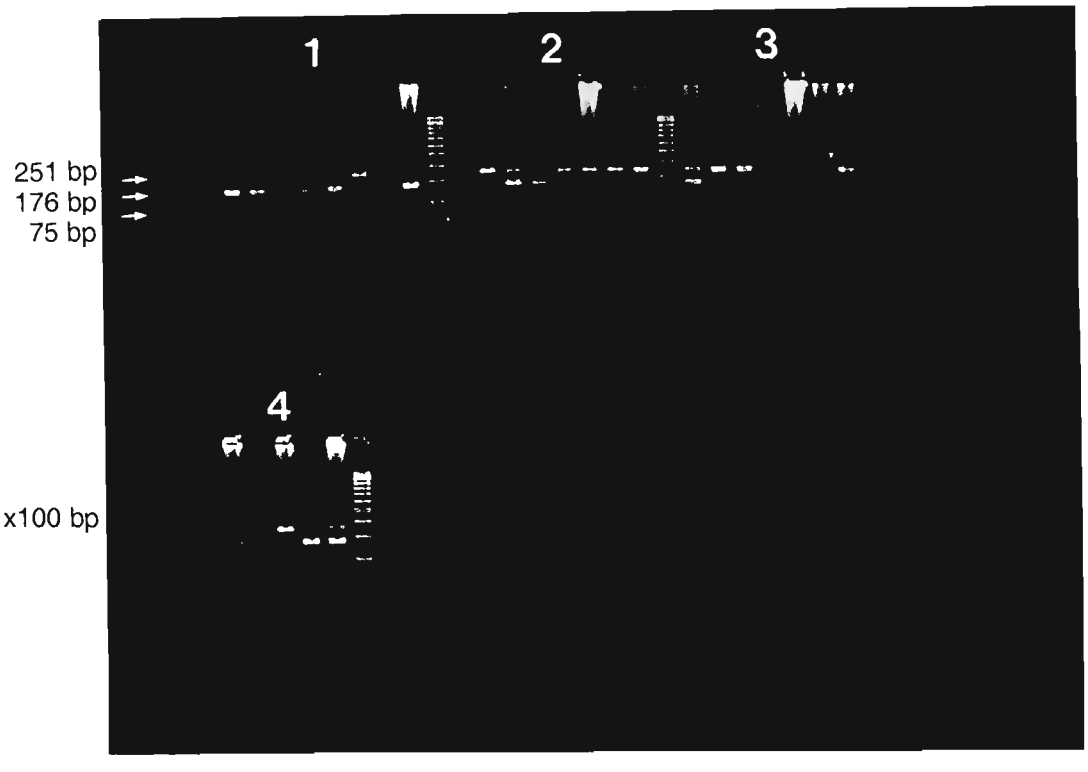

FIG 10: **AGT G6→A CORE PROMOTER PCR: PROBANDS AND REPEATS**

After the digestion with Eag 1 enzyme the PCR yielded three products: 75, 176 and 251 bp fragments corresponding to GG, AG and AA genotypes respectively.

AT1-PCR
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**110**	1.3	**143**	3 min 94^0
MgCl	0.6	**66**	0.9	**99**	45 sec 94^{0}C
DNTP 1.25M	1	**110**	1.3	**143**	30 sec 60^{0}C
Taq	0.02	**2.2**	0.03	**3.3**	60 sec 72^{0}C
AT-1 (25µM)	0.1	**11**	0.13	**14.3**	10 min 72^{0}C
AT-2 (25µM)	0.1	**11**	0.13	**14.3**	
H_2O	7.18	**789.8**	4.18	**459.8**	
DNA-template	0		3		
TOTAL	10		13		
Dde 1 (µL)	0.2	**22**	0.2	**220**	
Buffer Nr.3	2	**220**	2.3	**253**	37^{0}C forever
H_20	7.8	**858**	7.8	**858**	

CODE: 1 = AA ; 2 = AC ; 3 = CC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	1	1	1	1	1	1	1	1	1	0	1
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	1	1	1	1	1	1	1	1	1	(0)	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	1	1	1	1	2	1	1	1	1	1	1	1
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	1	1	1	1	2	1	1	1	1	1	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	1	1	1	1	0	1	1	2	1	0	1	1
F	7	15	23	34	41	56	87	130	147	167	177	188
	1	1	1	1	1	2	1	1	1	(1)	1	1
G	8	16	24	35	42	57	94	133	156	168	178	189
	1	1	1	1	1	1	1	2	1	(1)	(0)	1
H	9	17	25	36	43	60	93	136	158	169	179	190
	1	1	1	2	1	1	2	1	(1)	1	1	1

FIG 11:

(See page 158) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AT1-PCR:

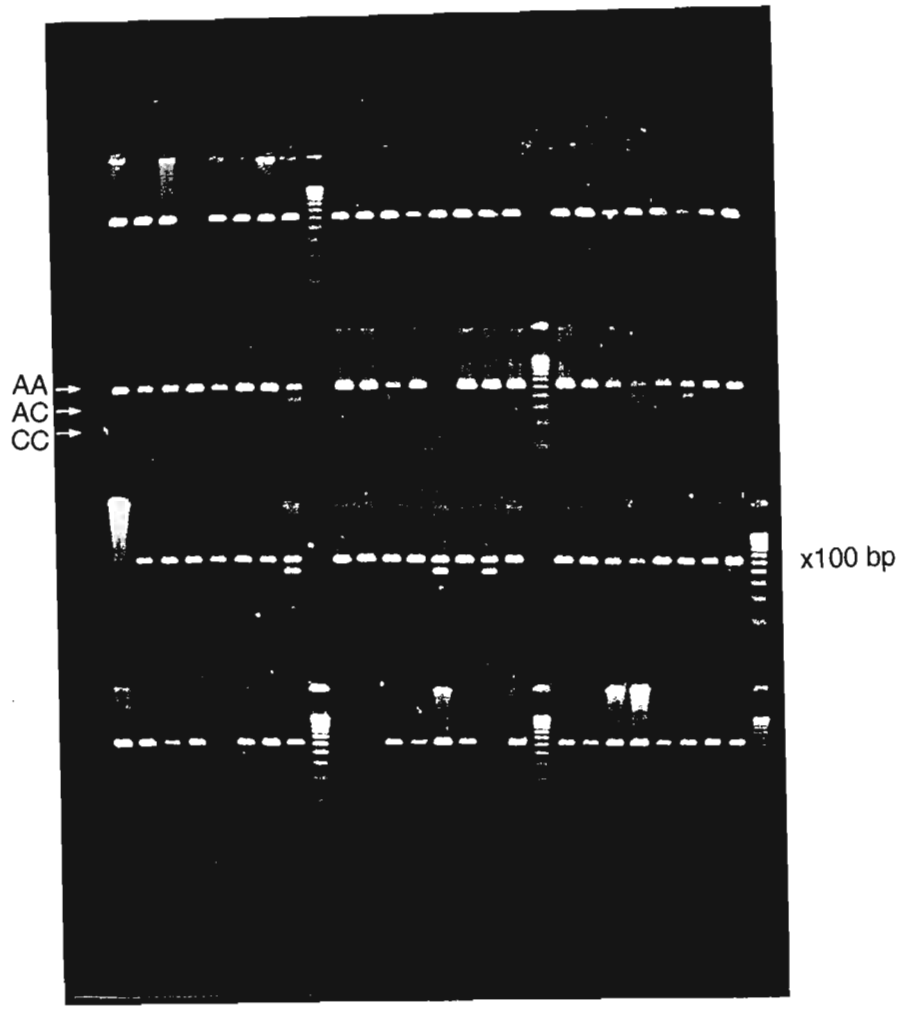

FIG 11: **AT1 PCR: PROBANDS**

After digestion with Dde I restriction endonuclease restriction the resulting fragments were size fractionated on 2% agarose gels yielding bands corresponding to AA, AC, and CC genotypes.

AT1-PCR
DNA:CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **140** tubes
Buffer	1	**140**	1.3	**182**	3 min 94^0
MgCl	0.6	**84**	0.9	**126**	45 sec 94^0C
dNTP 1.25M	1	**140**	1.3	**182**	45 sec 60^0C
Taq	0.02	**2.8**	0.03	**4.2**	60 sec 72^0C
AT-1 (25µM)	0.1	**14**	0.13	**18.2**	10 min 72^0C
AT-2 (25µM)	0.1	**14**	0.13	**18.2**	
H_2O	7.18	**1005.2**	4.18	**585.2**	
DNA-template	0		3		
TOTAL	10		13		
Dde 1 (µL)	0.2	**28**	0.2	**28**	
Buffer Nr.3	2	**280**	2.3	**322**	37^0C forever
H_20	7.8	**1092**	7.8	**1092**	

CODE: 1 = AA ; 2 = AC ; 3 = CC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1 2	9 1	17 1	25 1	33 1	40 (0)	48 1	A012 1	A080 1	A119 1	A161 1	20-2 2
B	2 2	10 1	18 1	26 1	H2O 0	41 1	49 1	A005 1	A098 1	A120 1	A162 (0)	23-2 2
C	3 1	11 2	19 2	27 1	34 1	42 1	50 1	A019 (0)	A104 1	A123 1	A166 2	27-2 3
D	4 1	12 (0)	20 1	28 2	35 1	43 2	H2O 0	A024 2	A106 1	A149 1	A171 1	50-2 3
E	5 1	13 1	21 1	29 1	36 1	44 1	A006 2	B003 1	A108 1	A150 1	A205 (0)	62-2 2
F	6 (0)	14 1	22 1	30 1	37 1	45 1	A004 1	B004 1	A109 1	A154 2	A170 1	64-2 3
G	7 1	15 1	23 (0)	31 2	38 (0)	46 1	A008 2	B005 1	A110 1	A157 1	A086 2	73-2 3
H	8 1	16 1	24 1	32 1	39 (0)	47 1	A010 1	A064 1	H2O 0	A160 1	A006 1	74-2 2

FIG 12:

(See page 160) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AT1-PCR:

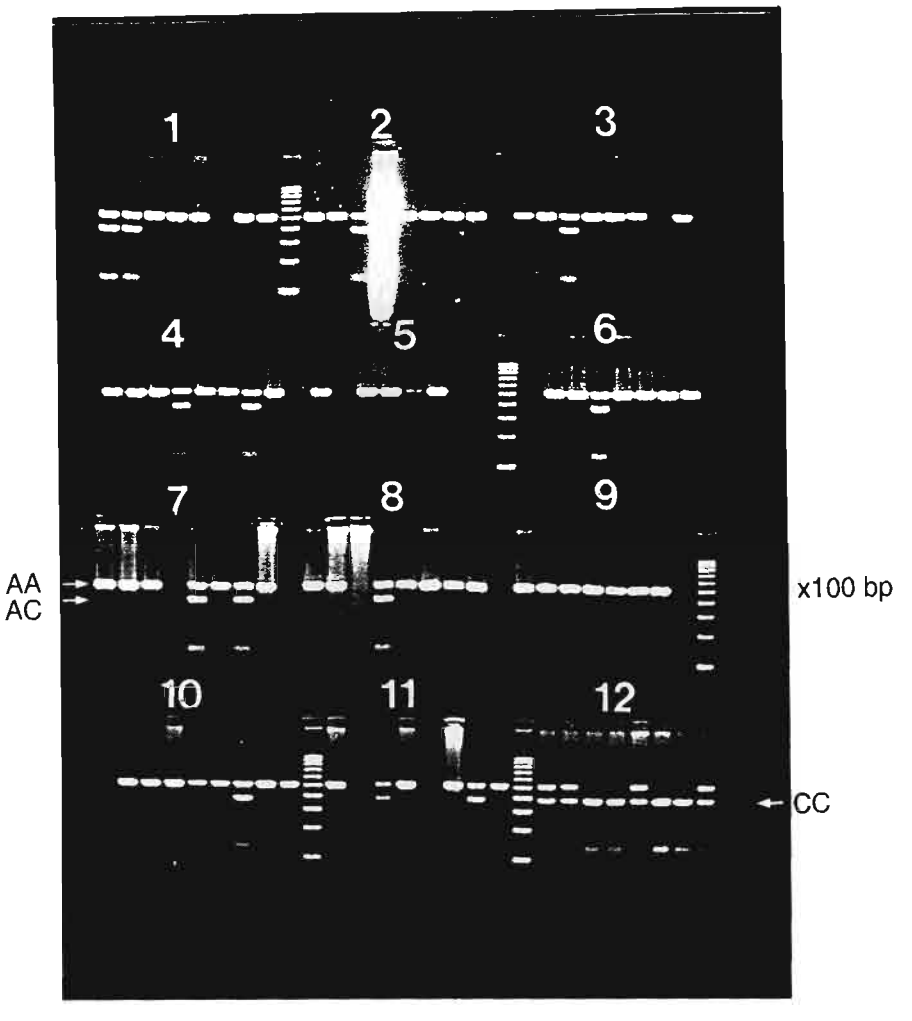

FIG 12: **AT1 PCR: CONTROLS & BFU HETEROZYGOTES**

After digestion with Dde I restriction endonuclease restriction the resulting fragments were size fractionated on 2% agarose gels yielding bands corresponding to AA, AC, and CC genotypes. Row 12 shows positive controls for heterozygotes (AC) and the rare CC genotypes.

AT1-PCR
DNA: PROBANDS + REPEATS

	Mastermix: Dry	110 tubes	30 tubes	
Buffer	1	110	30	3 min 94^0
MgCI	0.6	66	18	45 sec 94^{0}C
dNTP 1.25M	1	110	30	45 sec 60^{0}C
Taq	0.02	2.2	0.6	60 sec 72^{0}C
AT-1 (25µM)	0.1	11	3.0	10 min 72^{0}C
AT-2 (25µM)	0.1	11	3.0	
H_2O	7.18	789.8	215.4	
DNA-template	0			
TOTAL	10			
Dde 1 (µL)	0.2	22	6	
Buffer Nr.3	2	220	60	37^{0}C forever
H_20	7.8	858	234	

CODE: 1 = AA ; 2 = AC ; 3 = CC.

	1	2	3	4
A	191 1	H2O 0	223 1	116 (0)
B	193 1	203 1	224 1	153 1
C	193 2	204 1	225 1	197 1
D	195 2	206 2	H2O 0	198 2
E	196 1	209 1	208 (2)	6 (2)
F	199 2	220 1	148 (0)	
G	200 (0)	221 1	207 (1)	
H	202 1	222 2	28 1	

1	2	3
A158 1	A207 1	C040 0
A167 1	A116 (2)	A019 1
A168 1	A006 2	A162 0
A172 0	C006 2	A205 0
A178 1	C012 1	
A200 0	C023 2	
A208 2	C038 0	
A148 0	C039 0	

FIG 13:

(See page 162) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-4 above & 1-3 below). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

AT1-PCR:

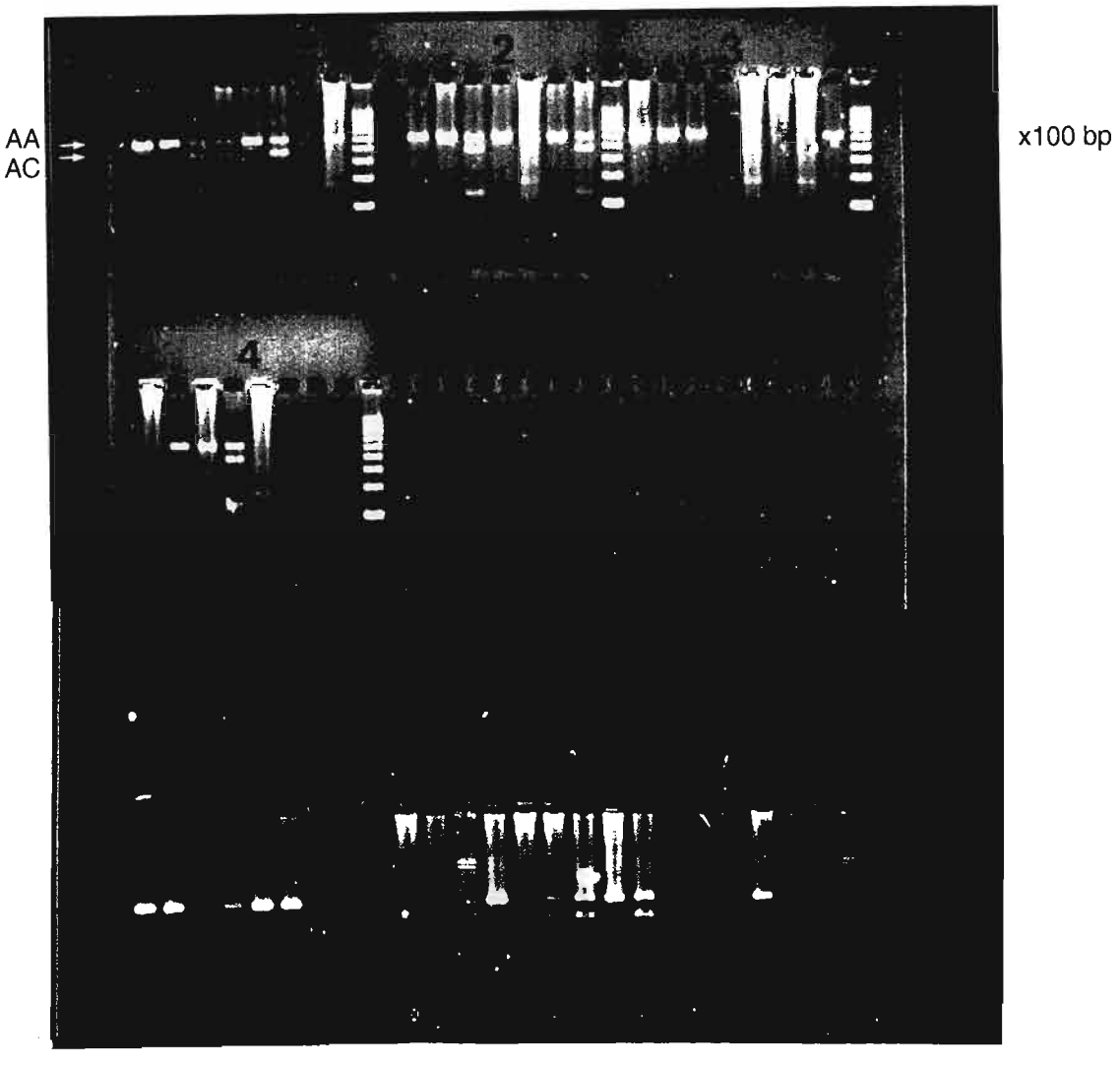

FIG 13: **AT1 PCR: PROBAND & REPEATS**
Probands *(top)* and Repeats *(below)*

After digestion with Dde I restriction endonuclease restriction the resulting fragments were size fractionated on 2% agarose gels yielding bands corresponding to AA, AC, and CC genotypes.

CYP11β2-PCR
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	1	**210**	2.5	**525**	3 min 94^0 C
MgCI	0.75	**157.5**	1.8	**378**	45 sec 94^{0}C
DNTP 1.25M	1	**210**	2.5	**525**	45 sec 52^{0}C
Taq	0.03	**6.3**	0.03	**6.3**	60 sec 72^{0}C
Primer 171 (50µM)	0.2	**42**	0.5	**105**	
Primer 304 (50µM)	0.2	**42**	0.5	**105**	10 min 72^{0}C
H_2O	6.82	**1432.2**	12.17	**2555.7**	
DNA-template	0		5		
TOTAL	10		25		
RESTRICTION:					
Neb 2	2	**420**		**1 = TT**	
HAE 3 Enzyme	0.1	**21**		**2 = TC**	2 Hrs 37^0 C
H_2O	7.9	**1659**		**3 = CC**	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	1	3	2	2	2	1	1	2	2	0	2
B	3	11	19	27	38	46	65	102	140	163	172	181
	2	2	2	2	2	2	3	1	2	1	2	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	1	1	2	2	3	2	1	1	1	1	1	3
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	2	3	2	2	1	2	1	0	1	1	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	0	1	2	1	0	3	1	1	1	0	2	2
F	7	15	23	34	41	56	87	130	147	167	177	188
	3	1	2	0	2	1	1	1	2	1	3	3
G	8	16	24	35	42	57	94	133	156	168	178	189
	2	2	1	2	2	2	2	2	2	2	2	2
H	9	17	25	36	43	60	93	136	158	169	179	190
	1	2	2	1	3	3	2	1	1	1	1	1

FIG 14:

(See page 164) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

CYP11β2 PCR:

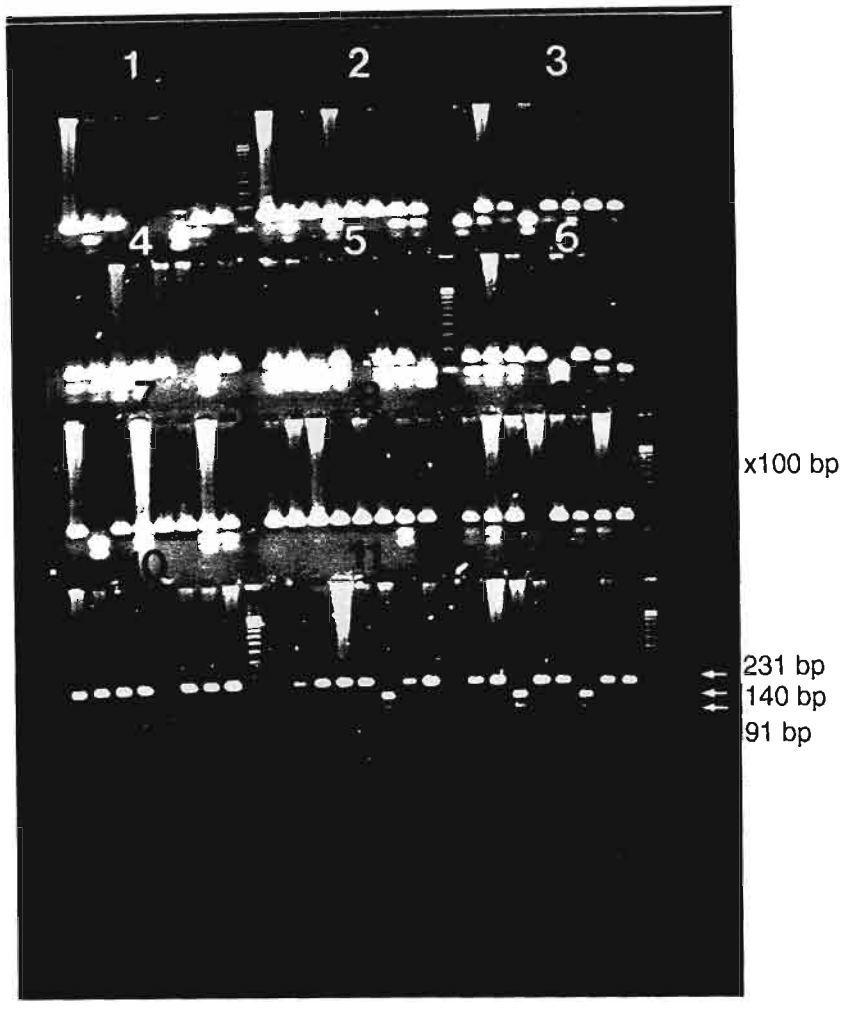

FIG 14: **CYP11β2 PCR for Aldosterone Synthase Polymorphism: PROBANDS**

After digestion with HAE 3 enzyme the reaction yielded three products: 231, 140 and 91 bp fragments corresponding to the TT, CT and CC genotypes respectively.

CYP11β2-PCR
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	1	**210**	2.5	**525**	3 min 94^0 C
MgCI	0.75	**157.5**	1.8	**378**	45 sec 94^{0}C
DNTP 1.25M	1	**210**	2.5	**525**	45 sec 52^{0}C
Taq	0.03	**6.3**	0.03	**6.3**	60 sec 72^{0}C
Primer 171 (50µM)	0.2	**42**	0.5	**105**	
Primer 304 (50µM)	0.2	**42**	0.5	**105**	10 min 72^{0}C
H_2O	6.82	**1432.2**	12.17	**2555.7**	
DNA-template	0		5		
TOTAL	10		25		
RESTRICTION:					
Neb 2	2	**420**		**1 = TT**	
HAE 3 Enzyme	0.1	**21**		**2 = TC**	2 Hrs 37^0 C
H_2O	7.9	**1659**		**3 = CC**	

	1	2	3	4	5	6	7	8	9	10	11
A	1 1	9 1	17 2	25 1	33 2	40 1	48 2	A012 2	A080 1	A119 2	A161 3
B	2 1	10 1	18 2	26 1	H2O 0	41 2	49 1	A005 1	A098 2	A120 2	A162 0
C	3 2	11 3	19 2	27 1	34 3	42 1	50 1	A019 2	A104 2	A123 2	A166 2
D	4 1	12 2	20 2	28 1	35 2	43 2	H2O 0	A024 1	A106 2	A149 1	A171 2
E	5 1	13 2	21 2	29 2	36 1	44 2	A006 2	B003 1	A108 3	A150 2	A205 1
F	6 2	14 2	22 2	30 2	37 1	45 2	A004 2	B004 1	A109 1	A154 1	A170 1
G	7 2	15 2	23 1	31 2	38 0	46 2	A008 3	B005 1	A110 3	A157 2	A086 2
H	8 2	16 2	24 2	32 1	39 1	47 2	A010 1	A064 0	H2O 0	A160 2	6 CO 2

FIG 15:

(See page 166) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

CYP11β2 PCR:

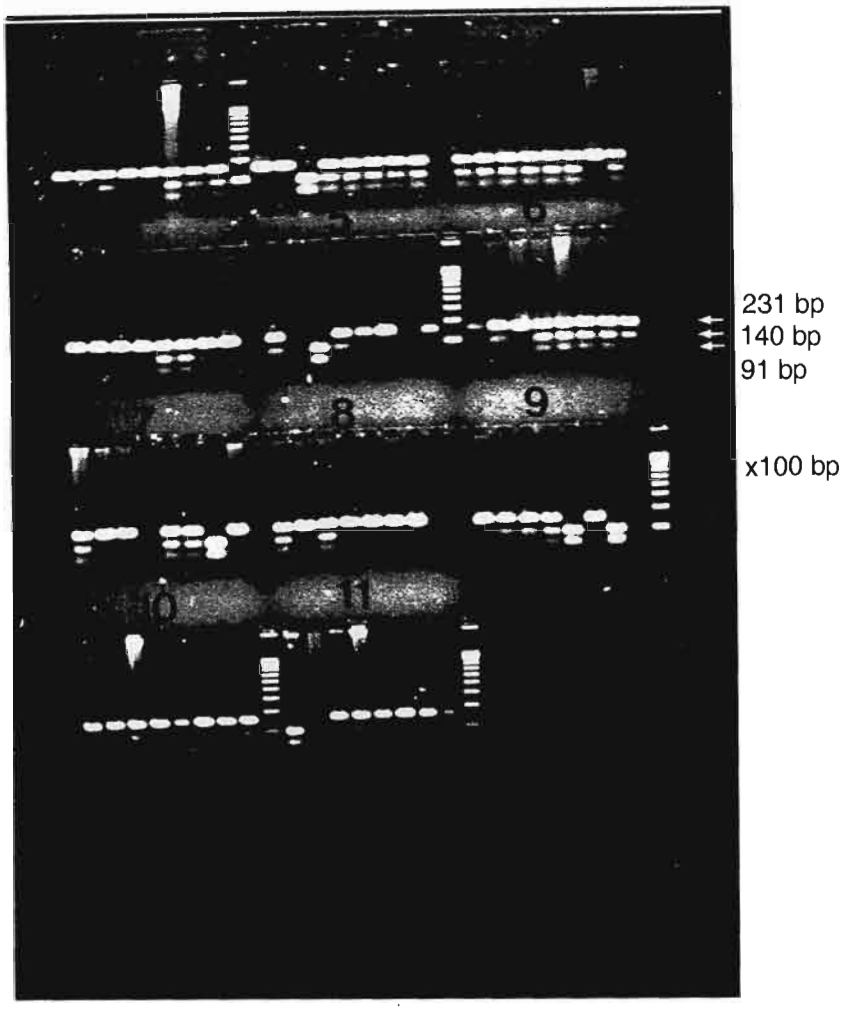

FIG 15 : **CYP11β2 PCR for Aldosterone Synthase Polymorphism: CONTROLS**

After digestion with HAE 3 enzyme the reaction yielded three products: 231, 140 and 91 bp fragments corresponding to the TT, CT and CC genotypes respectively.

CYP11β2-PCR
DNA: PROBANDS + REPEATS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **50** tubes
Buffer	1	**50**	2.5	**125**	3 min 94^0 C
MgCl	0.75	**37.5**	1.8	**90**	45 sec 94^{0}C
DNTP 1.25M	1	**50**	2.5	**125**	45 sec 52^{0}C
Taq	0.03	**1.5**	0.03	**1.5**	60 sec 72^{0}C
Primer 171 (50µM)	0.2	**10**	0.5	**25**	
Primer 304 (50µM)	0.2	**10**	0.5	**25**	10 min 72^{0}C
H_2O	6.82	**341**	12.17	**608.5**	
DNA-template	0		5		
TOTAL	10		25		
RESTRICTION:					
Neb 2	2	**100**		**1 = TT**	
HAE 3 Enzyme	0.1	**5**		**2 = TC**	2 Hrs 37^0 C
H_2O	7.9	**395**		**3 = CC**	

	1	2	3	4	5
A	191 PR 1	H2O 0	223 PR 1	116 PR 2	38 CO 2
B	192 PR 3	203 PR 1	224 PR 3	153 PR 1	3 CO 3
C	193 PR 2	204 PR 2	225 PR 3	197 PR 1	162 CO 0
D	195 PR 1	206 PR 1	H2O 0	198 PR 2	163 PR 1
E	196 PR 2	209 PR 2	208 PR 3	6 PR 1	
F	199 PR 2	220 PR 1	148 PR 2	5 PR 2	
G	200 PR 0	221 PR 2	207 PR 1	34 CO 1	
H	202 PR 2	222 PR 2	28 PR 2	145 PR 0	

FIG 16:

(See page 168) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-5). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

CYP11β2 PCR:

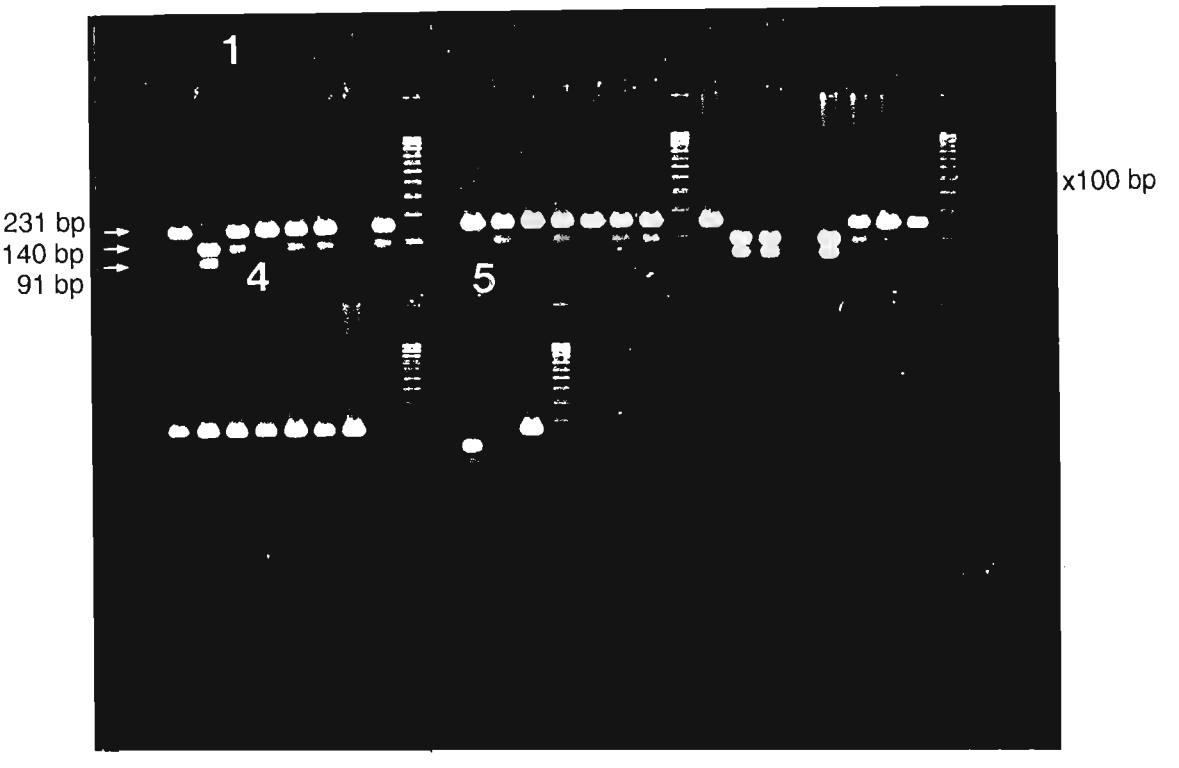

FIG 16: **CYP11β2 PCR FOR ALDOSTERONE SYNTHASE POLYMORPHISM: PROBANDS & REPEATS**

After digestion with HAE 3 enzyme the reaction yielded three products: 231, 140 and 91 bp fragments corresponding to the TT, CT and CC genotypes respectively.

11β-HSD2 - PCR
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	2	**420**	2.5	**525**	3 min 94^0 C
MgCI	0.75	**157.5**	0.75	**157.5**	30 sec 94^{0}C
DNTP 1.25M	1	**210**	1	**210**	30 sec 63^{0}C
Taq	0.03	**6.3**	0.03	**6.3**	30 sec 72^{0}C
DMSO	1	**210**	1	**210**	
Primer HSD 540A (5μM)	0.4	**84**	4	**840**	10 min 72^{0}C
Primer HSD 870B (5μM)	0.4	**84**	4	**840**	
H_2O	14.42	**3028.2**	6.72	**1411.2**	
DNA-template	0		5		
TOTAL	20		25		

RESTRICTION:				
ALu 1 Enzyme	0.2	**42**	**1 = GG**	4 Hrs 37^0 C
NE Buffer 2	3	**630**	**2 = GA**	
H_2O	6.8	**1428**	**3 = AA**	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	1	1	1	1	1	1	1	1	1	0	1
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	1	1	1	1	1	1	1	1	1	1	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	1	1	1	1	1	1	1	1	1	1	1	1
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	1	1	1	1	1	1	1	0	1	1	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	1	1	1	1	0	1	1	1	1	0	1	1
F	7	15	23	34	41	56	87	130	147	167	177	188
	1	1	1	1	1	1	1	1	0	1	1	1
G	8	16	24	35	42	57	94	133	156	168	178	189
	1	1	1	1	1	1	1	1	1	1	1	1
H	9	17	25	36	43	60	93	136	158	169	179	190
	1	1	1	1	1	1	1	2	1	1	1	1

FIG 17:

(See page 170) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

11β-HSD2 – PCR:

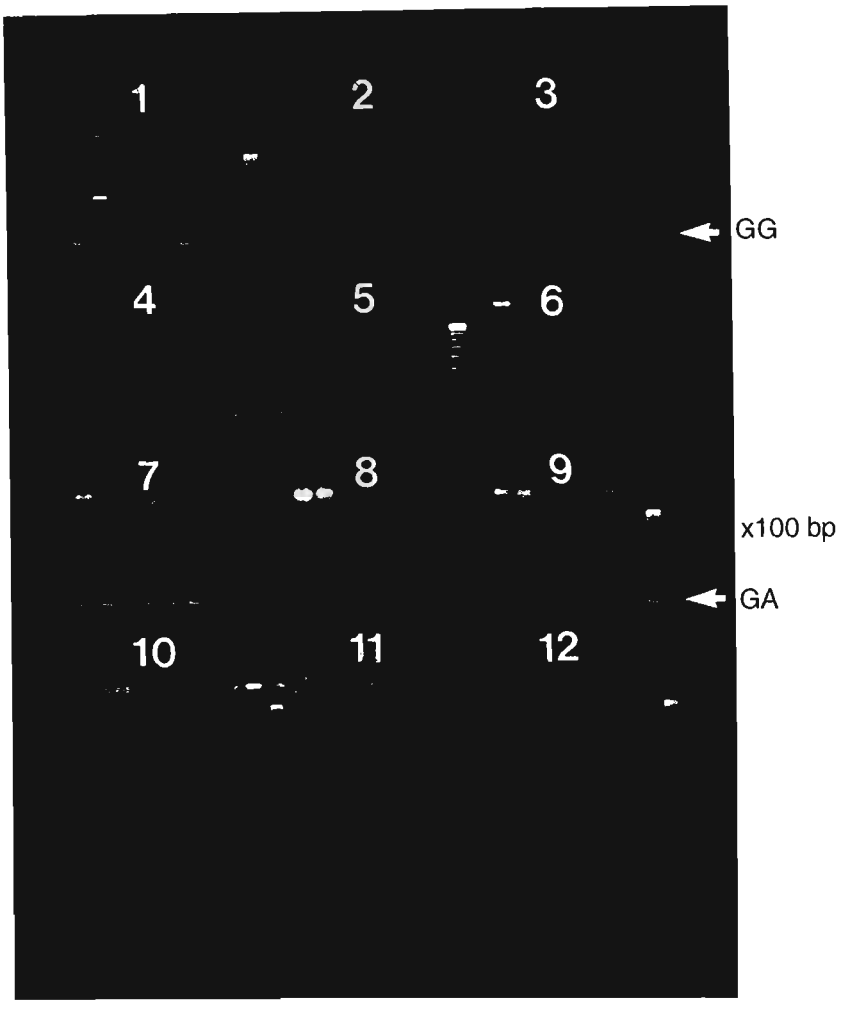

FIG 17: **PCR For 11β-Hydroxysteroid Dehydrogenase Polymorphism: PROBANDS**

After digestion with Alu-1 enzyme the PCR yielded 2 products corresponding to GG and AG genotypes. Only one proband coded for the heterozygote (GA No. 136).

11β-HSD2 - PCR
DNA: PROBANDS + REPEATS + BFU HETEROZYGOTES

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **80** tubes
Buffer	2	**160**	2.5	**200**	3 min 94^0 C
MgCI	0.75	**60**	0.75	**60**	30 sec 94^{0}C
DNTP 1.25M	1	**80**	1	**80**	30 sec 63^{0}C
Taq	0.03	**2.4**	0.03	**2.4**	30 sec 72^{0}C
DMSO	1	**80**	1	**80**	
Primer HSD 540A (5μM)	0.4	**32**	4	**320**	10 min 72^{0}C
Primer HSD 870B (5μM)	0.4	**32**	4	**320**	
H_2O	14.42	**1153.6**	6.72	**537.6**	
DNA-template	0		5		
TOTAL	20		25		
RESTRICTION:					
ALu 1 Enzyme	0.2	**16**		**1 = GG**	
NE Buffer 2	3	**240**		**2 = GA**	4 Hrs 37^0 C
H_2O	6.8	**544**		**3 = AA**	

	1	2	3	4	5	6	7	8
A	191 PR 1	H2O 0	223 PR 1	116 PR 1	140 Z 2	46 PR 1	35 CO 1	123 CO 1
B	192 PR 1	203 PR 1	224 PR 1	153 PR 1	328 Z 2	144 PR 1	36 CO 0	149 CO 0
C	193 PR 1	204 PR 1	225 PR 1	197 PR 1	377 Z 2	145 PR 0	38 CO 0	161 CO 1
D	195 PR 1	206 PR 1	H2O 0	198 PR 1	378 Z 2	147 PR 0	39 CO 0	162 CO 0
E	196 PR 1	209 PR 1	208 PR 1	6 PR 1	380 Z 2	172 PR 1	40 CO 0	205 CO 0
F	199 PR 1	220 PR 1	148 PR 1	14 Z 2	H2O 0	178 PR 1	41 CO 0	6 CO 1
G	200 PR 0	221 PR 1	207 PR 1	67 Z 2	3 PR 1	180 PR 1	48 CO 0	
H	202 PR 1	222 PR 1	28 PR 1	137 Z 2	44 PR 1	33 CO 1	108 CO 1	

FIG 18:

(See page 172) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-8). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

11β-HSD2 – PCR:

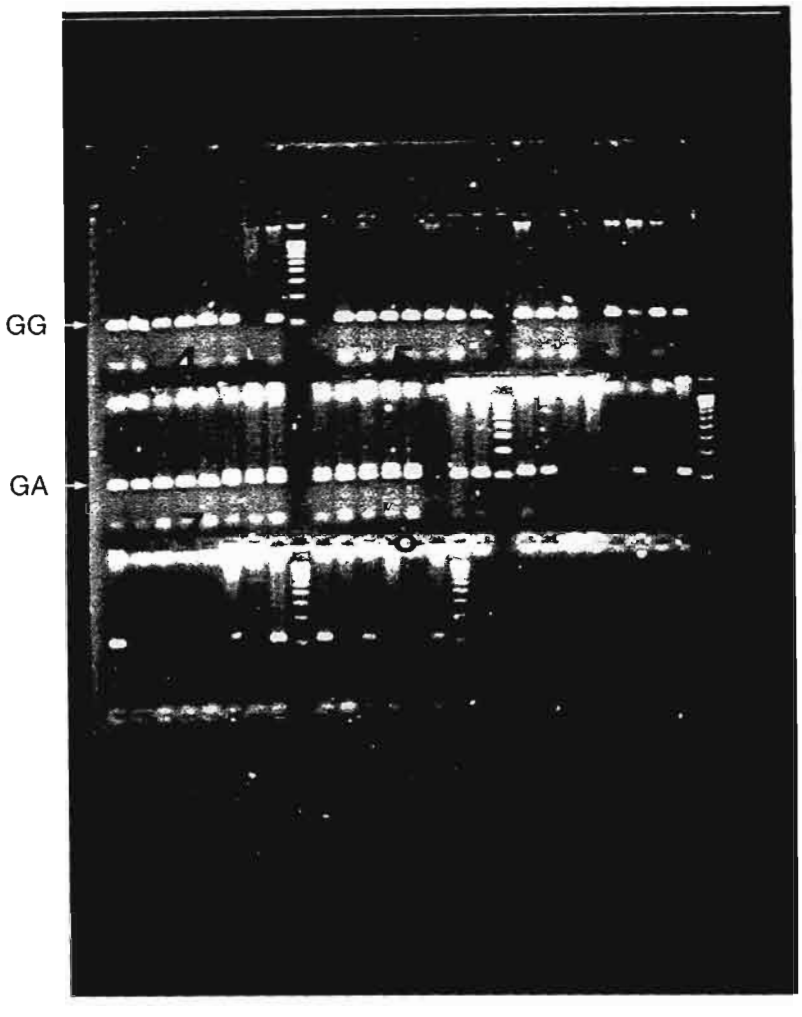

***FIG 18*:** **PCR for 11β-Hydroxysteroid Dehydrogenase Polymorphism: PROBANDS & REPEATS & BFU HETEROZYGOTES**

After digestion with Alu-1 enzyme the PCR yielded 2 products corresponding to GG and AG genotypes. Since the rare – 534A allele was not identified the PCR reaction known from BFU heterozygotes as positive controls (coded yellow) and the results of the PCR confirmed.

11β-HSD2 - PCR
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **210** tubes
Buffer	2	**420**	2.5	**525**	3 min 94° C
MgCI	0.75	**157.5**	0.75	**157.5**	30 sec 94°C
DNTP 1.25M	1	**210**	1	**210**	30 sec 63°C
Taq	0.03	**6.3**	0.03	**6.3**	30 sec 72°C
DMSO	1	**210**	1	**210**	
Primer HSD 540A (5µM)	0.4	**84**	4	**840**	10 min 72°C
Primer HSD 870B (5µM)	0.4	**84**	4	**840**	
H_2O	14.42	**3028.2**	6.72	**1411.2**	
DNA-template	0		5		
TOTAL	20		25		
RESTRICTION:					
ALu 1 Enzyme	0.2	**42**		**1 = GG**	
NE Buffer 2	3	**630**		**2 = GA**	4 Hrs 37° C
H_2O	6.8	**1428**		**3 = AA**	

	1	2	3	4	5	6	7	8	9	10	11
A	1 1	9 1	17 1	25 1	33 1	40 0	48 0	A012 1	A080 1	A119 1	A161 1
B	2 1	10 1	18 1	26 1	H2O 0	41 0	49 1	A005 1	A098 1	A120 1	A162 0
C	3 1	11 1	19 1	27 1	34 1	42 1	50 1	A019 1	A104 1	A123 1	A166 1
D	4 1	12 1	20 1	28 1	35 1	43 1	H2O 0	A024 1	A106 1	A149 0	A171 1
E	5 1	13 1	21 1	29 1	36 0	44 1	A006 1	B003 1	A108 1	A150 1	A205 0
F	6 1	14 1	22 1	30 1	37 1	45 1	A004 1	B004 1	A109 1	A154 1	A170 1
G	7 1	15 1	23 1	31 1	38 0	46 1	A008 1	B005 1	A110 1	A157 1	A086 1
H	8 1	16 1	24 1	32 1	39 0	47 1	A010 1	A064 1	H2O 0	A160 1	6 CO 1

FIG 19:

(See page 174) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

11β-HSD2 – PCR:

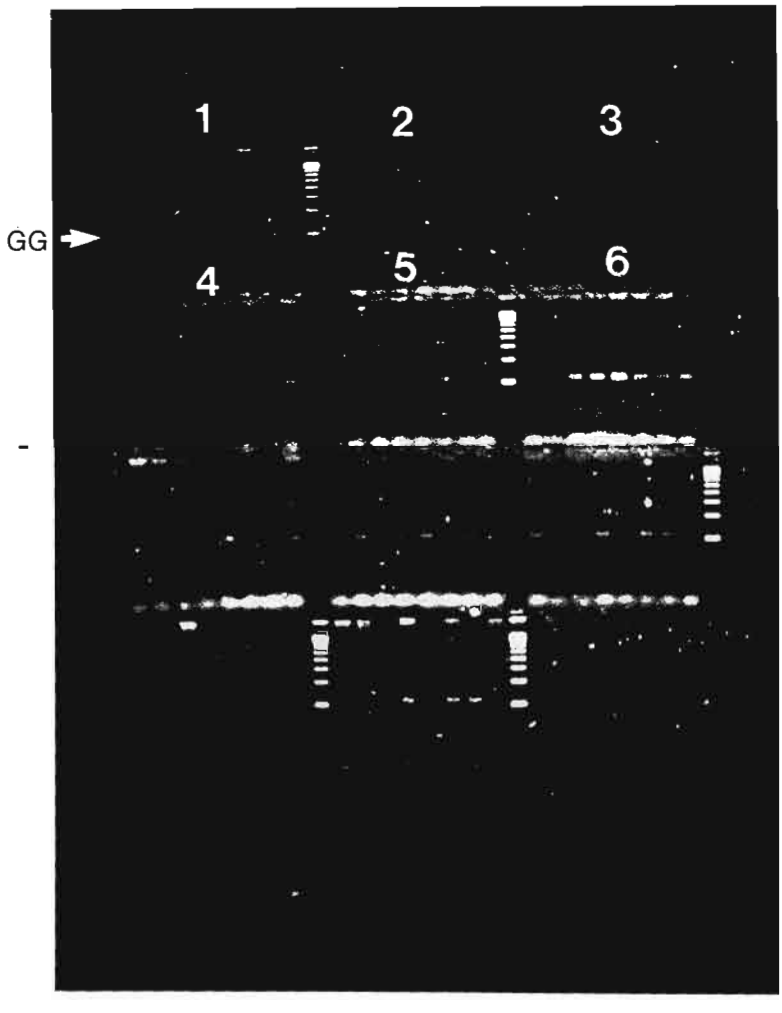

FIG 19: **PCR for 11β-Hydroxysteroid Dehydrogenase Polymorphism: CONTROLS**

After digestion with Alu-1 enzyme the PCR yielded 2 products corresponding to GG and AG genotypes. However, only the GG genotypes was identified.

TGF-β1 PCR:
DNA: PROBANDS

	Mastermix: Dry	Dry
Buffer	1	220
MgCI (25Mm)	0.6	132
dNTP 1.25M	0.25	55
Taq 5U/µl	0.05	11
TSG β1 (20µM)	0.05	11
TSG β2 (20µM)	0.045	9.9
TSG β3 (20µM)	0.075	
H_2O	7.93	16.5
DNA-template	0	1744.6
TOTAL	10	2200

For **220** tubes

3 min 94^0
45 sec 94^0C
45 sec 68^0C
60 sec 72^0C
10 min 72^0C

CODE: 1 = CC ; 2 = CT ; 3 = TT.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	10	18	26	37	44	63	99	137	159	H20	180
	1	1	1	1	1	1	1	1	1	1	0	1
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	1	1	1	1	1	1	1	1	1	0	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	1	1	0	1	1	1	1	1	1	1	1	1
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	1	1	1	1	1	1	1	0	1	1	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	1	1	1	1	0	1	1	1	1	0	1	1
F	7	15	23	34	41	56	87	130	147	167	177	188
	1	1	1	1	1	1	1	1	1	1	1	1
G	8	16	24	35	42	57	94	133	156	168	178	189
	1	1	1	1	1	1	1	1	1	1	0	1
H	9	17	25	36	43	60	93	136	158	169	179	190
	1	1	1	1	1	1	1	1	1	1	1	1

FIG 20:

(See page 176) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

TGF-β1 PCR:

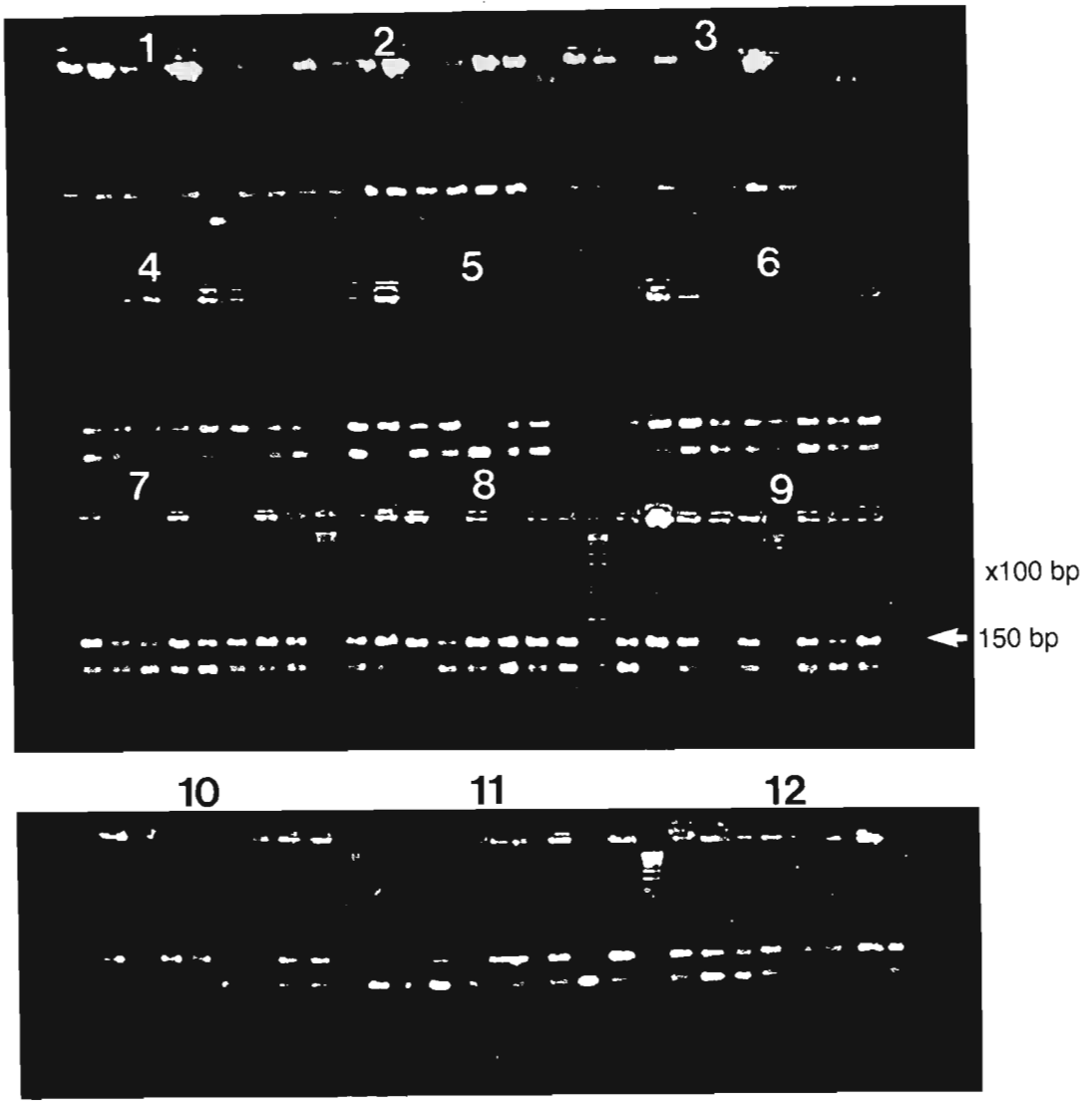

FIG 20: **TGF-β1 PCR :MUTAGENICALLY-SEPARATED PCR ASSAY PROBANDS**

Three primers were used in a single reaction mix to yield two products. In the presence of Thr 263 a PCR product of 150 bp was amplified, whereas a 129 bp fragment was amplified in the presence of the Ile 263 variant. Since no individual homozygous for the rare allele (Ile 263) was identified the PCR was repeated using known heterozygotes and the results of the PCR confirmed.

TGF-β1 PCR:
DNA: PROBANDS & REPEATS

	Mastermix: Dry	Dry
Buffer	1	60
MgCl (25Mm)	0.6	36
dNTP 1.25M	0.2	15
Taq 5U/µl	0.05	3
TSG β1 (20µM)	0.05	3
TSG β2 (20µM)	0.045	2.7
TSG β3 (20µM)	0.075	4.5
H_2O	7.93	475.8
DNA-template	0	
TOTAL	10	2200

For **60** tubes

3 min 94^0
45 sec 94^0C
45 sec 68^0C
60 sec 72^0C
10 min 72^0C

CODE: 1 = CC ; 2 = CT ; 3 = TT.

	1	2	3	4	5	6
A	191 PR 1	H2O 0	223 PR 1	116 PR 1	145 PR 0	40 CO 0
B	192 PR 1	203 PR 1	224 PR 1	153 PR 1	163 PR 1	150 CO 1
C	193 PR 1	204 PR 1	225 PR 1	197 PR 1	172 PR 0	162 CO 0
D	195 PR 1	206 PR 1	H2O 0	198 PR 1	178 PR 0	205 CO 1
E	196 PR 1	209 PR 1	208 PR 1	6 PR 1	200 PR 0	
F	199 PR 1	220 PR 1	148 PR 1	205 B 2	36 CO 0	
G	200 PR 0	221 PR 1	207 PR 1	223 B 2	38 CO 0	
H	202 PR 1	222 PR 1	28 PR 1	291 B 2	39 CO 0	

FIG 21:

(See page 178) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-6). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

TGF-β1 PCR:

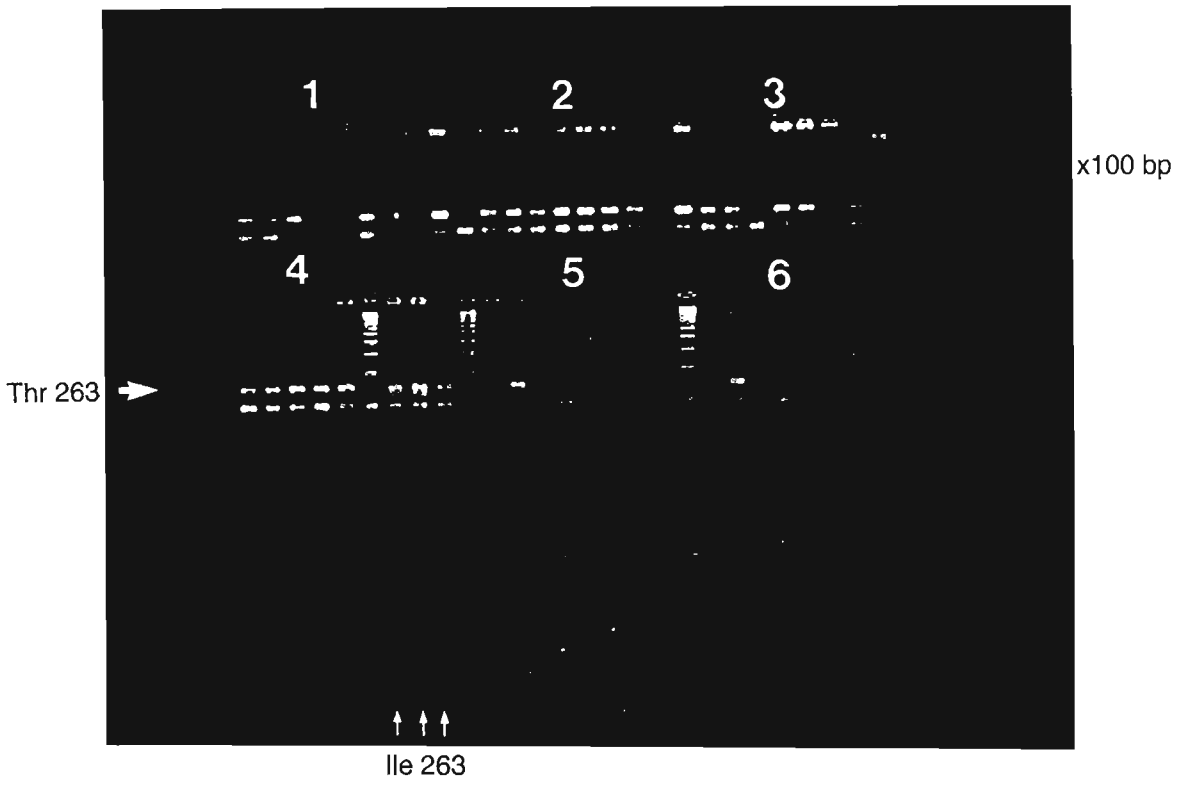

FIG 21: **TGF-β1 PCR: MUTAGENICALLY-SEPARATED PCR ASSAY PROBANDS & REPEATS:**

Three primers were used in a single reaction mix to yield two products. In the presence of Thr 263 a PCR product of 150 bp was amplified, whereas a 129 bp fragment was amplified in the presence of the Ile 263 variant. Since no individual homozygous for the rare allele (Ile 263) was identified the PCR was repeated using known heterozygotes and the results of the PCR confirmed.

TGF-β1 PCR:
DNA: CONTROLS

	Mastermix: Dry	Dry
Buffer	1	110
MgCI (25Mm)	0.6	66
dNTP 1.25M	0.25	27.5
Taq 5U/μl	0.05	5.5
TSG β1 (20μM)	0.05	5.5
TSG β2 (20μM)	0.045	4.95
TSG β3 (20μM)	0.075	8.25
H_2O	7.93	872.3
DNA-template	0	
TOTAL	10	

For **110** tubes

3 min 94^0
45 sec 94^0C
45 sec 68^0C
60 sec 72^0C
10 min 72^0C

	1	2	3	4	5	6	7	8	9	10	11
A	1	9	17	25	33	40	48	A012	A080	A119	A161
	1	1	1	1	1	0	1	1	1	1	1
B	2	10	18	26	H2O	41	49	A005	A098	A120	A162
	1	1	1	1	0	1	1	1	1	1	0
C	3	11	19	27	34	42	50	A019	A104	A123	A166
	1	1	1	1	1	1	1	1	1	1	1
D	4	12	20	28	35	43	H2O	A024	A106	A149	A171
	1	1	1	1	1	1	0	1	1	1	1
E	5	13	21	29	36	44	A006	B003	A108	A150	A205
	1	1	1	1	0	1	1	1	1	1	1
F	6	14	22	30	37	45	A004	B004	A109	A154	A170
	1	1	1	1	1	1	1	1	1	1	1
G	7	15	23	31	38	46	A008	B005	A110	A157	A086
	1	1	1	1	0	1	1	1	1	1	1
H	8	16	24	32	39	47	A010	A064	H2O	A160	006 CO
	1	1	1	1	0	1	1	1	0	1	1

FIG 22:

(See page 180) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

TGF-β1 PCR:

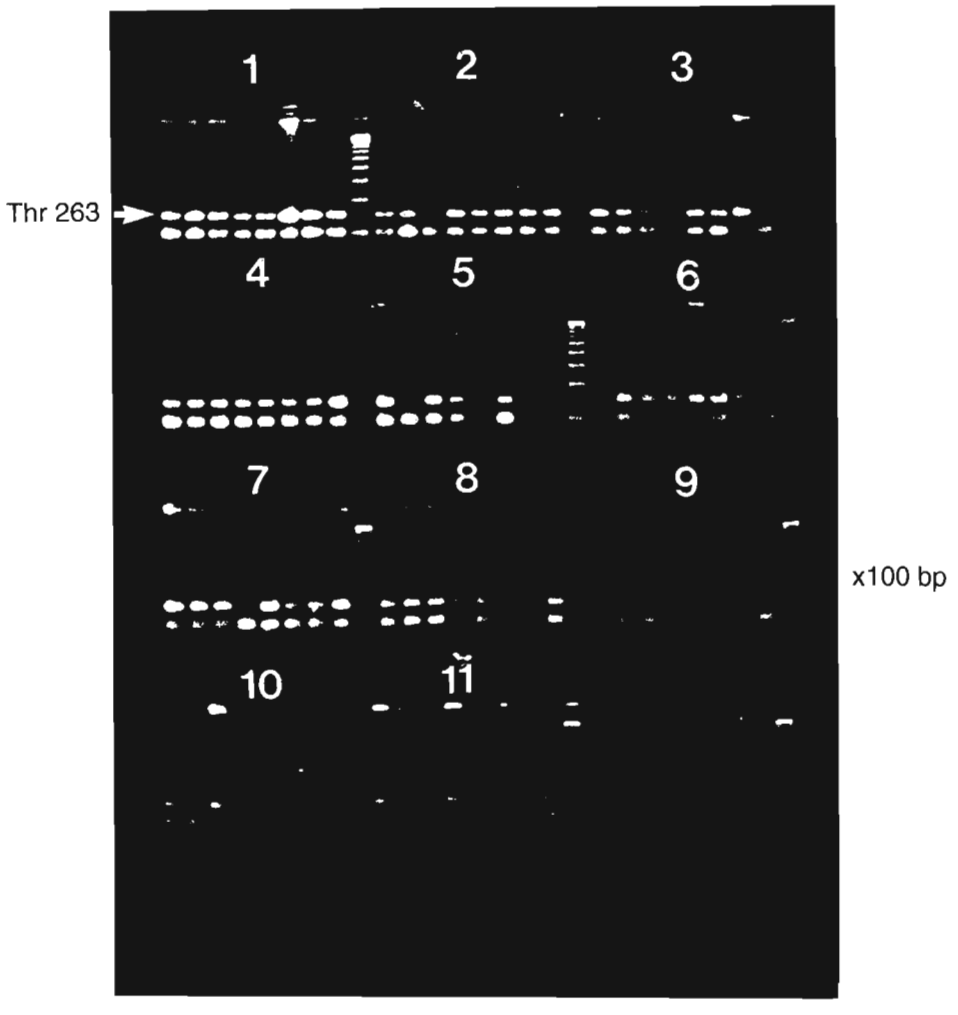

FIG 22: **TGF-β1 PCR : MUTAGENICALLY-SEPARATED PCR ASSAY: CONTROLS**

Three primers were used in a single reaction mix to yield two products. In the presence of Thr 263 a PCR product of 150 bp was amplified, whereas a 129 bp fragment was amplified in the presence of the Ile 263 variant. Since no individual homozygous for the rare allele (Ile 263) was identified the PCR was repeated using known heterozygotes and the results of the PCR confirmed.

MTHFR-PCR
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **220** tubes
Buffer	1	**220**	1.3	**286**	3 min 94^0 C
MgCI	0.6	**132**	0.9	**198**	15 sec 94^{0}C
DNTP 1.25M	1.6	**352**	1.3	**286**	45 sec 58^{0}C
Taq	0.03	**6.6**	0.03	**6.6**	45 sec 72^{0}C
MTHFR-S (6µM)	1	**220**	0.13	**28.6**	
MTHFR-AS(6µM)	1	**220**	0.13	**28.6**	10 min 72^{0}C
H_2O	4.77	**1049.4**	1.77	**389.4**	
DNA-template	0				
TOTAL	10		13		
RESTRICTION:					
Hinf 1	0.1	**22**	0.2	**44**	
Buffer Nr.2	2	**440**	2.3	**506**	20 Hrs 37^0 C
H_2O	7.9	**1738**	7.9	**1738**	

CODE: 1 = TT ; 2 = TC ; 3 = CC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2	10	18	26	37	44	63	99	137	159	H2O	180
	1	3	1	1	1	1	1	1	1	1	0	1
B	3	11	19	27	38	46	65	102	140	163	172	181
	1	1	1	1	1	1	1	1	1	2	0	1
C	4	12	20	30	39	49	77	103	144	164	174	184
	2	2	1	1	1	1	1	1	1	1	1	1
D	H2O	13	21	31	40	54	78	105	145	165	175	186
	0	1	2	1	1	1	2	1	0	1	2	1
E	5	14	22	33	H2O	55	85	111	146	H2O	176	187
	1	1	1	1	0	1	1	1	1	0	2	1
F	7	15	23	34	41	56	87	130	147	167	177	188
	1	1	1	1	1	1	2	1	1	1	2	1
G	8	16	24	35	42	57	94	133	156	168	178	189
	1	1	1	2	2	1	1	1	1	1	1	2
H	9	17	25	36	43	60	93	136	158	169	179	190
	2	2	1	1	2	2	1	1	1	1	1	1

FIG 23:

(See page 182) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

MTHFR-PCR:

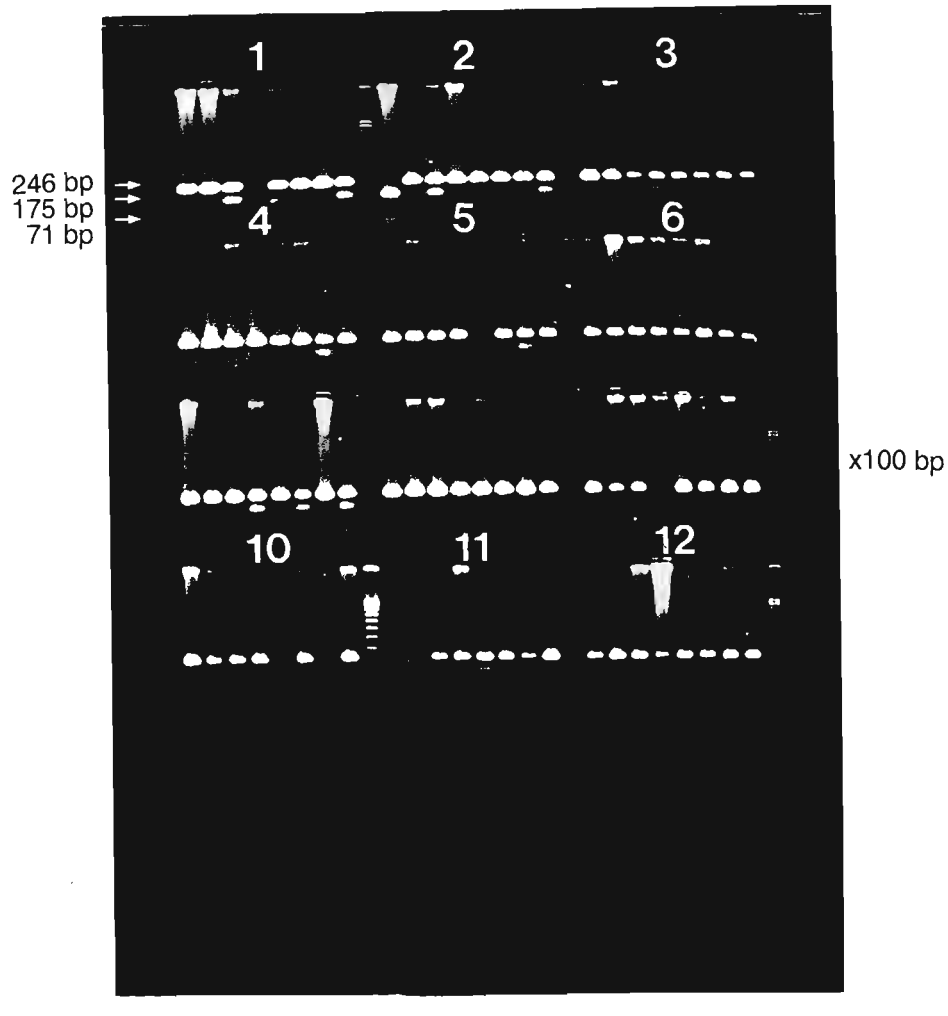

FIG 23: **MTHFR C677T PCR: PROBANDS**

After digestion with Hinf I restriction endonuclease the PCR yielded three products: 246, 175, 71 bp fragments corresponding to the CC, CT and TT genotypes respectively.

MTHFR-PCR
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **220** tubes
Buffer	1	**220**	1.3	**286**	3 min 94^0 C
MgCl	0.6	**132**	0.9	**198**	15 sec 94^{0}C
DNTP 1.25M	1.6	**352**	1.3	**286**	45 sec 58^{0}C
Taq	0.03	**6.6**	0.03	**6.6**	45 sec 72^{0}C
MTHFR-S (6µM)	1	**220**	0.13	**28.6**	
MTHFR-AS(6µM)	1	**220**	0.13	**28.6**	10 min 72^{0}C
H_2O	4.77	**1049**	1.77	**389.4**	
DNA-template	0				
TOTAL	10		13		
RESTRICTION:					
Hinf 1	0.1	**22**	0.2	**44**	
Buffer Nr.2	2	**440**	2.3	**506**	20 Hrs 37^0 C
H_2O	7.9	**1738**	7.9	**1738**	

CODE: 1 = CC; 2 = TC; 3 = TT.

	1	2	3	4	5	6	7	8	9	10	11
A	1 2	9 1	17 1	25 1	33 1	40 0	48 1	A012 1	A080 1	A119 3	A161 1
B	2 2	10 1	18 1	26 1	H2O 0	41 1	49 1	A005 1	A098 3	A120 1	A162 0
C	3 1	11 1	19 1	27 1	34 1	42 1	50 1	A019 1	A104 1	A123 2	A166 1
D	4 1	12 1	20 1	28 1	35 2	43 1	H2O 0	A024 1	A106 1	A149 2	A171 1
E	5 1	13 1	21 1	29 2	36 0	44 1	A006 1	B003 1	A108 1	A150 1	A205 1
F	6 1	14 1	22 1	30 2	37 1	45 1	A004 1	B004 1	A109 1	A154 1	A170 1
G	7 1	15 2	23 1	31 2	38 0	46 2	A008 2	B005 2	A110 1	A157 1	A086 1
H	8 1	16 1	24 2	32 1	39 0	47 1	A010 1	A064 1	H2O 0	A160 1	6 CO 1

FIG 24:

(See page 184) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

MTHFR-PCR:

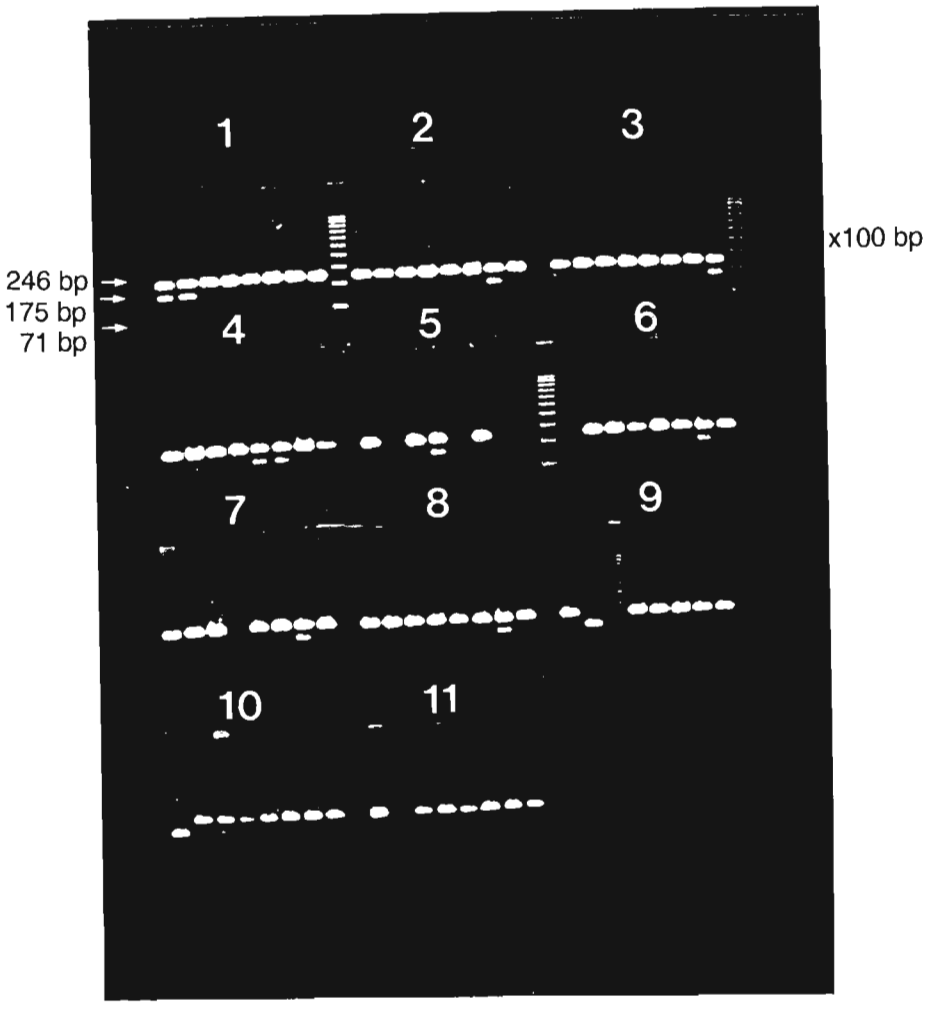

FIG 24: **MTHFR C677T PCR: CONTROLS**

After digestion with Hinf I restriction endonuclease the PCR yielded three products: 246, 175, 71 bp fragments corresponding to the CC, CT and TT genotypes respectively.

MTHFR-PCR
DNA: PROBANDS + REPEATS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **50** tubes
Buffer	1	**50**	1.3	**65**	3 min 94^0 C
MgCI	0.6	**30**	0.9	**45**	15 sec 94^{0}C
DNTP 1.25M	1.6	**80**	1.3	**65**	45 sec 58^{0}C
Taq	0.03	**1.5**	0.03	**1.5**	45 sec 72^{0}C
MTHFR-S (6µM)	1	**50**	0.13	**6.5**	
MTHFR-AS(6µM)	1	**50**	0.13	**6.5**	10 min 72^{0}C
H_2O	4.77	**238.5**	1.77	**88**	
DNA-template	0				
TOTAL	10		13		
RESTRICTION:					
Hinf 1	0.1	**5**	0.2	**10**	
Buffer Nr.2	2	**100**	2.3	**115**	20 Hrs 37^0 C
H_2O	7.9	**395**	7.9	**395**	

CODE: 1 = TT ; 2 = TC ; 3 = CC.

	1	2	3	4	5
A	191 PR 1	H2O 0	223 PR 2	116 PR 1	172 PR 1
B	192 PR 2	203 PR 1	224 PR 1	153 PR 2	36 CO 1
C	193 PR 1	204 PR 1	225 PR 1	197 PR 1	38 CO 0
D	195 PR 1	206 PR 1	H2O 0	198 PR 1	39 CO 0
E	196 PR 1	209 PR 1	208 PR 2	6 PR 1	40 CO 0
F	199 PR 1	220 PR 1	148 PR 0	5 PR 1	98 CO 3
G	200 PR 0	221 PR 1	207 PR 1	145 PR 0	162 CO 0
H	202 PR 1	222 PR 1	28 PR 1	168 PR 1	163 PR 2

FIG 25:

(See page 186) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-5). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

MTHFR-PCR:

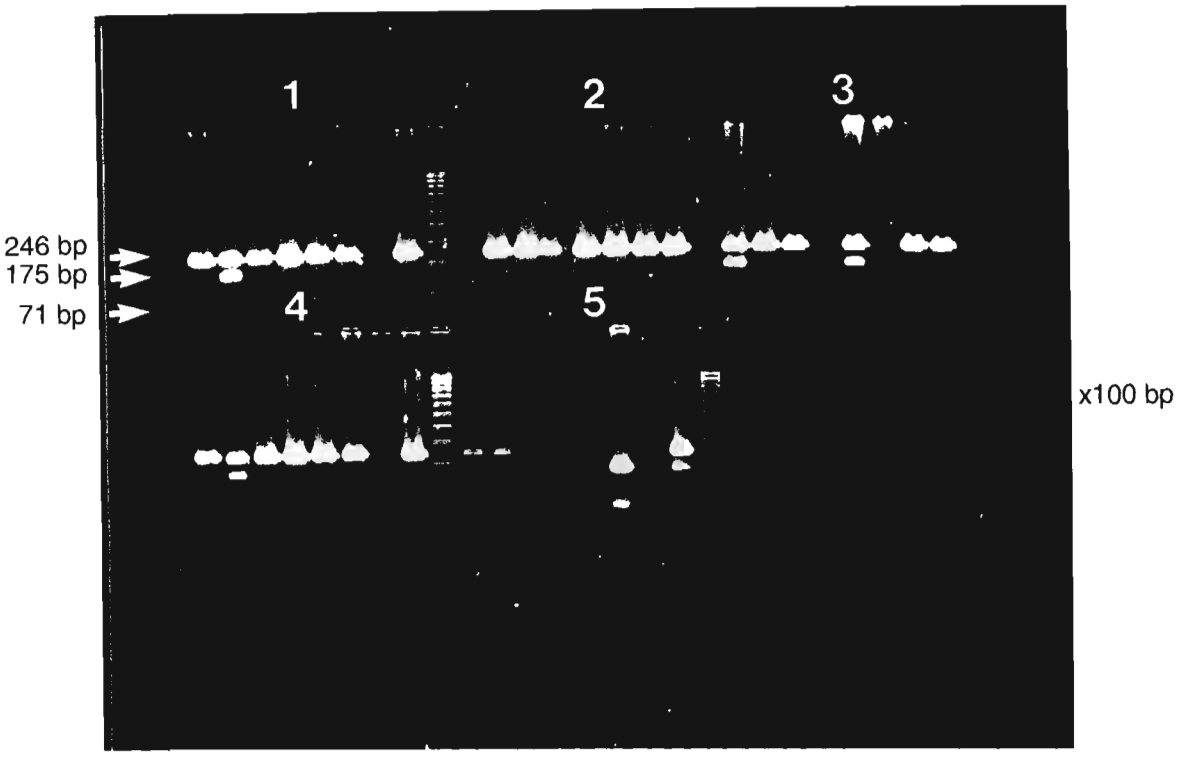

FIG 25: **MTHFR C677T PCR: PROBAND REPEATS**

After digestion with Hinf I restriction endonuclease the PCR yielded three products: 246, 175, 71 bp fragments corresponding to the CC, CT and TT genotypes respectively.

G-PROTEIN-PCR
DNA: PROBANDS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**110**	1.5	**165**	3 min 94^0
MgCl	0.75	**82.5**	1.2	**132**	45 sec 94^{0}C
DNTP 1.25M	1	**110**	1.5	**165**	45 sec 60^{0}C
Taq	0.02	**2.2**	0.03	**3.3**	60 sec 72^{0}C
GPROT 1+2 (20µM)	0.26	**28.6**	0.4	**44**	10 min 72^{0}C
H_2O	6.97	**766.7**	5.37	**90.7**	
DNA-template	0		5		
TOTAL	10		15		
DSE D 1 (µL)	0.6	**66**	0.6	**66**	
Buffer Nr.Y	1.8	**198**	2.2	**242**	2 Hrs 60^0 C
BSA 1: 10	1	**110**	1.0	**110**	
H_2O	6.6	**726**	6.2	**682**	

CODE: 1 = TT ; 2 = TC ; 3 = CC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 2	10 3	18 2	26 2	37 2	44 2	63 0	99 2	137 2	159 3	H2O 0	180 0
B	3 3	11 2	19 2	27 2	38 3	46 3	65 1	102 3	140 3	163 2	172 2	181 3
C	4 2	12 2	20 2	30 3	39 3	49 3	77 3	103 2	144 3	164 2	174 2	184 3
D	H2O 0	13 3	21 2	31 2	40 3	54 3	78 2	105 3	145 2	165 3	175 3	186 3
E	5 3	14 2	22 1	33 3	H2O 0	55 2	85 2	111 2	146 3	H2O 0	176 3	187 2
F	7 2	15 3	23 3	34 2	41 2	56 0	87 3	130 2	147 3	167 2	177 2	188 1
G	8 2	16 2	24 3	35 2	42 3	57 2	94 3	133 3	156 3	168 2	178 (3)	189 3
H	9 3	17 3	25 3	36 2	43 3	60 2	93 3	136 2	158 2	169 3	179 1	190 3

FIG 26:

(See page 188) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-12). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

G-PROTEIN PCR:

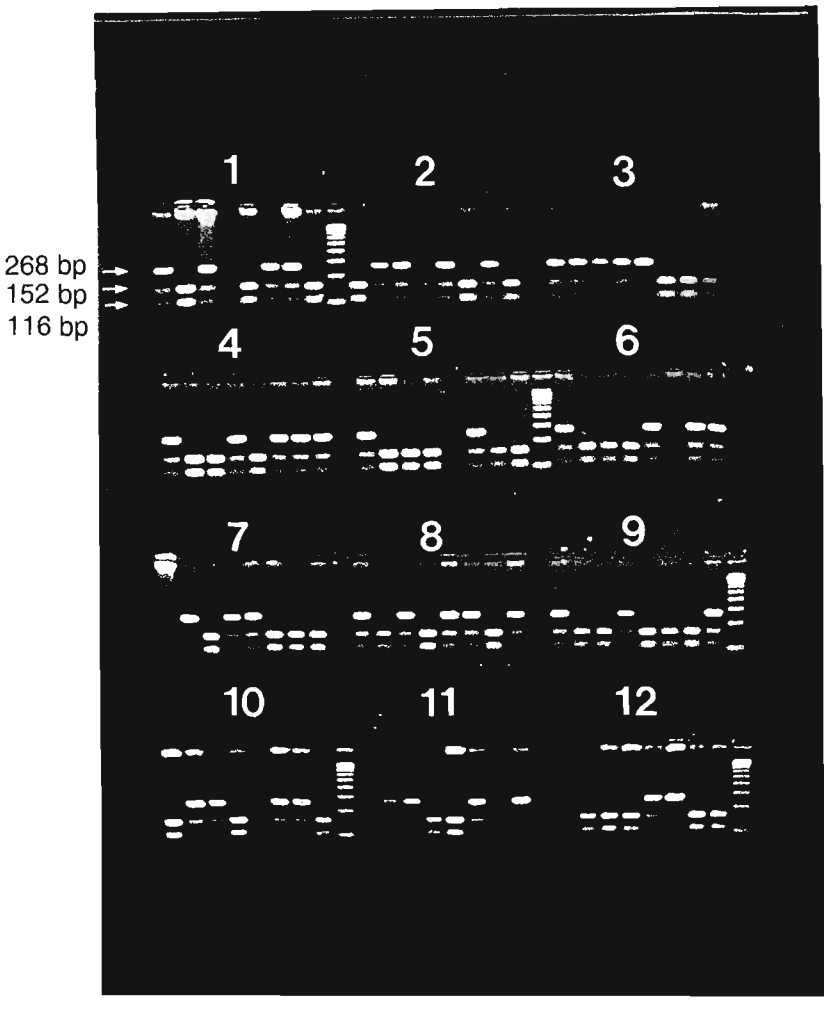

FIG 26: **G-PROTEIN: PROBANDS**

After digestion with BSE DI restriction endonuclease the PCR yielded 3 products: 268, 152 116 bp fragments corresponding to the TT, TC and CC genotypes respectively.

G-PROTEIN-PCR
DNA: PROBANDS & REPEAT CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**50**	1.5	**75**	3 min 94^0
MgCI	0.75	**37.5**	1.2	**60**	45 sec 94^{0}C
DNTP 1.25M	1	**50**	1.5	**75**	45 sec 60^{0}C
Taq	0.02	**1**	0.03	**1.5**	60 sec 72^{0}C
GPROT 1+2 (20µM)	0.26	**13**	0.4	**20**	10 min 72^{0}C
H_2O	6.97	**348.5**	5.37	**268.5**	
DNA-template	0		5		
TOTAL	10		15		
DSE D 1 (µL)	0.6	**30**	0.6	**30**	
Buffer Nr.Y	1.8	**90**	2.2	**110**	2 Hrs 60^0 C
BSA 1: 10	1	**50**	1.0	**50**	
H_2O	6.6	**330**	6.2	**310**	

CODE: 1 = TT ; 2 = TC ; 3 = CC.

	1	2	3	4	5	6
A	191 PR 1	H2O 0	223 PR 3	116 PR 3	180 PR 2	160 CO 0
B	192 PR 1	203 PR 3	224 PR 2	153 PR 3	6 CO 3	205 CO 2
C	193 PR 3	204 PR 3	225 PR 3	197 PR 3	18 CO 2	6 CO 3
D	195 PR 1	206 PR 2	H2O 0	198 PR 3	23 CO 2	
E	196 PR 3	209 PR 3	208 PR 3	6 PR 3	36 CO 0	
F	199 PR 1	220 PR 2	148 PR 2	56 PR 3	38 CO 0	
G	200 PR 0	221 PR 2	207 PR 3	63 PR 3	39 CO 0	
H	202 PR 2	222 PR 1	28 PR 3	178 PR 3	40 CO 0	

FIG 27:

(See page 190) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-6). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

G-PROTEIN PCR:

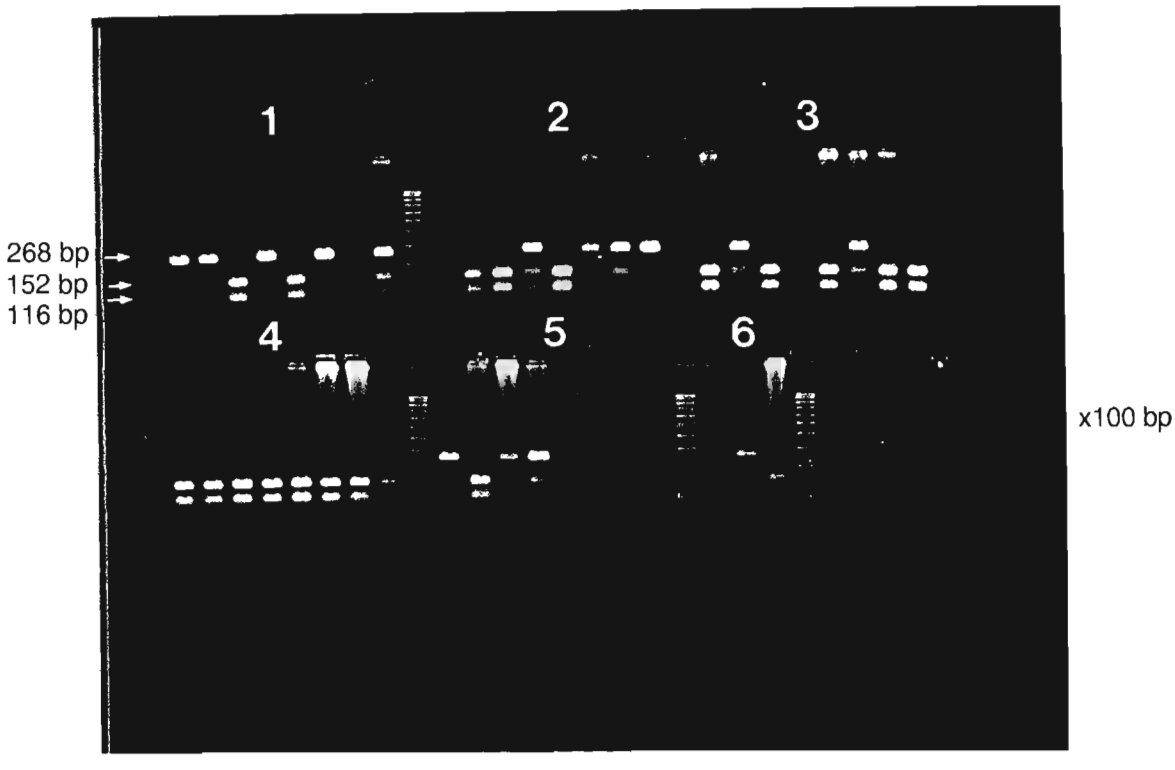

FIG 27: **G-PROTEIN: PROBANDS & REPEATS**

After digestion with BSE DI restriction endonuclease the PCR yielded 3 products: 268, 152 116 bp fragments corresponding to the TT, TC and CC genotypes respectively.

G-PROTEIN-PCR
DNA: CONTROLS

	Mastermix: Dry	Dry	Liquid	Master: Liquid	For **110** tubes
Buffer	1	**210**	1.5	**315**	3 min 94^0
MgCI	0.75	**157.5**	1.2	**252**	45 sec 94^{0}C
DNTP 1.25M	1	**210**	1.5	**315**	45 sec 60^{0}C
Taq	0.02	**4.2**	0.03	**6.3**	60 sec 72^{0}C
GPROT 1+2 (20µM)	0.26	**54.6**	0.4	**84**	10 min 72^{0}C
H_2O	6.97	**1463.7**	5.37	**1127.7**	
DNA-template	0		5		
TOTAL	10		15		
DSE D 1 (µL)	0.6	**126**	0.6	**126**	
Buffer Nr.Y	1.8	**378**	2.2	**462**	2 Hrs 60^0 C
BSA 1: 10	1	**210**	1.0	**210**	
H_2O	6.6	**1386**	6.2	**1302**	

CODE: 1 = TT ; 2 = TC ; 3 = CC.

	1	2	3	4	5	6	7	8	9	10	11
A	1	9	17	25	33	40	48	A012	A080	A119	A161
	3	2	3	3	3	0	3	2	2	3	3
B	2	10	18	26	H2O	41	49	A005	A098	A120	A162
	3	3	0	3	0	2	3	3	3	3	3
C	3	11	19	27	34	42	50	A019	A104	A123	A166
	2	2	2	2	3	3	2	1	3	3	1
D	4	12	20	28	35	43	H2O	A024	A106	A149	A171
	2	2	1	2	3	2	0	2	3	3	2
E	5	13	21	29	36	44	A006	B003	A108	A150	A205
	2	2	3	3	0	3	3	2	3	2	0
F	6	14	22	30	37	45	A004	B004	A109	A154	A170
	0	2	3	3	3	2	2	2	2	3	3
G	7	15	23	31	38	46	A008	B005	A110	A157	A086
	2	2	0	1	0	2	1	3	3	2	2
H	8	16	24	32	39	47	A010	A064	H2O	A160	A006
	3	3	3	1	0	3	3	3	0	0	3

FIG 28:

(See page 192) - 2% Agarose gel electrophoresis showing DNA profile as per table above (lanes 1-11). The molecular weight marker is a 100 bp ladder. Control samples are indicated as water (H_2O).

G-PROTEIN PCR:

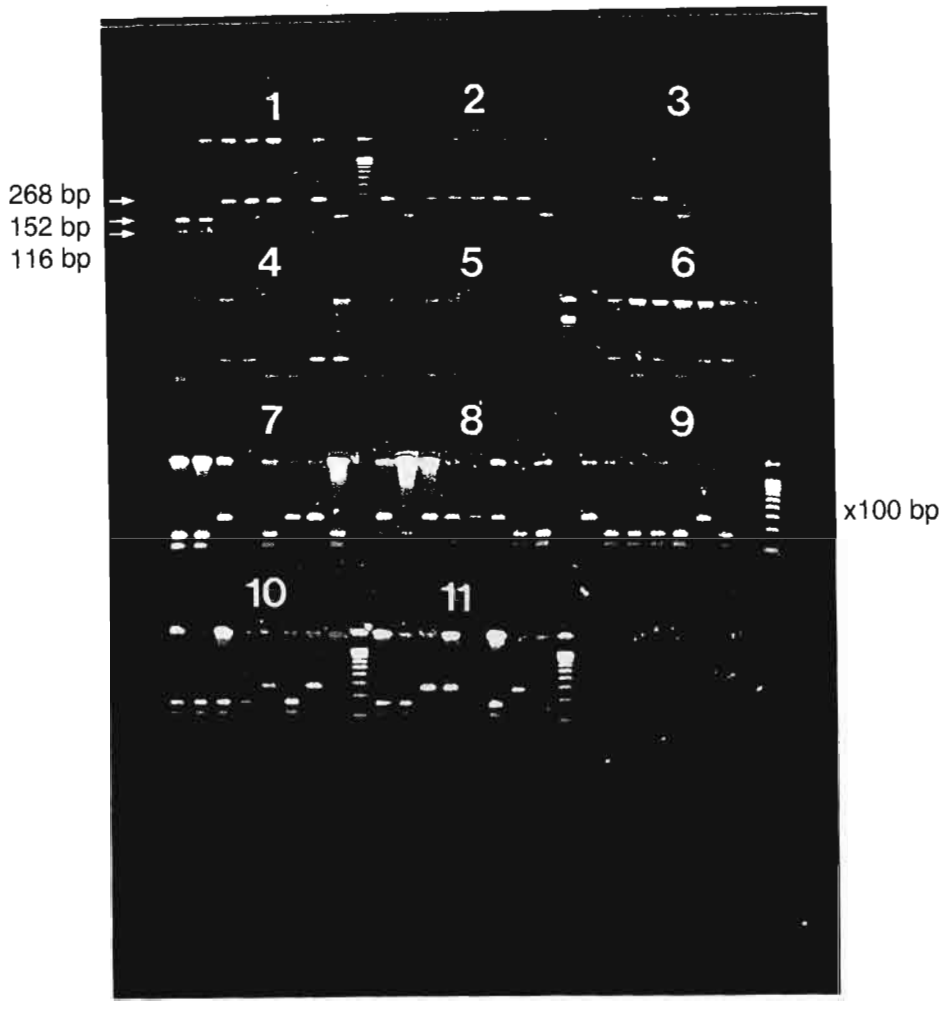

FIG 28: **G-PROTEIN: CONTROLS**

After digestion with BSE DI restriction endonuclease the PCR yielded 3 products: 268, 152 116 bp fragments corresponding to the TT, TC and CC genotypes respectively.

ACE	controls (O)	cases (O)	sum				
II	25	44	69				
ID	34	55	89				
DD	17	16	33				
	76	115	191				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
II	27.46	41.54	6.03	6.03	0.22	0.15	
ID	35.41	53.59	2.00	2.00	0.06	0.04	
DD	13.13	19.87	14.97	14.97	1.14	0.75	
	76.00	115.00	23.00	23.00	1.42	0.94	
X^2 =	**2.35**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
II	25	23	0.14	II	44	44	0.00
ID	34	38	0.34	ID	55	54	0.02
DD	17	15	0.21	DD	16	16	0.01
n=	**76**	**76**	**0.69**	n=	**115**	**115**	**0.03**
fI	**0.55**			f1	**0.62**		
fD	**0.45**			f3	**0.38**		
	I	D					
cases	143	87	230				
controls	84	68	152				
	227	155	382	X^2 =	**1.81**	DF = 1	

AGT	controls (O)	cases (O)	sum				
1	32	44	76				
2	32	50	82				
3	11	18	29				
	75	112	187				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	30.48	45.52	2.31	2.31	0.08	0.05	
2	32.89	49.11	0.79	0.79	0.02	0.02	
3	11.63	17.37	0.40	0.40	0.03	0.02	
	75.00	112.00	3.49	3.49	0.13	0.09	
X^2 =	**0.22**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	32	31	0.05	1	44	43	0.05
2	32	35	0.19	2	50	53	0.17
3	11	10	0.17	3	18	17	0.13
n=	**75**	**75**	**0.41**	n=	**112**	**112**	**0.35**
f1	**0.64**			f1	**0.62**		
f3	**0.36**			f3	**0.38**		
	1	3					
cases	138	86	224				
controls	96	54	150				
	234	140	374	X^2 =	**0.22**	DF = 1	

TGF-B	controls (O)	cases (O)	sum				
1	78	114	192				
2	1	1	2				
3	1	1	2				
	80	116	196				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	78.37	113.63	0.13	0.13	0.00	0.00	
2	0.82	1.18	0.03	0.03	0.04	0.03	
3	0.82	1.18	0.03	0.03	0.04	0.03	
	80.00	116.00	0.20	0.20	0.08	0.06	
X^2 =	**0.14**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	78	77	0.01	1	114	113	0.01
2	1	3	1.28	2	1	3	1.30
3	1	1	0.00	3	1	1	0.00
n =	**80**	**81**	**1.30**	n =	**116**	**117**	**1.31**
f1 =	**0.98**			f1 =	**0.99**		
f3 =	**0.02**			f3 =	**0.01**		
	1	3					
cases	229	3	232				
controls	157	3	160				
	386	6	392	X^2 =	**0.21**	DF = 1	
				Fisher's exact (number of cells < 5)			
				p =	0.69		
For the purposes of the Hardy-Weinberg calculation, the 0 value for the CC and the							
TT genotype was replaced by 1. The allelic frequencies remained the same							

AT1	controls (O)	cases (O)	sum				
1	65	100	165				
2	15	16	31				
3	0	0	0				
	80	116	196				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	67.35	97.65	5.51	5.51	0.08	0.06	
2	12.65	18.35	5.51	5.51	0.44	0.30	
3	0.00	0.00	0.00	0.00			
	80.00	116.00	11.02	11.02			
X^2 =	**0.88**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	65	66	0.01	1	100	101	0.00
2	15	14	0.15	2	16	15	0.08
3	0	1	0.70	3	0	1	0.55
n =	**80**	**80**	**0.86**	n =	**116**	**116**	**0.64**
f1 =	**0.91**			f1 =	**0.93**		
f3 =	**0.09**			f3 =	**0.07**		
	1	3					
cases	216	16	232				
controls	145	15	160				
	361	31	392	X^2 =	**0.80**	DF = 1	
For the purposes of the Hardy-Weinberg calculation, the 0 value for the CC genotype							
was replaced by 1. The statistical significance for the allelic frequencies was kept the							
same.							

CYP11B2	controls (O)	cases (O)	sum				
1	31	48	79				
2	42	54	96				
3	7	15	22				
	80	117	197				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	32.08	46.92	1.17	1.17	0.04	0.02	
2	38.98	57.02	9.09	9.09	0.23	0.16	
3	8.93	13.07	3.74	3.74	0.42	0.29	
	80.00	117.00	14.00	14.00	0.69	0.47	
X^2 =	**1.16**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	31	36	0.71	1	48	48	0.00
2	42	32	3.20	2	54	54	0.00
3	2	1	1.00	3	15	1	0.02
n =	**75**	**69**	**4.91**	n =	**117**	**103**	**0.02**
f1 =	**0.69**			f1 =	**0.64**		
f3 =	**0.31**			f3 =	**0.36**		
	1	3					
cases	150	84	234				
controls	104	56	160				
	254	140	394	X^2 =	**0.03**	DF = 1	
				p =	0.97		

11BHSD2	controls (O)	cases (O)	sum				
1	76	115	191				
2	1	1	2				
3	1	1	2				
	78	117	195				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	76.40	114.60	0.16	0.16	0.00	0.00	
2	0.80	1.20	0.04	0.04	0.05	0.03	
3	0.80	1.20	0.04	0.04	0.05	0.03	
	78.00	117.00	0.24	0.24	0.10	0.07	
X^2 =	**0.17**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	76	75	0.01	1	115	114	0.01
2	1	3	1.28	2	1	3	1.30
3	1	1	0.00	3	1	1	0.00
n =	**78**	**79**	**1.29**	n =	**117**	**118**	**1.31**
f1 =	**0.98**			f1 =	**0.99**		
f3 =	**0.02**			f3 =	**0.01**		
	1	3					
cases	231	3	234				
controls	153	3	156				
	384	6	390	X^2 =	**0.25**	DF = 1	
				Fisher's exact (number of cells < 5)			
				p =	0.69		

MTHFR	controls (O)	cases (O)	sum				
1	64	94	158				
2	14	21	35				
3	2	1	3				
	80	116	196				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	64.49	93.51	0.24	0.24	0.00	0.00	
2	14.29	20.71	0.08	0.08	0.01	0.00	
3	1.22	1.78	0.60	0.60	0.49	0.34	
	80.00	116.00	0.92	0.92	0.50	0.35	
X^2 =	**0.85**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	64	63	0.02	1	94	94	0.00
2	14	16	0.24	2	21	21	0.00
3	2	1	1.00	3	1	1	0.02
n =	**80**	**80**	**1.26**	n =	**116**	**116**	**0.02**
f1 =	**0.89**			f1 =	**0.90**		
f3 =	**0.11**			f3 =	**0.10**		
	1	3					
cases	209	23	232				
controls	142	18	160				
	351	41	392	X^2 =	**0.18**	DF = 1	
				p =	0.83		

G-PROT	controls (O)	cases (O)	sum				
1	6	9	15				
2	32	47	79				
3	42	61	103				
	80	117	197				
	controls (E)	cases (E)	cont (O-E)^2	cas (O-E)^2	co (O-E)^2/E	ca (O-E)^2/E	
1	6.09	8.91	0.01	0.01	0.00	0.00	
2	32.08	46.92	0.01	0.01	0.00	0.00	
3	41.83	61.17	0.03	0.03	0.00	0.00	
	80.00	117.00	0.04	0.04	0.00	0.00	
X^2 =	**0.00**	DF=2					
Hardy-Weinberg Equilibrium?							
	controls				cases		
	observed	expected	X^2		observed	expected	X^2
1	6	6	0.00	1	9	9	0.00
2	32	32	0.00	2	47	47	0.00
3	42	42	0.00	3	61	61	0.00
n =	**80**	**80**	**0.00**	n =	**117**	**117**	**0.00**
f1=	**0.28**			f1 =	**0.28**		
f3=	**0.73**			f3 =	**0.72**		
	1	3					
cases	65	169	234				
controls	44	116	160				
	109	285	394	X^2 =	**0.00**	DF = 1	