Investigations of Renin-Angiotensin Aldosterone System (RAAS) genes in hypertrophy in Hypertrophic cardiomyopathy (HCM) founder families.

Ruben Earl Ashley Cloete

Thesis presented for approval for the Master's degree of Science in Biomedical Sciences at the Faculty of Health Sciences, at the University of Stellenbosch in 2008.

Promoter: Prof Valerie A. Corfield
Co-promoter: Prof Johanna C. Moolman-Smook
Co-promoter: Prof Lize van der Merwe

March 2008

i

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature…………………………………….. Date………………………………….

ABSTRACT

In hypertrophic cardiomyopathy (HCM), an autosomal dominant disorder, hypertrophy is variable within and between families carrying the same causal mutation, suggesting a role for modifier genes. Associations between left ventricular hypertrophy and left ventricular pressure overload suggested that sequence variants in genes involved in the Renin-Angiotensin Aldosterone System (RAAS) may act as hypertrophy modifiers in HCM, but some of these studies may have been confounded by, amongst other things, lack of adjustment for hypertrophy covariates.

To investigate this hypothesis, twenty one polymorphic loci spread across six genes (*ACE1*, *AGT*, *AGTR1*, *CYP11B2*, *CMA* and *ACE2*) of the RAAS were genotyped in 353 subjects from 22 South African HCM-families, in which founder mutations segregate. Genotypes were compared to 17 echocardiographically-derived hypertrophic indices of left ventricular wall thickness at 16 segments covering three longitudinal levels. Family-based association was performed by quantitative transmission disequilibrium testing (QTDT), and mixed effects models to analyse the X-linked gene *ACE2,* with concurrent adjustment for hypertrophy covariates (age, sex, systolic blood pressure (BP), diastolic BP, body surface area, heart rate and mutation status).

Strong evidence of linkage in the absence of association was detected between polymorphisms at *ACE1* and posterior and anterior wall thickness (PW and AW, respectively) at the papillary muscle level (*pap*) and apex level (*apx*). In single-locus analysis, statistically significant associations were generated between the *CYP11B2* rs3097 polymorphism and PW at the mitral valve level (*mit*) and both PW*pap* and inferior wall thickness (IW)*pap*. Statistically significant associations were generated at three *AGTR1* polymorphisms, namely, between rs2640539 and AW*mit*, rs 3772627 and anterior interventricular septum thickness at *pap* and rs5182 and both IW*pap* and AW*apx*. Furthermore, mixed effects model detected statistically significant association between the *ACE2* rs879922 polymorphism and both posterior interventricular septum thickness and lateral wall thickness at *mit* in females only.

These data indicate a role for RAAS gene variants, independent of hypertrophy covariates, in modifying the phenotypic expression of hypertrophy in HCM-affected individuals.

OPSOMMING

Hipertrofiese kardiomiopatie (HCM), 'n autosomale dominante afwyking, toon hoogs variërende hipertrofie binne en tussen families wat dieselfde siekte-veroorsakende mutasie het, hierdie dui op die moontlike betrokkenheid van geassosieerde modifiserende gene. Assosiasies tussen linker ventrikulêre hipertrofie en linker ventrikulêre druk-oorlading stel voor dat volgorde variasies in gene betrokke in die Renin-Angiotensin Aldosteroon Sisteem (RAAS) mag optree as hipertrofie modifiseerders in HCM. Sommige van hierdie soort studies is egter beperk omdat hulle nie gekompenseer het vir kovariante van hipertrofie nie.

Om hierdie hipotese te ondersoek, is die genotipe bepaal by een-en-twintig polimorfiese lokusse, verspreid regoor ses RAAS gene (*ACE1*, *AGT*, *AGTR1*, *CYP11B2*, *CMA* and *ACE2*), in 353 kandidate vanuit 22 Suid-Afrikaanse HCM-families in wie stigter mutasies segregeer. Genotipes was vergelyk met 17 eggokardiografies afgeleide hipertrofiese indekse van linker ventrikulêre wanddikte by 16 segmente wat oor drie longitudinale vlakke strek. Familie-gebaseerde assosiasies was bestudeer deur kwantitatiewe transmissie disequilibrium toetsing (QTDT) en gemengde effek modelle om die X-gekoppelde geen *ACE2* te analiseer, met gelyktydige kompensasie vir hipertrofie kovariate (ouderdom, geslag, sistoliese bloed druk (BP), diastoliese BP, liggaamsoppervlak area, hartritme en mutasie-status).

Sterk indikasies van koppeling in die afwesigheid van assosiasie is waargeneem tussen *ACE1* lokusse en posterior wanddikte (PW) asook anterior wanddikte (AW) by die papillêre spier vlak (*pap*) en die apeks vlak (*apx*). In enkel-lokus analises is statisties-betekenisvolle assosiasies gevind tussen die *CYP11B2* rs3097 polimorfisme en PW by die mitraalklep vlak (*mit*) en beide die PW*pap* en inferior wanddikte (IW)*pap*. Statisties-betekenisvolle assosiasies was verder gevind by drie *AGTR1* polimorfismes, naamlik, tussen rs2640539 polimorfisme en AW*mit*, rs3772627 en die anterior interventrikulêre septumdikte (aIVS) by die *pap* en rs5182 by beide die IW*pap* en AW*apx*. Gemengde-effek modelle het verder assosiasies aangetoon tussen die *ACE2* rs879922 polimorfisme en die posterior interventrikulêre septumdikte en die laterale wanddikte by die *mit,* slegs in vrouens.

Hierdie data dui op 'n kovariaat-onafhanklike rol vir RAAS genetiese variante in die modifisering van die fenotipiese uitdrukking van hipertrofie in HCM-geaffekteerde individue.

ACKNOWLEDGEMENTS

I would like to thank the following individuals for their tremendous contributions during the course of this study.

- To God, for giving me the strength and power to do this project with much enthusiasm and with great passion.

- To Prof Valerie Corfield, for encouraging and persuading me to undertake this project as well as her guidance and support in the final preparation of this thesis.

- To Prof Hanlie Moolman-Smook, for her patience, understanding and good nature during the course of this study. Her encouragement and scientific input as well as final oversee of this thesis.

- To Prof Lize van der Merwe, for doing most of the statistical analysis and her assistance to interpret the statistical results.

- To Prof Paul Brink, for his advice during preparation for oral presentations.

- To colleagues/friends in lab 4036 (the MAGIC lab) and offices in the department thank you for your kindness, generosity and humility.0

LIST OF ABBREVIATIONS

2D-echo	: 2D-echocardiography
2-DLVH	: two dimensional left ventricular hypertrophy score
3β-HSD	: 3β-hydroxysteroid dehydrogenase
AA	: amino-acid
ACE1	*:* angiotensin-1 converting enzyme
ACE2	: angiotensin converting enzyme 2
ACEI	: ACE inhibitor
ACTC	: cardiac actin
ADH	: aldosterone hormone
AGT	: angiotensinogen
AGTR1	: angiotensin 2 type-1 receptor
aIVS	: anterior interventricular septum
aIVSmit	: anterior interventricular septum thickness at level of mitral valve
aIVSpap	: anterior interventricular septum thickness at level of papillary muscles
AMP	: adenosine mono-phosphate
AMPK	: 5'-AMP-activated protein kinase
ANG (1-7)	: angiotensin fragments 1 to 7
ANGI	: angiotensin I
ANGII	: angiotensin II
APM	: affected pedigree member
ASREA	: allele specific restriction enzyme analysis
AV	: aortic valve
AW	: anterior wall
AWapx	: anterior wall thickness at level of apex
AWmit	: anterior wall thickness at level of mitral valve
AWpap	: anterior wall thickness at level of papillary muscles
BDKRB2	: bradykinin B2 receptor
BMI	: body mass index
BMR	: bone marrow
bp	: base pair

BP	: blood pressure
BRN	: brain
BSA	: body surface area
C	: cluster
cAMP	: cyclic adenosine mono-phosphate
CHD	: coronary heart disease
CMA	: cardiac chymase
CRP3	: muscle LIM protein
CSWT	: cardiac septal wall thickness
cTnT	: cardiac troponin T
CVD's	: cardiovascular diseases
CWT	: cumulative wall thickness
CWT-score	: cumulative wall thickness score
CYP11A	: cholesterol desmolase
CYP11B2	: aldosterone synthase
CYP21	: 21-hydroxylase
D	: deletion
D'	: Lewontins standardised disequilibrium coefficient
DBP	: diastolic blood pressure
DCC	: data coordination center
DCM	: dilated cardiomyopathy
ddNTPs	: dideoxy-nucleotide-triphosphates
DMSO	: dimethylsulfoxide
DNA	: deoxyribonucleic acid
dNTPs	: deoxy-nucleotide triphosphates
E	: environment
EDN1	: endothelin 1
EDTA	: ethylene-diamine-tetra-acetic acid
EHT	: essential hypertension
ESTs	: expressed-sequence tags
Ex	: exon

Exo1	:	exonuclease 1
F	:	frequency
FHCM	:	familial hypertrophic cardiomyopathy
Fig	:	figure
G	:	genetic
GC	:	guanine-cytosine
H	:	HapMap
HapMap	:	haplotype maps
HCM	:	hypertrophic cardiomyopathy
HDL	:	high density lipoprotein
Hg	:	mercury
HPLC	:	high pressure liquid chromatography
HR	:	heart rate
HRT	:	heart
HT	:	hypertension
HWE	:	Hardy-Weinberg equilibrium
I	:	insertion
IBD	:	identical-by-descent
IC	:	intron 2 conversion
ICM	:	ischaemic cardiomyopathy
IGF2	:	insulin-like growth factor 2
IL6	:	interleukin-6
IVS	:	interventricular septum thickness
IVSapx	:	interventricular septum thickness at level of apex
IW	:	inferior wall
IWmit	:	inferior wall thickness at level of mitral valve
IWpap	:	inferior wall thickness at level of papillary muscles
JG	:	juxtaglomerular
Kb	:	kilobases
KDN	:	kidney
LA	:	left atrium

LAMP2	: linked lysosome-associated membrane protein
LD	: linkage disequilibrium
LNG	: lung
LOD scores	: logarithm of the odds
LV	: left ventricle
LVH	: left ventricular hypertrophy
LVM	: left ventricle mass
LVMI	: left ventricle mass index
LVOT	: left ventricular outflow tract
LVPW	: left ventricular posterior wall
LVR	: liver
LVWT	: left ventricle wall thickness
LW	: lateral wall
LWapx	: lateral wall thickness at level of apex
LWmit	: lateral wall thickness at level of mitral valve
LWpap	: lateral wall thickness at level of papillary muscles
M	: molar
MCMC	: Markov Chain Monte-Carlo permutation tests
MI	: myocardial infarction
min	: minute
mIVS	: maximum interventricular septum thickness
mIVSmit	: maximum interventricular septum thickness at level of mitral valve
mIVSp	: maximum interventricular septum thickness at level of papillary muscles
ml	: milliliter
MLINK	: Microsoft Linkage format
mLVWT	: maximum left ventricle wall thickness
mLVWTapx	: maximum left ventricle wall thickness at level of apex
mLVWTmit	: maximum left ventricle wall thickness at level of mitral valve
mLVWTpap	: maximum left ventricle wall thickness at level of papillary muscles
MLYCD	: malonyl-CoA decarboxylase
mm	: millimetres

mM	: millimolar
MONICA	: Monitoring of trends and determinants in CVD in Augsburg
mPWT	: maximum posterior wall thickness
MR	: mineralocorticoid receptor
MRC	: Medical Research Council
MSL	: skeletal muscle
MV	: mitral valve
MWT	: maximum wall thickness
MYBPC3	: myosin binding protein C3 gene
MYH6	: α-myosin heavy chain 6
MYH7	: myosin heavy chain gene 7
MYL2	: myosin regulatory light chain 2
MYL3	: myosin essential light chain 3
N.D	: not determined
NAR	: Nucleic Acid Research
NCBI	: The National Centre for Biotechnology Information
NEB	: New England Biolabs
ng	: nanograms
NO	: nitric oxide
Nsyn	: non-synonomous
^{o}C	: degree celsius
PAGE	: polyacrylamide gel electrophoresis
PCR	: polymerase chain reaction
PEDSTATS	: pedigree statistics
PFKFB2	: 6-phosphofructo-2-kinase/fructose-2,6-biphosphate
PI3K	: phosphoinositide 3-kinase
pIVS	: posterior interventricular septum
pIVSmit	: posterior interventricular septum thickness at level of mitral valve
pIVSpap	: posterior interventricular septum at level of papillary muscles
PKD	: protein kinase D
PNC	: pancreas

PRA	: plasma renin activity
PRKAG2	: 5'-AMP-activated protein kinase subunit γ2
PST	: prostate
PubMed	: medical publication database
p-value	: probability value
PW	: posterior wall
PWapx	: posterior wall thickness at level of apex
PWmit	: posterior wall thickness at level of mitral valve
PWpap	: posterior wall thickness at level of papillary muscles
QTDT	: quantitative transmission disequilibrium test
QTL	: quantitative trait loci
RAAS	: renin-angiotensin aldosterone system
RNA	: ribonucleic acid
rpm	: revolutions per minute
Rs	: NCBI accession number for SNP
RSA	: Republic of South Africa
RVOT	: right ventricular outflow tract
SAP	: shrimp alkaline phosphatase
SB	: di-sodium tetraborate-decahydrate
SBP	: systolic blood pressure
SCD	: sudden cardiac death
SDS	: sodium dodecyl-sulphate
Sec	: seconds
SF-1	: steroidogenic transcription factor
SNPs	: single nucleotide polymorphisms
SPC	: spinal cord
SPL	: spleen
SWT	: septal wall thickness
Syn	: synonomous
TA	: annealing temperature
TATA box	: short modular sequence of DNA (T = A) pairs

TBE	: tris, boric acid and EDTA buffer
TD	: denaturing temperature
TDT	: transmission disequilibrium test
TE	: extension temperature
TG	: triglyceride
TGF-β	: transforming growth factor β
TMS	: thymus
TNF-α	: tumor necrosis factor α
TNNC1	: cardiac troponin C
TNNI3	: troponin I
TNNT2	: troponin T gene 2
TnT	: troponin T
TPM1	: α-tropomyosin
TTN	: titin
U	: unit
UCSC	: University of California Santa Cruz
UCT	: University of Cape Town
US	: University of Stellenbosch
USA	: United States of America
UTR	: untranslated region
UV	: ultra violet
V	: volts
WPW	: Wolf-Parkinson-White syndrome
WT	: wild type
Y	: years
αADD	: α-adducin
βMHC	: cardiac β-myosin heavy chain gene
μg	: microgram
μl	: microliter

Amino Acid Abbreviations

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Cys	C	Cysteine
Gln	Q	Glutamine
His	H	Histidine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Nucleotide Abbreviations

A	: adenine
C	: cytosine
G	: guanine
T	: tyrosine

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

CHAPTER 1

1. Introduction:

1.1. Left ventricular hypertrophy (LVH)

Left ventricular hypertrophy (LVH) is a condition recognised echocardiographically by an increase in left ventricle mass, marked thickening of the interventricular septum (IVS) and the posterior wall of the left ventricle (LV) (figure 1.1) (Czubryt and Olson, 2004). Heart muscle overgrowth is considered to be the major predictor of morbidity and mortality, secondary to age (Levy et al., 1990). Levy et al., (1988) and Koren et al., (1991) implicated LVH as being a precursor of morbidity and mortality, and noted its frequent occurrence in older people and individuals with hypertension, obesity, myocardial infarction (MI) and valve diseases. Furthermore, it has been shown that LVH acts independently of other cardiovascular risk factors such as smoking and hypercholesterolaemia (Koren et al., 1991; Ghali et al., 1992).

Left ventricular hypertrophy is associated with many common complex and some rarer inherited disease states. Previous tenets suggested that LVH is a result of left ventricular pressure overload (Grossman et al., 1975; Ganau et al., 1992). However, it has been observed that the degree of LVH and blood pressure (BP) do not show a positive correlation (Grossman et al., 1975; Drayer et al., 1983). In addition, it has been demonstrated that some individuals with LVH have normal BP, suggesting that factors other than haemodynamic overload may play a role in hypertrophy development (Levy et al., 1988). Additionally, not all hypertrophy is harmful and two forms have been recognised, pathological hypertrophy and physiological hypertrophy, such as occurs in "athlete's heart" in response to exercise, and these conditions have been shown to differ from one another (Granger et al., 1985).
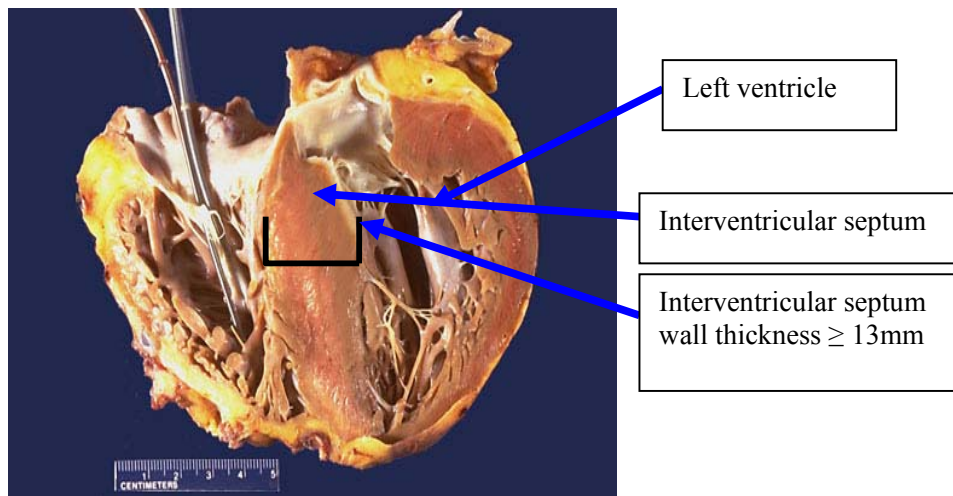
Figure 1.1: Cross section through a hypertrophied heart showing the relationship between interventricular septum thickness and left ventricle size. Reproduced from http://www-medlib.med.utah.edu/WebPath/CVHTML/CV169.html

1.2 Hypertrophic cardiomyopathy (HCM)

One of the inherited heart diseases in which LVH occurs, is hypertrophic cardiomyopathy (HCM), an inherited heart muscle disease; in turn, HCM serves as a model to elucidate the molecular mechanisms involved in hypertrophy development within susceptible individuals (Moolman et al., 1997). At the molecular level, HCM is considered to be a disease of the sarcomere (Moolman-Smook et al., 2003). The hypertrophy which is the hallmark of HCM mostly affects the IVS and the LV, although right ventricular hypertrophy also occurs (Wigle et al., 2001). At the clinical level, the disease is characterised by arrhythmias, impaired exercise tolerance and sudden cardiac death (SCD) in young individuals under the age of 35 years old (Denfield and Garson, 1990).

The pathological hypertrophy observed in HCM is associated with decreased muscle compliance due to defective sarcomere functioning and/or structure (Wigle et al., 1995). Additionally, the tissue pathology of hypertrophied hearts differs between HCM and exercise-induced hearts (Gregory et al., 1983; Brink et al., 1996). HCM is classified at histological level by myofibrillar disarray and interstitial fibrosis (Davies et al., 1984; Tanaka et al., 1986).

The patterns of LVH found in HCM patients are either concentric or asymmetric and vary in extent from apical to basal regions of the LV (Wigle et al., 1985). Typical (classical) HCM, characterised by asymmetrical hypertrophy, occurs more frequently in HCM patients than apical hypertrophy and most often affects the IVS and, to some extent, the posterior walls of the LV, resulting in a narrow outflow tract (Wigle et al., 1995). Apical HCM is characterised by a spade-shaped ventricular cavity and is said to be a genetically and clinically different form of HCM because familial occurrence of apical HCM is rare and it is associated with a good clinical prognosis (Wigle et al., 1995). Hypertrophy in HCM in general varies in a range from none or minimal (less than 11mm in adults being termed normal), to massive hypertrophy (the latter defined as maximum ventricular wall thickness of 35mm or more in adults), which is adjusted for age in children (Spirito et al., 1997).

The physical examination for HCM may or may not reveal physical signs of cardiac hypertrophy (e.g., pressure loaded apex beat, fourth heart sound) nor signs of obstruction (e.g., jerky pulse, ejection systolic murmur which varies on squatting or Valsalva manoeuvre), if hypertrophic obstructive cardiomyopathy is present (in ~ 20% of cases). The electrocardiographic and echocardiographic abnormalities are found on a special examination for electrical changes in the heart rhythm and measurement of the extent and localisation of hypertrophy by means of a two-dimensional echocardiography (2D-echo). Both techniques are considered compulsory for effective diagnosis, with each procedure having its own advantages and disadvantages. For diagnosis of HCM in adults, an 2D-echo measurement of the left ventricular wall thickness (LVWT) having a value of equal or greater than 13mm is considered clinically affected, while values of LVWT between 11mm and 13mm are considered clinically equivocal (Maron et al., 1981). According to Spirito et al., (1994) and Maron et al., (1995), a value of 13mm is a good indicator of HCM, considering endurance athletes rarely exceed this level. However, 2D-echo is unable to detect HCM in pre-pubescent children and at all ages misdiagnosis is a possibility because hypertrophy could be acquired due to other causes (Bachinski and Roberts, 1996).

To accurately capture the extent and localisation of hypertrophy within the myocardium, three composite hypertrophy-score measures have been advocated. The first scoring system used for quantitative evaluation of the extent of LVH is the semi-quantitative point score method (ranges 0 to 10) developed by Wigle et al., (1985), because maximum left ventricular wall thickness measured by 2D-echo may not truly reflect the extent of hypertrophy, or involvement of the distal (apical) half of the septum or lateral walls. A maximum of 10 points is given; 1 to 4 points for septal hypertrophy based on magnitude of thickness, 2 points for extension of hypertrophy beyond the level of the papillary muscles (basal two thirds of the septum), 2 points for extension of hypertrophy to the apex (total septal involvement), and 2 points for extension of hypertrophy into the lateral wall.

The extent of hypertrophy is quantified by a second score method the Spirito-Maron score (Spirito and Maron et al., 1990). This is calculated by dividing the LV into four regions, the anterior and posterior ventricular septum with the anterolateral and posterior left ventricular walls. The Spirito-Maron score is calculated by adding the measurements of maximum wall thickness (MWT) obtained in each of the four left ventricular regions. Maximal wall thickness is defined as the sum of the greatest wall thickness observed in the mitral valve level or papillary level of the short-axis planes in any of the four segments.

The third quantitative hypertrophy score more recently proposed by Forissier et al., (2005) is the two-dimensional left ventricular hypertrophy score (2-DLVH), a determination of the sum of the MWT obtained in the four regions of the LV. The segments correspond to the anterior, posterior ventricular septum and the anterolateral and posterior left ventricular free walls. Additionally, Forissier et al., (2005) validated the 2-DLVH score as having a higher diagnostic value for familial HCM screening than the conventional criteria of MWT suggested by Charron et al., (1997), particularly in young adolescents. Even though, the 2-DLVH score was measured, it was not considered a parameter in the present study, because it has not been verified in hypertrophy score-derived studies.

1.2.1. Molecular genetics of HCM

The prevalence of HCM within the population of the United States of America (USA) is estimated to be 1 in 500 individuals (Maron et al., 1984) but its prevalence has not been reported in the South African population. Numerous investigations have indicated that HCM may be either sporadic or inherited and it appears that each form represents approximately 50% of the cases (Maron et al., 1984). Familial hypertrophic cardiomyopathy (FHCM) is a genetically heterogeneous disease, inherited in an autosomal dominant pattern. However, a proportion of the HCM cases are probably caused by *de novo* germline mutations and thus affected individuals could transmit the disease to their offspring (i.e. FHCM) (reviewed by Maron et al., 2002). Presently, more than 400 different HCM-causative mutations have been identified in 14 different genes, 11 of the genes encoding sarcomeric proteins (figure 1.2) (Ho and Seidman et al, 2006).

A hallmark of HCM is clinical heterogeneity, a phenomenon manifesting amongst individuals of both the same and/or different families (intrafamilial and interfamilial variability), respectively (Epstein et al., 1992a; Epstein et al., 1992b; Fananapazir and Epstein, 1994; Posen et al., 1995; Moolman et al., 1997). This clinical variability ranges from asymptomatic to severe forms of hypertrophy with a high risk of SCD, and is associated with different symptoms such as shortness of breath, angina, presyncope, syncope, mitral valve regurgitation and arrhythmias (Brachfeld and Gorlin et al., 1959; Brent et al., 1960).

Molecular genetic studies have facilitated the identification of four prevalent FHC-causative genes, which code for β-myosin heavy chain (*MYH7*) on chromosome 14q12, cardiac troponin T (*TNNT2*) on chromosome 1q32, α-tropomyosin (*TPM1*) on chromosome 15q2 and cardiac myosin binding protein C (cardiac *MYBPC3*) on chromosome 11q11 (Geisterfer-Lowrance et al., 1990; Thierfelder et al., 1994; Bonne et al., 1995; Watkins et al., 1995).
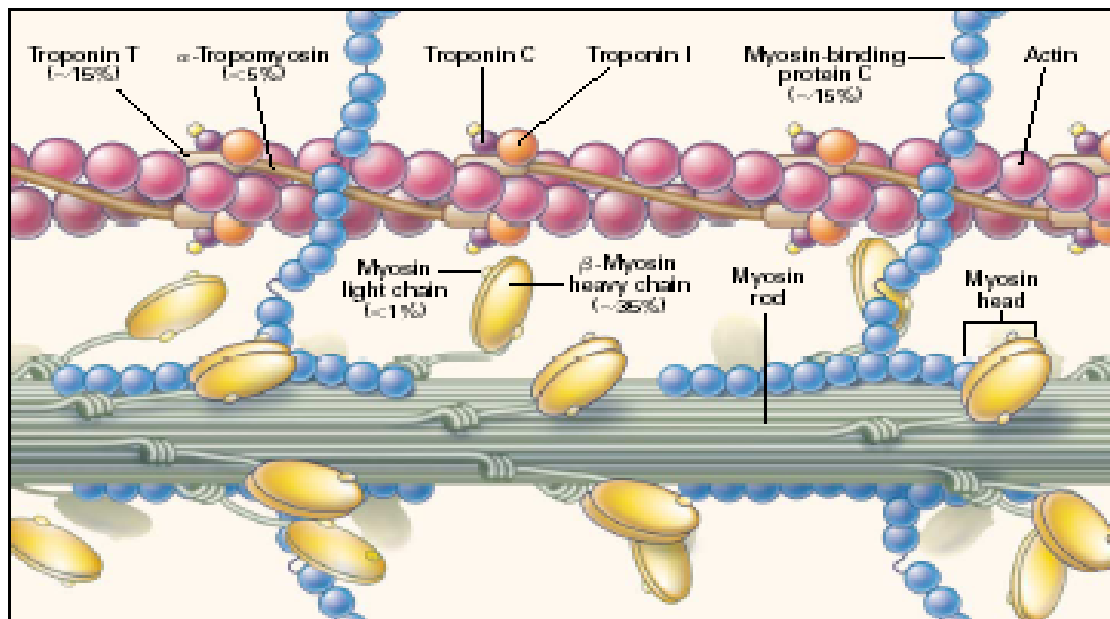
Figure 1.2: Diagrammatic representation of the sarcomere showing sarcomeric proteins in which the majority of HCM-causing mutations have been described (the myosin light chain is present but not indicated is its two forms, namely, myosin essential light chain and myosin regulatory light chain and not indicated is α-myosin heavy chain and titin). Taken from Spirito et al., 1997.

In 1995, Marian and Roberts suggested that a small proportion of unknown genes, in which mutations could cause HCM, excluding the known genes with mutations that account for 50-70% of all FHCM related cases, may still exist. Other less prevalent HCM-causative genes identified to date are *ACTC, TNNI3, MYL3, MYL2, MYH6, TNNC1* and *TTN*. These genes encode the sarcomeric proteins cardiac actin, troponin I, myosin essential light chain (MELC), myosin regulatory light chain (MRLC), α-myosin heavy chain, cardiac troponin C and titin, respectively (Poetter et al., 1996; Kimura et al., 1997; Mogensen et al., 1999; Satoh et al., 1999). Additionally, the *PRKAG2* that encodes the γ2–subunit of a non-sarcomeric protein, 5'-AMP-activated protein kinase (AMPK), was found to cause HCM associated with Wolf-Parkinson-White syndrome (WPW) (Gollob et al., 2001; Arad et al., 2002). Subsequently, two additional non-sarcomeric proteins, namely, muscle LIM protein and X-linked lysosome-associated membrane protein, encoded by *CRP3* and *LAMP2*, respectively, were identified as containing HCM-causative mutations (Schmitt et al., 2003b). Listed in table 1.1 are the known sarcomeric and non-sarcomeric genes in which HCM-causative mutations have been described.

Table 1.1: HCM-causative genes, chromosomal loci and their sub-cellular localisation.

Causative gene	Locus	Sub-cellular localisation	Reference
MYH7	14q12	sarcomere	1
MYH6	14q13	sarcomere	2
MYBPC3	11p11	sarcomere	3
TNNT2	1q32	sarcomere	4
TNNI3	19q13	sarcomere	5
TNNC1	3p21	sarcomere	6
TPM1	15q22	sarcomere	4
MYL2	12q23-q24	sarcomere	7
MYL3	3p21	sarcomere	7
ACTC	15q14	sarcomere	8
TTN	2q31	sarcomere	9
CRP3	11p15	non-sarcomere	10
PRKAG2	7q36	non-sarcomere	11
LAMP2	X	non-sarcomere	12

Abbreviations: MYH7- cardiac β-myosin heavy chain gene; MYH6 - cardiac α-myosin heavy chain gene; MYBPC3 - cardiac myosin binding protein C gene; TNNT2 - cardiac troponin T gene; TNNI3 - cardiac troponin I; TNNC1 - cardiac troponin C; TPM1 - cardiac tropomyosin gene; MYL2 - ventricular myosin regulatory light chain; MYL3 – myosin essential light chain; ACTC - cardiac actin; TTN - titin; CRP3 - cardiac muscle lim protein gene; PRKAG2 - 5'-AMP-activated protein kinase, gamma-2 subunit gene; LAMP2 - linked lysosome-associated membrane protein. References: 1) Geisterfer-Lowrance et al., 1990, Vikstrom and Leinwand, 1996; 2) Niimura et al., 2002; 3) Bonne et al., 1995, Watkins et al., 1995; 4) Thierfelder et al., 1994; 5) Kimura et al., 1997; 6) Hoffmann et al., 2001; 7) Poetter et al., 1996; 8) Olson et al., 2000; 9) Satoh et al., 1999; 10) Geier et al., 2003; 11) Blair et al., 2001, Gollob et al., 2001 and Arad et al., 2002; 12) Arad et al., 2005.

Worldwide, it has been calculated that three predominant FHC-causing genes, *MYH7*, *MYBPC3* and *TNNT2*, account for ~70 % of FHC cases, subdivided into ~35% caused by mutations in *MYH7*, ~20% caused by mutations in *MYBPC3*, ~15% caused by mutations in *TNNT2*, an additional ~3% is due to mutations in *TPM1* (Watkins et al., 1995). In South Africa (SA), unlike the rest of the world, where unique private mutations, each having an independent origin, are the "rule", molecular genetic studies identified unique founder mutations, two in *MYH7* (Ala797Thr and Arg403Trp) and one in *TNNT2* (Arg92Trp), each accounting for 25%, 5% and 11% of HCM cases, respectively (Moolman-Smook et al., 2000). The identical-by-descent (IBD) origin of these HCM

mutations, indicating that individuals harbour the same disease-causing mutation inherited from a common ancestor, was confirmed by haplotype analysis (Moolman-Smook et al., 1999).

1.2.2. Clinical variability in HCM

Initially, Watkins et al., (1992) and Fananapazir et al., (1994) reported a correlation between phenotypic expression of HCM and specific mutations in *MYH7*. It has also been demonstrated that mutations in *MYH7*, *TNNT2* and *MYBPC3* are associated with varying degrees of penetrance, in terms of the extent and distribution of hypertrophy and the occurrence of SCD, which is variable amongst HCM-mutation carriers (Posen et al., 1995; Watkins et al., 1995; Moolman et al., 1997). Furthermore, Watkins et al., (1995) and Moolman et al., (1997) suggested that the position of the mutation within a gene affects the phenotype, as discussed in the following paragraphs.

Missense mutations in *MYH7* such as Arg403Gln, Arg453Cys and Arg719Trp are associated with variable risk of SCD and overt hypertrophy, while a missense mutation in *TNNT2* (Arg92Trp) is associated with a high frequency of SCD with subtle to undetectable hypertrophy (see figures 1.3 and 1.4). In contrast, several protein truncation mutations in *MYBPC3* (intervening sequence intron (IVS7) +1G>A, IVS20 -2A>G and IVS27 +1G>A) is associated with a broad spectrum of HCM phenotypes, including life-threatening arrhythmias present in affected individuals (Erdmann et al., 2001). Missense mutations, insertion/deletion and splice junction mutations in *MYBPC3* account for approximately 20% to 25% of all HCM cases (Erdmann et al., 2001). For individuals with *MYBPC3* missense mutations, the prognosis is favourable with a late onset of disease (Charron et al., 1998; Niimura et al., 1998), while individuals with frameshifts and deletion mutations present a more severe phenotype with a high risk of SCD (Erdmann et al., 2001).
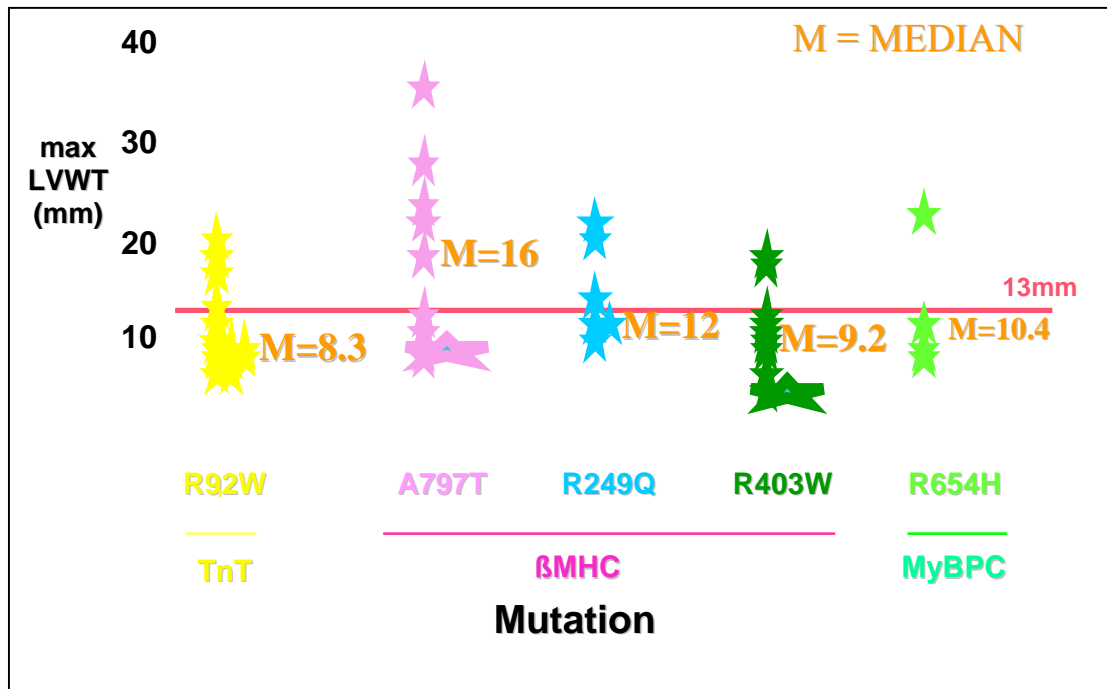
Figure 1.3: Comparison of the degree of hypertrophy (left ventricular wall thickness) in family members with single distinct HCM-causing mutations in different sarcomeric protein coding genes (compiled from the data of Watkins et al., 1995 and Moolman et al., 1997). Abbreviations used: LVWT - left ventricular wall thickness, mm - millimetres, TnT - troponin T, βMHC - β-myosin heavy chain and MYBPC - myosin binding protein C. The clinical diagnostic criteria for HCM mutation carriers was measured for a maximum left ventricular wall thickness (MLVWT) ≥ 13mm [indicated by bold red line]. Above and on the line were considered as clinically affected and below unaffected.

Previous views recognised *TNNT2* mutations as being associated with relatively mild hypertrophy in diseased individuals with high SCD (Watkins et al., 1995; Moolman et al., 1997). However, the *TNNT2* missense mutation Phe110Glu segregating in Japanese families is associated with a benign form of hypertrophy with no incidence of SCD (Watkins et al., 1995; Anan et al., 1998). Furthermore, the supporting evidence of molecular genetic studies revealed that, in some cases, a quarter of genetically-positive individuals showed no clinical symptoms of disease (Dausse et al., 1993; Carrier et al., 1997). These findings suggest that the variability of phenotype within and amongst HCM families harbouring the same mutation is indicative of possible multifactorial contributors to disease presentation and that the inheritance pattern in HCM is not purely a monogenic disease.
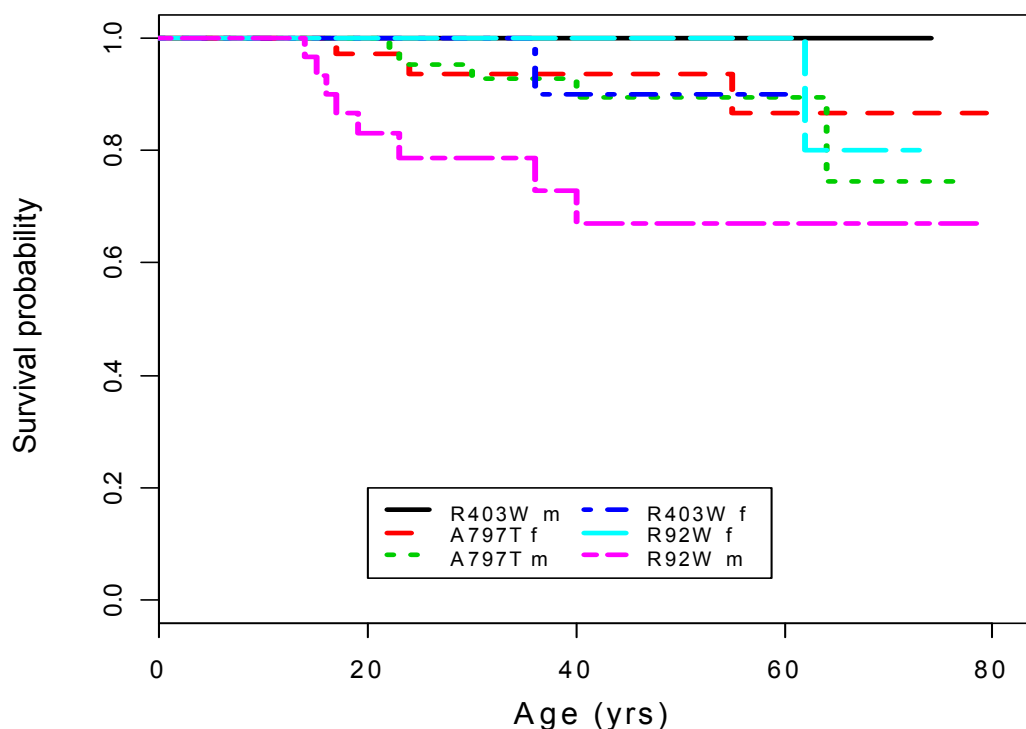
Figure 1.4: Kaplan-Meier survival curves
These curves demonstrate the probability of a male or female person, belonging to a family in which a distinct HCM-causing founder mutation is segregating, being alive at a given age. Abbreviations used: m - male, f - female, R403W - Arg403Trp in *MYH7*, R92W - Arg92Trp in *TNNT2*, A797T - Ala797Thr in *MYH7*.

1.3. Candidate gene modifiers

1.3.1. Establishing the role of genetic modifiers of clinical phenotypes

As discussed in the preceding sections, phenotypic variability is a prominent feature of HCM, even among individuals of the same family harboring the same disease-causative mutation (Watkins et al., 1995; Moolman et al., 1997). It has been suggested that phenotypic variability is caused by genetic background and/or environmental factors, which together, or separately, may influence disease expression (Patel et al., 2000). For example, Fananapazir and Epstein et al., (1994) demonstrated that the *MYH7* Arg403Gln mutation, that is generally associated with malignant HCM, exhibited a benign outcome in one Korean family. Similarly, two related individuals harboring the same *TNNI3* Lys183 deletion mutation exhibited two distinct forms of HCM, namely, apical HCM and typical (classical) HCM (Kimura et al., 1997). Investigations of the heritability of cardiac size in monozygotic and dizygotic twin studies indicated that genetic background

contributes to the variability in cardiac size irrespective of other influences (Adams et al., 1985). Subsequently, Schunkert et al., (1999a) used echocardiographic measurements to demonstrate that siblings of LVH subjects had a higher risk for LVH development than subjects who did not have a LVH-affected sibling.

Genetic background has been shown to be a contributor to HCM phenotype demonstrated in transgenic animal models (Sebkhi et al., 1999; Semsarian et al., 2001). Moreover, the same group of Sebkhi and colleagues (1999) performed a whole genome linkage scan and identified regions on chromosome 3 in rats that independently co-segregate with LV weight (termed quantitative trait loci (QTL)) and which affect differences in LV mass (LVM) in two inbred normotensive rat strains. Additionally, Semsarian et al., (2001) illustrated that the variability in cardiac hypertrophy can be influenced by genetic background in α-MHC$^{403/+}$ knockout inbred and outbred mouse models. The α-MHC$^{403/+}$ missense mutation in mice is equivalent to the human *MYH7* Arg403Trp mutation. They observed strain dependent differences in the degree of variability in cardiac hypertrophy and SCD irrespective of body weight and exercise (Semsarian et al., 2001). On the basis of these findings it can therefore be suggested that similar, unknown, QTLs could play a role in hypertrophy development in humans, either independently, or by influencing the phenotypic response to mutationally altered sarcomere proteins.

1.3.2. Candidate gene modifiers of HCM phenotype

Numerous studies performed in Caucasian individuals with HCM have assessed the role of several candidate genes as hypertrophy modifiers of the condition HCM (Table 1.2) (Ishanov et al., 1997). Many of these genes encode proteins that are components of the renin-angiotensin aldosterone system (RAAS), including angiotensin-1 converting enzyme encoded by *ACE1*, angiotensinogen encoded by *AGT*, angiotensin II type I receptor encoded by *AGTR1*, cardiac chymase encoded by *CMA*, bradykinin B2 receptor encoded by *BDKRB2* and aldosterone synthase encoded by *CYP11B2* (Marian et al., 1993; Pfeufer et al., 1996; Brugada et al., 1997; Yamada et al., 1997; Erdmann et al., 1998; Patel et al., 2000). Other implicated genes include those that encode trophic factors,

namely, endothelin 1 *(EDN1)*, tumor necrosis factor α *(TNF-α)* and insulin-like growth factor 2 (*IGF2*) (Brugada et al., 1997; Patel et al., 2000).

Although some of the genes (*ACE1*, *TNF-α*, *BDKRB2* and *AGTR1*) reported to be associated with hypertrophy maintained significant association in replication studies, only a minority account for the variability in the HCM phenotype (Marian et al., 2002). For example, in one study only the uncommon *A*-allele of the functional promoter region *TNF*-α-308 *G/A* polymorphism was found to be associated with increased LVH in HCM patients when compared to other functional variants of the candidate modifier genes transforming growth factor-β1 (*TGFβ1*), *CYP11B2*, interleukin-6 (*IL6*) and *IGF-2* (Patel et al., 2000). These results suggests a role for *TNF-α* as a potential modifier gene for HCM (Patel et al., 2000).

Previously, many of the candidate genes were considered as hypertrophy modifiers because of their regulatory role in control of BP and involvement in cell growth (Brugada et al., 1997; Patel et al., 2000). Additionally, *ACE1* has been implicated as a potential modifier of HCM. The insertion/deletion (I/D) polymorphism has also been shown to be associated with various other cardiovascular diseases (CVD) (Perkins et al., 2005), and higher plasma ACE1 levels have been observed in HCM or hypertensive patients (Rigat et al., 1990; Lechin et al., 1995). The first observation was made by Marian et al., (1993), when they illustrated an association between the I/D polymorphism of *ACE1* and the risk of SCD in HCM patients. Moreover, they observed that the *DD* genotype was more common in HCM families with a high incidence of SCD compared to other families with the *II* genotype (Marian et al., 1993). Subsequently, (Tesson et al., 1997) reported an association found between the *DD* genotype and cardiac hypertrophy expression, specifically in French and South African HCM individuals harbouring the *MYH7* Arg403Gln gene mutation.

In summary, these data presented above only implicate *ACE1* and *TNF-α* as genetic modifiers in replication studies in the development of inherited HCM. In the next section the RAAS will be discussed in more detail.

Table 1.2: Candidate modifier genes for hypertrophic cardiomyopathy (HCM).

Gene	Variants	Study population	Type of study	Covariates	Results	References
Tumour Necrosis Factor α	-308 G/A	HCM-affectees	Case-only	None	-308A associated with LVH	1
Insulin-like Growth Factor 2	820G/A	HCM-affectees	Case-only	None	No association found	1
Transforming Growth Factor β	-509C/T	HCM-affectees	Case-only	None	No association found	1
		HCM-affectees	Case-only	None		
Interleukin 6	-174G/C	HCM-affectees	Case-only	None	No association found	1
<u>Angiotensinogen</u>	M235T, T174M and -6G/A	HCM-affectees	Case-only	None	-6 G/A no association	2
<u>Angiotensinogen</u>	M235T and T174M	HCM-affectees	Case-control	None	No association found	3
Endothelin 1	G8002A	HCM-affectees	Case-only	None	8002A associated with LVH	2
<u>Aldosterone synthase</u>	-344C/T	HCM-affectees	Case-only	None	No association found	1
<u>Angiotensin II receptor 1</u>	1166A/C	HCM-affectees	Case-only	age, gender, two polymorphisms, peak LV outflow tract gradient and plasma renin concentration	1166C associated with LVH	4
<u>Chymase A</u>	1625G/A and -1903G/A	HCM-affectees	Case-control	age and gender	No association found	5
<u>Angiotensin-1 Converting Enzyme</u>	I/D	HCM-affectees	Case-only	age, sex, weight, height, BSA, BMI and *ACE1* genotypes	DD associated with LVH	6
<u>Angiotensin-1 Converting Enzyme</u>	I/D	HCM-affectees	family-based study	None	DD associated with LVH	7
Bradykinin B2 receptor	T21M and -412C/G	CVD	Case-control	None	21M associated with HCM	8

The genes underlined are involved in hypertension and hypertrophy. Reproduced and adapted from Marian et al., 2002. Abbreviation used: CVD – cardiovascular disease, I/D – insertion/deletion. References: 1) Patel et al., 2000; 2) Brugada et al., 1997; 3) Yamada et al., 1997; 4) Osterop et al., 1998; 5) Pfeufer et al., 1996; 6) Lechin et al., 1995; 7) Tesson et al., 1997; 8) Erdmann et al., 1998.

1.4. Renin-Angiotensin Aldosterone System (RAAS)

1.4.1. What is RAAS?

The renin-angiotensin aldosterone system (RAAS) was previously considered to be an endocrine system that played an important role in regulation of BP, fluid balance and electrolyte homeostasis within the vasculature (Catt et al., 1970; Vane et al., 1974). However, subsequent evidence demonstrated that the RAAS is present and functional in a vast number of tissues such as the brain (Ganten et al., 1984), kidney (Deschepper et al., 1986) and heart (Schelling et al., 1991). Thus, the concept of tissue-based RAAS emerged (Dzau et al., 1993). Two forms of the RAAS occur in the human body, namely, plasma and tissue, with both involved at multiple levels of synthesis of angiotensin II (ANGII) (endocrine, autocrine and paracrine), which come into play during increased BP levels. One of the most important actions of the RAAS is its role in adaptive processes related to cardiac hypertrophy and angiogenesis (Dzau et al., 1993). The main effector peptide of this system is ANGII, which functions both as an autocrine and paracrine substance having vast effects on various glands and tissues. The two-enzyme cascade system regulated by angiotensin-converting enzyme (ACE1) and renin has been shown to play a substantial role in the pathogenesis of CVD (Campbell et al., 1987; Dzau et al., 1993).

In the first step of the system, angiotensin I (ANGI), an inactive decapeptide, is cleaved from the precursor substrate angiotensinogen (AGT) by renin. The second step involves the cleavage of ANGI by both ACE1 and cardiac chymase (CMA) to generate ANGII, an active vasoconstrictor peptide, and aldosterone-stimulating peptide via its binding to angiotensin II type 1 receptor (AGTRI); these interactions mediate downstream effects of ANGII (figure 1.5) (Urata et al., 1996).

In the following paragraphs the key components involved in the RAAS cascade, namely, renin, AGT, ACE1, CMA, angiotensin converting enzyme 2 (ACE2) and aldosterone synthase (CYP11B2) will be discussed.

A critical component of the RAAS is AGT, an α-2 globulin produced in the liver that is the substrate involved in first step of the RAAS cascade (figure 1.5). AGT is also expressed in the brain, kidney, heart, vascular wall and adipose tissue (Campbell et al., 1986 and 1987; Dzau et al., 1987 and 1989; de Mello and Danser et al., 2000). This substrate ultimately determines the amount of ANGII produced, irrespective of both renin concentration and/or ACE1 activity (Reid et al., 1978).

In the second step of the cascade, renin, an aspartyl protease localised within the smooth muscle cell layer (in the vasculature) is biosynthesised in, and released from, the juxtaglomerular (JG) cells of the renal afferent arterioles (figure 1.5) (Higashimori et al., 1991; Carey and Siragy, 2003). Renin is encoded by a single gene that translates renin mRNA into preprorenin that is subsequently cleaved and processed in the Golgi-apparatus to active renin and released from JG cells. At the same time prorenin is released from the cell membrane and converted to active renin by a trypsin-like activating enzyme (Hsueh et al., 1991). Renin, the initiating enzyme of the tightly regulated RAAS cascade is involved in a rate-limiting step in the production of ANGI from AGT.

A cascade enzyme, the dipeptidyl-carboxypeptidaseI/kinase II ecto-enzyme ACE1, has been found to be synthesised by adipose, cardiac and vascular tissue (figure 1.5) (Cooper et al., 1997; Baker et al., 1992). In humans, two forms of ACE1 are expressed, a somatic and germinal form. The somatic form is synthesised by adipose, cardiac and vascular tissue expressed on the surface of endothelial cells of lung vessels and various other cell types (monocytes, T lymphocytes and adipocytes); the germinal form is found exclusively in the testes (Fleming et al., 2006). ACE1, similar to renin, is a rate-limiting enzyme in the RAAS pathway, and hydrolyses ANGI to generate the effector peptide ANGII (figure 1.5) (Danser et al., 1992; Muller et al., 1998).
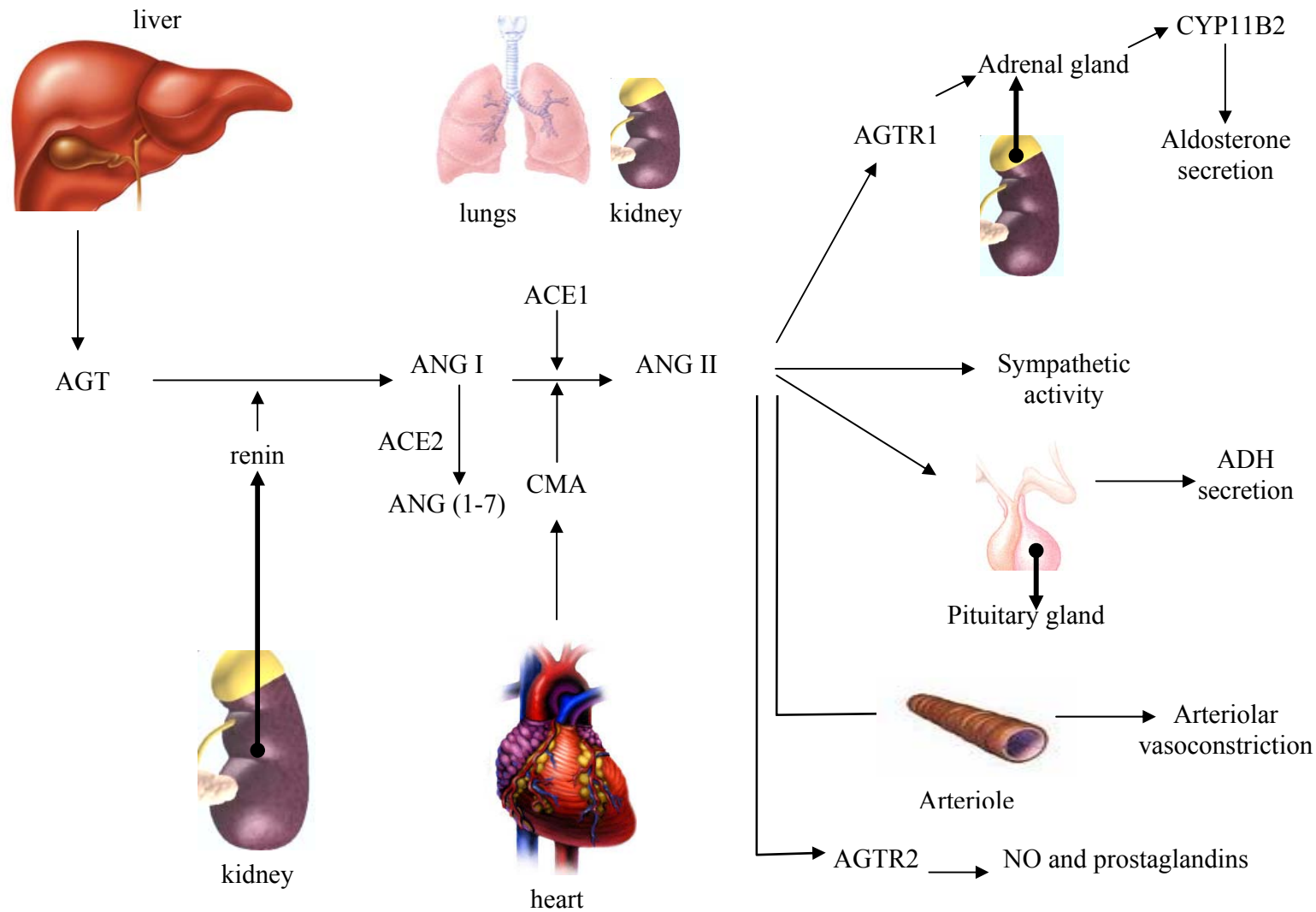
Figure 1.5: Schematic illustration of circulating and tissue-based RAAS and its effects on various organs. RAAS is initiated by a low perfusion pressure in the juxtaglomerular apparatus. Abbreviations used: AGT - angiotensinogen; ANGI - angiotensin I; ANGII - angiotensin II; ACE1 - angiotensin-converting enzyme; ACE2 - angiotensin-converting enzyme 2; CMA - cardiac chymase; AGTR1 receptor - angiotensin II receptor type I; AGTR2 receptor - angiotensin II receptor type II; CYP11B2 - aldosterone synthase; ADH - aldosterone hormone; NO - nitric oxide and ANG (1-7) - angiotensin fragments 1 to 7.

Another enzyme involved in the generation of ANGII, is CMA, a serine protease (figure 1.5). CMA belongs to the chymotrypsin family of enzymes that are located within mast cells of various tissues of both human and animal species (Fleming et al., 2006). It is suggested that CMA is responsible for >80% of human heart and >60% of human artery ANGII formation (Petrie et al., 2001; Borland et al., 2005). ANGII has been implicated in the development of CVDs, particularly in cardiac hypertrophy and heart failure (Takai et al., 2004). Interestingly, ANG1-7, a heptapeptide fragment of ANGI generated by ACE2, a monocarboxypeptidase, antagonises the actions of ANGII (Schiavone et al., 1988; Tipnis et al., 2000). Moreover, ANGII is able to increase BP, while ANG1-7 decreases BP in hypertensive animals and reduces vascular cell wall growth (Benter et al., 1995; Freeman et al., 1996).

Aldosterone, a mineralocorticoid, is synthesised in the adrenal glomerulosa cells from cholesterol and occurs via the actions of four cytochrome P450 enzymes; cholesterol desmolase (CYP11A), 21-hydroxylase (CYP21), CYP11B2 (18-oxidase) and 3β-hydroxysteroid dehydrogenase (3β-HSD) (figure 1.5) (Silvestre et al., 1998). Even though it is known that most of the circulating aldosterone is manufactured from cholesterol in the adrenal gland, the cardiac tissue in humans and rats has been shown to contain the molecular machinery for aldosterone synthesis and response, producing both the co-expressed receptors and 11-β-hydroxysteriod dehydrogenase enzyme (Lombes et al., 1995, Silvestre et al., 1998). Further evidence exists for aldosterone synthesis and release from cardiac tissue from the infarcted hearts of rats (Silvestre et al., 1999). Additionally, an experiment in cultured rat aortic smooth muscle cells demonstrated inhibition of vascular smooth muscle cell proliferation when aldosterone receptor antagonists were present, thus providing evidence for the role of aldosterone in controlling vasculature structure and function (Xiao et al., 2000).

In summary, the RAAS which produces ANGII has been identified in cardiomyocytes, endothelial cells, as well as vascular smooth muscle cells (Fleming et al., 2006). Koch-Weser et al., (1964) illustrated that both circulating and locally generated ANGII are able to induce vasoconstriction and chronotropic actions on the heart. Local RAAS, or tissue-

based RAAS, has been found to be involved in both the maintenance of cardiovascular structure and repair. Evidence for this comes from experiments of *in vivo* gene transfer of ACE1 into uninjured rat carotid arteries (Morishita et al., 1994). The investigators observed vascular remodeling resulting in vascular hypertrophy independent of systematic and haemodynamic effects (Morishita et al., 1994). In addition, other reports demonstrated that overexpression of ACE1 results in morphological changes associated with atrial arrhythmias and sudden death (Xiao et al., 2004).

1.4.2. RAAS and the cardiovascular system

The role of RAAS components in the pathogenesis of CVD will be discussed in this section.

ANGII

The RAAS has become the focus of many cardiovascular studies, largely due to the trophic factor ANGII's involvement in CVD. Schelling and colleagues (1991) observed that this effector peptide of the RAAS may be a potential growth factor triggering the chronic cardiovascular hypertrophy process. The effects exerted by ANGII are enhanced by the facilitation of noradrenaline release from the sympathetic nerve endings. ANGII induces various pathologies such as cardiac hypertrophy, inflammation and fibrosis in the heart by increasing endothelin-1, transforming growth factor (TGF-β), oxidative stress and cytokines (Bader et al., 2004).

Additionally, using transgenic animal models, Schelling et al., (1991) demonstrated that mouse fibroblasts grew in a dosage-related manner promoted by ANGII. Several studies have shown trophic effects of ANGII in both vascular smooth muscle cells and cardiac myocytes (Naftilan 1989a and 1989b; Taubman et al., 1989). Several researchers have also shown that blocking the endothelin receptor with receptor specific antagonists reduces the release of ANGII and endothelin-1 in rat hearts in which hypertrophy would normally be induced due to haemodynamic overload and in mechanical stretch-induced cultured cardiomyocytes of neonatal rats pretreated with endothelin-1 receptor antagonists (Ito et al., 1994; Arai et al., 1995; Yamazaki et al., 1996). The

cardiomyocytes were stretched by plating them on culture dishes with stretch frames attached to a silicone dish. The stretch frame is designed to mechanically expand by turning a horizontal thumb screw, thus increasing the length of the dish (Komuro et al., 1990). From Schelling and colleagues' (1991) observations, it has been suggested that the next step forward will be to develop transgenic and knockout animal models to study the aberrant expression of local RAAS components (Stec et al., 1998; Bader et al., 2000; Lim et al., 2001; Patel et al., 2001). The above findings reveal the role of ANGII in promoting CVD in animal models.

ACE1 and *AGT* transgenic animal models

Schunkert et al., (1990) demonstrated a correlation between ACE1-activity and elevated mRNA levels in a study done in rats that developed LVH due to long term experimental aortic stenosis, generated by placing a metal clip on the ascending aorta via thoracic incision (termed aortic banding). Prior to the study of Schunkert et al., (1990), Linz and colleagues (1989) demonstrated that ACE1 inhibition induced the regression of cardiac hypertrophy in rats (Linz et al., 1989). Transgenic rat models created to overexpress ACE1 demonstrated no change in cardiac morphology unless they were subjected to pressure overload due to aortic banding (Tian et al., 2004). However, mice that exclusively overexpressed *AGT*, maintained normal BP levels but still developed hypertrophy (Mazzolai 1998).

Summary of studies of the role of RAAS in CVD

In summary, from these studies, it was evident that the RAAS plays an important role in the development of cardiac hypertrophy, prompting numerous investigations to identify new drug targets to interfere with ANGII formation and possibly other components of the system (Shirani et al., 2000; Spirito et al., 2000; Marian et al., 2002).

1.4.3. Role of RAAS in Hypertension and LVH

1.4.3.1 RAAS and Hypertension

The RAAS is an important system known to be associated with the development of abnormally high BP in humans. Evidence to support its role in the development of high

BP is the excessive amounts of aldosterone production observed in hypertensive patients with adrenal hyperplasia and adenomas (Stowasser et al., 2001). Additional evidence for its pathophysiology is the active release of renin in renovascular hypertension (Laragh et al., 1986). However, it has been observed that within many hypertensive individuals the plasma renin activity (PRA) is normal (Folkow et al., 1982). Similarly, in essential hypertension (EHT) (which is defined as an increased BP of which the cause is unknown or undefined) of subjects who have a normal aldosterone concentration, the renin levels have also been shown to be normal, thus suggesting a downstream abnormality in the RAAS pathway, somewhere from AGT to aldosterone formation (Fisher et al., 1999).

Conversely, hypertensive subjects of African ancestry showed a decreased PRA without changes in plasma aldosterone concentrations (Cohen et al., 1982). To determine if the low PRA was due to genetic variation within *CYP11B2*, three polymorphisms, -344C/T, Arg173Lys and an intron 2 conversion (IC), were investigated and found not to be associated with EHT in black Afro-Caribbean origin subjects from South-West London (Zhu et al., 2003).

In both hypertensive parents and their offspring, increased plasma AGT concentrations have been observed (Watt et al., 1992). According to several investigators, BP often correlates with plasma AGT concentrations (Walker et al., 1979; Bennett et al., 1993; Bloem et al., 1995). Harrap et al., (1996) suggested that an elevated ANGII concentration predisposes an individual to develop hypertension.

A substantial amount of evidence favours the role of the RAAS in hypertension. However, no single gene is considered to control the activity of this RAAS. This is supported by various studies using animal models each implicating a RAAS gene involved in control of BP (Rapp et al., 1989; Kim et al., 1995; Yu et al., 1998). For example, it has been found that in mice a reduction in BP was observed when the two isoforms of the ACE1 gene were totally inactivated by an insertional mutation within exon 14 (Krege et al., 1995). Although to date no monogenic form of hypertension exists that influences other components of the RAAS, investigators hypothesise that RAAS

gene variants might be important in the development of EHT (Tiago et al., 2002 and 2003).

In summary, the RAAS is involved at multiple molecular levels to regulate BP, however, gene variants that moderate RAAS activity are likely to affect BP and left ventricular wall size, which in turn, could have effects on phenotypic risk factors for CVD (Tiago et al., 2002).

1.4.3.2. RAAS and LVH

LVH is considered a major independent risk factor for CVD (Koren et al., 1991). The assumption was made on the basis of certain clinical characteristics observed in LVH patients, which included the presence of various pathologies such as cardiac fibrosis (Weber et al., 1994), apoptosis (Sharov et al., 1996) and impaired coronary haemodynamics (Marcus et al., 1981). However, the mechanisms that generate LVH in CVD patients are unclear, even though there remains a direct relationship between regression of LVH during treatment and reduction in cardiac events (Liebson and Serry, 2000). According to Korner and Jennings (1998), LVH is due to increased BP, whereby the heart muscle compensates for increased cardiac load. However, there is a poor correlation between BP and cardiac mass (Korner and Jennings, 1998). Additionally, in patients undergoing antihypertensive treatment decreased BP does not always result in a similar reduction in left ventricle mass (LVM), although the prognosis is suggested to be better within these patients (Liebson and Serry, 2000). Several investigators observed unfavourable effects of regressing LVH within animal and human models, by failing to reduce LVM when treated with the antihypertensive drug, hydralazine (Fogari et al., 1995; Norton et al., 1997; Yamazaki et al., 1999; Tsotetsi et al., 2001).

These observations implied the presence of variability in LVM in hypertensive patients (Liebson and Serry, 2000) and indicate that factors independent of haemodynamic effects (BP) should also be examined as potential hypertrophy determinants.

As shown in cultured cells stimulated by ANGII, the RAAS mediates its effects on cardiac growth independently of its actions on BP and induces cardiomyocyte hypertrophy (Sadoshima and Izumo, 1993) through both reactive oxygen species (Takemoto et al., 2001) and calcineurin-dependent pathways (Olson and Molkentin, 1999). In 11 patients (6 males and 5 females) with HCM and 8 healthy asymptomatic individuals, a 4.5-fold increased myocardial aldosterone level was observed in HCM patients versus controls and increased *CYP11B2* mRNA levels was also seen in the subjects with HCM, while the cAMP level was normal in both cases and controls (Tsybouleva et al., 2004).

In a study by Schmitt et al., (2003a), the effects of transverse aortic banding on two strains of transgenic mice with and without the cardiac *MYH6* Arg403Gln missense mutation (equivalent to the human *MYH7* Arg403Gln mutation) were assessed. They found that in transverse aortic banded mice that were not subjected to treatment with the calcineurin-inhibitor cyclosporin A, the hypertrophic response to hypertrophy was uniform. When cyclosporin A (an agent that is known to amplify hypertrophy induced by this sarcomere mutation [Arg403Gln]) (Fatkin et al., 2000) was added to banded mice harbouring either the wild type (WT) (+/+) or the missense (+/-) mutation, it resulted in an augmented hypertrophic response in both groups (Schmitt et al., 2003a). Consequently, the authors predicted that both hypertension and HCM act independently of one another and not synergistically.

In cultured rat cardiac myocytes and fibroblasts, CYP11B2 provoked expression of hypertrophic and profibrotic effects via activation of protein kinase D (PKD) and upregulation of phosphoinositide 3-kinase (PI3K) (Tsybouleva et al., 2004). Additionally, Tsybouleva et al., (2004) demonstrated that in a cardiac troponin T (cTnT)-Q92 transgenic mouse model of human HCM (in humans, *TNNT2* Arg92Trp mutation), blockade with mineralocorticoid receptor (MR) antagonist spironolactone inhibited PKD (a mediator of hypertrophic effect of aldosterone) and PI3K (a mediator of profibrotic effects). This resulted in aldosterone normalised myocardial collagen content and attenuated myocyte disarray phenotypes. The myocytic disarray phenotypes were similar

to those shown by Varnava et al., (2001) and Silvestre et al., (1998) to associate with SCD and heart failure in humans and mouse models of HCM. These studies implicate aldosterone as a molecular link between sarcomeric gene mutations and cardiac phenotypes as demonstrated in human hearts, cultured cells and a genetic animal model of HCM.

Direct role for RAAS in LVH

Thus, there is mounting evidence for the RAAS's role in LVH, the first being an independent relationship between plasma ACE levels and aldosterone concentrations with either LVH or indices of cardiac growth (Schunkert et al., 1996, 1997). The second line of evidence was the finding by Harrap et al., (1996), which indicated a relationship between plasma ANGII concentrations and LVM in healthy young adults. Numerous data implicate the RAAS as a mediator of LVM by having either direct cellular effect on cardiac growth (Olson and Molkelin, 1999; Takemoto et al., 2001) and/or indirect effects via circulating components of the RAAS on LVM (Harrap et al., 1996; Schunkert et al., 1996, 1997). This evidence has prompted researchers to investigate the role of RAAS gene variants on the development of LVH.

Mechanism of RAAS inducing LVH

The mechanism of RAAS's effects on LVH occurs via an interaction between direct cellular/molecular pathways and indirect haemodynamic changes (Sadoshima and Izumo, 1993; Harrap et al., 1996). Both these effects are considered interdependent and synergistically induce marked LVM changes. Thus, one change is not sufficient to modify LVM as shown in previous preclinical studies (Sen et al., 1974; Sen and Tarazi, 1983; Frohlich and Sasaki, 1990). It is assumed that genetic polymorphisms within the RAAS system could potentially modify RAAS activity in the presence of hypertension and thus produce marked effects on LVM (Tiago et al., 2002). If the RAAS is not the primary cause then it might be a contributor to LVH, a characteristic often associated with HCM. The potential effects of RAAS activation in cardiac hypertrophy include myocardial fibrosis, diastolic dysfunction (Weber et al., 1994), myocyte necrosis (Tan et al., 1991; Ollivier and Bouchet, 1992), myocyte slippage, cardiac dilatation (Mann and

Spinale, 1998) and vasoconstriction (Hall et al., 1986; Hall et al., 1990; Hall and Gayton, 1996).

Some of the risk factors involved in CVD development are known and the RAAS is considered a mediator of these factors leading up to LVH (Tiago et al., 2002). Other studies have sought to determine the genetic factors involved in the development of hypertension and LVH, using genetic approaches.

The following sections will introduce the concept of genetic association studies, linkage disequilibrium (LD), population stratification and using family members as controls in genetic studies, because of their relevance to the present case-control association study in which the role of RAAS as a modifier of the hypertrophic phenotype in HCM was investigated.

Genetic association analyses/ Case-control association studies

Association studies aim to demonstrate a statistical difference in the distribution of allelic variants of selected genes within affected (cases) and unaffected (control) individuals. The studied individuals are included irrespective of their family members' disease status in these studies. Genetic association analyses are usually conducted in a population-based setting using larger sample sizes to gain adequate power (Silverman and Palmer, 2000).

For association studies one of two approaches can be used. The first approach is based on an "*a priori*" candidate gene hypothesis whereby variants within a specific gene are investigated based on known physiological, biochemical or pharmacological evidence. These studies offer increased power to detect genes of moderate effect, while taking into account the current information known about the tissues, proteins and potential genes involved in the pathogenesis of a condition. The second approach, known as a genome-wide association analysis, involves screening the entire genome in search of the causal genetic variant(s), without prior consideration of pathophysiological mechanisms. This systematic approach is regarded as being unbiased because it also involves no prior

assumptions regarding the localisation of possible susceptibility variants (Hirschhorn and Daly, 2005).

The success of both methods relies on the association found between the selected marker and the susceptibility allele due to a phenomenon known as linkage disequilibrium (LD).

Linkage disequilibrium (LD)

LD is the non-random statistical association of alleles at single linked loci, which co-segregate with a high frequency across a population. LD can be used to investigate human evolution and genetic aetiology of complex disorders (Jorde et al., 1995; Kidd et al., 1998) and can aid in the identification of susceptibility loci, for example, in a group of affected individuals that are descended from a single founder individual. It is considered that, over a restricted period of time, in a group descended from a common ancestor, or single founder individual, a low fraction of meiotic recombination events will have occurred, thereby defining a commonly inherited DNA-region that possesses the susceptibility alleles. In case-control association studies, LD patterns give information on the genetic distance over which signals of causation may be generated. Risch and Merikangas, (1996) regarded LD as a tool to identify the neighborhood surrounding the susceptibility allele. However, there are several caveats that should be considered when performing an association study, one being population stratification (see below).

Population stratification

A study is said to have population stratification, if the allele frequencies between cases and controls differ due to diversity in background of the population (Cardon and Palmer, 2003). Population stratification normally occurs when there are differences in disease prevalence between cases and controls and variation in allele frequencies between population groups (Cardon and Palmer, 2003). In case-control association studies, false positives may occur due to even the slightest differences in genetic ancestry between cases and controls. This is because the source population under investigation might comprise of different clusters of subpopulations resulting in spurious associations. For example any allele which by chance has a higher frequency in the subpopulation that

possesses a larger disease risk than in the control group might appear to have an association with the disease (Wacholder et al., 2002). Alternatively, population stratification may result in a false negative finding: if the frequency of the associated allele is by chance, low in a subgroup of people in which the disease is more prevalent, than in the control group the association with the disease may be masked (Deng et al., 2001). However, population stratification can be avoided by using family members as controls (Thomas and White, 2002); this rationale was implemented in the present study.

<u>Using family members as controls</u>

Transmission disequilibrium test analysis (TDT) makes use of parents and siblings as family-based controls. TDT allows the transmission pattern of selected marker alleles from heterozygous parents to affected offspring to be followed. If transmission frequency of the marker allele exceeds that which is expected by chance alone, the allele is assumed to be associated with the disorder in some way.

The approaches mentioned above have been used in several genetic association studies to determine the influence of genetic factors of the RAAS on the phenotypic expression of hypertension and LVH; as will be discussed in further detail in the following section.

1.4.4. RAAS genes in hypertension and LVH

As discussed in section 1.4.1, the RAAS is a complex integrated system mediating both cellular/molecular and haemodynamic effects on tissues and organs. Several studies have implicated RAAS gene variants as having independent effects on cardiac growth. Previously, polymorphisms within the RAAS genes have been extensively investigated for involvement in hypertension and a variety of other CVD's in case-control association studies (Bonnardeaux et al., 1994; Staessen et al., 1997). These polymorphisms include the *ACE1* insertion/deletion (I/D), *AGT* M235T, *AGTR1 A/C*1166 (an *A/C* transversion at nucleotide position 1166), *CMA A/G* exchange at position -1903 and *CYP11B2* C/T exchange at position -344 (Rigat et al., 1990; Jeunemaitre et al., 1992; Bonnardeaux et al., 1994; Pfeufer et al., 1996; Kupari et al 1998) (Table 1.2). Subsequently, several other polymorphisms located in the promoter, exonic or untranslated region (UTR) of *ACE1*,

AGT and *AGTR1* were identified, which were also examined for association with CVD's (Villard et al., 1996; Inoue et al., 1997; Ishigami et al., 1997; Sato et al., 1997). Concomitantly, researchers were prompted to investigate additional variants in other components/genes of the RAAS for possible associations.

The following section will provide more detail on the aforementioned genes investigated in previous association studies arranged chronologically in support of the present study.

1.4.4.1. *ACE1*

The *ACE1* gene located on chromosome 17q23.3 comprises 25 exons, 24 introns and a promoter region (figure 1.6). The 287 base pair (bp) I/D polymorphism in intron 16 is associated with approximately half of the plasma ACE1 level variability between individuals, as well as in variation with tissues (Rigat et al., 1990; Danser et al 1995). Other studies have confirmed a positive correlation between the D-allele of *ACE1* and increased enzyme activity (Watt et al., 1992; Nakai et al., 1994; Dessi-Fulgheri et al., 1995; Bloem et al., 1996; Fornage et al 1998).

<u>*ACE1* and Blood pressure</u>

Various studies performed to determine if *ACE1* is involved in human hypertension development have yielded conflicting results. However, some studies have shown positive results for a plausible role of the *ACE1* in gender-specific hypertension. Using a large population-based sample of 1445 men and 1650 women from the Framingham heart study, O'Donnell et al., (1998) confirmed a relationship between the D-allele of *ACE1* and both a diagnosis of EHT and increased BP in males. An additional case-control study supported a gender-based association between microsatellite markers in *ACE1* and EHT again in male subjects only (Fornage et al., 1998). The study recruited 1488 Caucasian siblings consisting of 814 males and 674 females subdivided into hypertensives (systolic BP (SBP) > 160 and diastolic BP (DBP) > 95mmHg) and normotensives (SBP $\leq$ 140 and DBP $\leq$ 90mmHg) (Fornage et al., 1998). Subsequently, a larger gender-specific study conducted in 5014 Japanese EHT patients (2340 men and 2674 women) confirmed Fornage and colleague's (1998) results (Higaki et al., 2000b).

In a study of 3596 Finnish children aged 6 to 8 years who had hypertensive parents, results suggested that polymorphisms in *ACE1* are associated with BP in offspring belonging to parents who had a positive history of hypertension, while *AGT* polymorphism(s) were not associated with BP (Tian et al., 1997). However, two other studies failed to demonstrate a relationship between *ACE1* genotypes and severity of BP changes in EHT in 343 Caucasian German subjects (Beige et al., 1997) and 50 untreated hypertensive subjects (Gharavi et al., 1997). Similarly, in French hypertensive patients (453 men and 326 women) the *ACE1* I/D variant were found not to be associated with hypertension (Tiret et al., 1998). The lack of association mentioned in the latter three studies could be due to small sample sizes used for analysis.
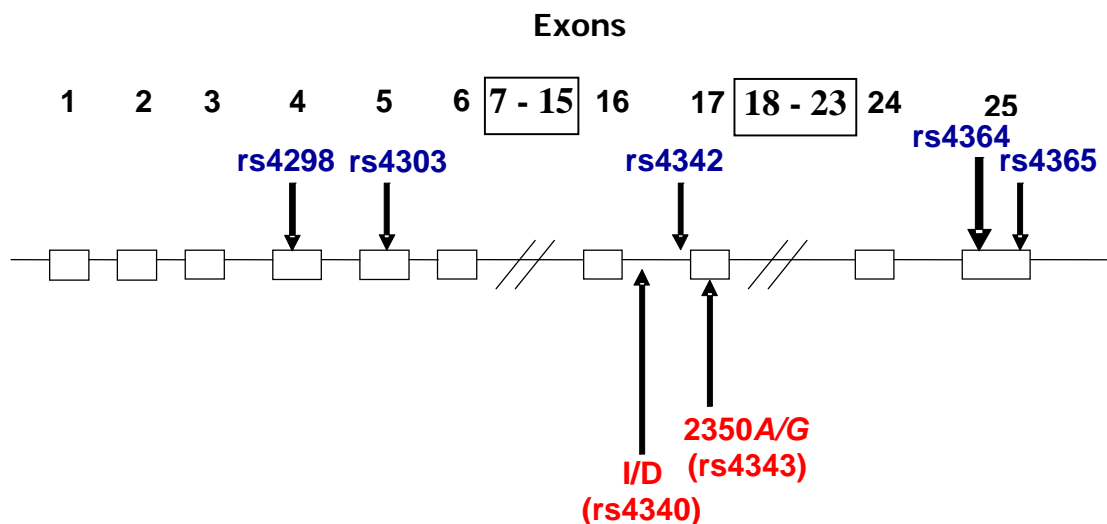
Figure 1.6: Genomic organisation of the human angiotensin-converting enzyme gene (*ACE1*) showing variants investigated in association studies. The locations of various polymorphisms being investigated for possible association with LVH (indicated by vertical black arrows). The markers highlighted in red indicate variants investigated in previous association studies and the blue highlighted markers indicate variants that have not yet been investigated (Table 1.2 section 1.3.2). Abbreviations: I/D – insertion/deletion; rs – SNP identification number in NCBI database.

In 5014 human Japanese hypertensive subjects, the D-allele was significantly associated with hypertension in men but not in women, even after adjustment for the effect of

confounding variables such as age, body mass index (BMI), smoking and drinking habits, total cholesterol, triglycerides, HDL-cholesterol, fasting plasma glucose and creatine (Higaki et al., 2000b). A case-control association study by Agachan and colleagues (2003) found a positive association between the *ACE1* I/D variant and EHT in 109 hypertensive subjects (SBP > 140 and DBP > 90mmHg) compared to 86 normotensives (SBP < 140 and DBP < 90mmHg) from Turkey.

ACE1 and LVH *per se*

The literature is filled with conflicting results, because associations have been reported for the *ACE1* D-allele with electrocardiographic or echocardiographic LVH *per se* (Schunkert et al., 1994; Gharavi et al., 1996), while other studies report no significant association of the D-allele with these conditions (as referred to below). The Schunkert et al's., (1994) study, which found an association between the *ACE1* D-allele and electrocardiographic LVH, recruited Caucasian subjects of Western European descent, consisting of 717 men and 711 women between the ages of 45 and 59 years, with LVH and a similar number of healthy controls matched for age, sex and BP status.

Additionally, in a population-based case-control association study of 430 randomly selected hypertensive and 426 control subjects, no association was found between the *ACE1* I/D polymorphism and LVM in subjects from Oula, Finland (Kauma et al 1998). The epidemiological study by Kauma et al., (1998) included hypertensive subjects who had age and sex-matched controls with statistical analysis performed by adjusting for the influence of covariates of hypertrophy such as age, BMI, SBP, gender, fasting insulin and physical activity.

Similarly, in a group of 87 Japanese patients (48 men and 39 women) randomly selected from the population, the *DD* genotype of the *ACE1* I/D variant was associated with hypertensive LVH in the concentric hypertrophy group (increased LVMI and relative wall thickness) (Ueno et al., 1999).

In 2000, a meta-analysis study found no association for the *ACE1* I/D polymorphism with either electrocardiographic or echocardiographic LVH (Kuznetsova et al., 2000). However, when other studies were performed in untreated hypertensive patients, a significant association was observed between *DD* typed homozygotes and LVMI (Iwai et al., 1994; Gharavi et al., 1996; Perticone et al., 1997). But in continuing association studies, Ortlepp et al., (2001) enrolled 105 patients with aortic stenosis and genotyped them for five RAAS polymorphisms, namely, *ACE1* I/D, *AGT* M235T, *AGTR1 A/C*1166, *CMA* -1905*A/G* and *CYP11B2* -344*C/T*. However, none of the investigated variants showed significant association with LVH in patients with aortic stenosis (Ortlepp et al., 2001). In contrast, Hernandez et al., (2003) genotyped 61 Caucasian male endurance athletes from the Canary Islands for the *ACE1* I/D polymorphism and found that *DD*-genotyped athletes had greater LVMI than *II* and *ID* athletes. The statistically significant association remained even after adjusting for the influence of confounding variables such as age, BMI, haemoglobin levels, years of training and BP.

An ensuing association study by Kuznetsova et al., (2004) recruited 221 nuclear Caucasian families (384 parents and 431 offspring) from Poland and Italy. Using a population and family-based approach, Kuznetsova and colleagues (2004) demonstrated by statistical analysis, adjusting for the effects of covariates, that the *ACE1* I/D and *AGTR2* 1675*G/A* variants independently influence LVM in men and are modulated by sodium intake.

A retrospective, case-control study recruited 180 Emiratus nationals consisting of 50 LVH patients and 130 controls between the ages of 43-72 years old, who were all non-alcohol users and non-smokers; thus reducing environmental confounders (Saeed et al., 2005). The two genotyped polymorphisms in *ACE1* I/D and 2350*G/A*, demonstrated an independent association with LVH in normotensive LVH patients (Saeed et al., 2005). However, the association was lost after adjusting for age and gender, but was regained when performing haplotype analyses, with the combined two polymorphisms yielding haplotype I/G that was significantly associated with LVH (Saeed et al., 2005). Similarly,

reports on association between the *ACE1* I/D polymorphism and hypertrophic or survival phenotypes in HCM are conflicting (Marian et al., 1993).

In population-based association studies, Yoneya et al., (1995) demonstrated in Japanese subjects that the D-allele was more common in family members of 80 HCM patients that had a high incidence of SCD compared to 88 of their unaffected children and their siblings. These results are discordant with those generated in a case-control study using 71 unrelated patients with nonfamilial HCM and 88 patients with nonfamilial DCM and 122 healthy control subjects from Japan (Yamada et al., 1997). Genotyping these cases and controls for *ACE1* I/D, *AGT* M235T and T174M polymorphisms failed to confirm any association between either of the polymorphisms and LVH (Yamada et al., 1997).

Lechin and colleagues (1995) enrolled 183 Caucasian patients with HCM, of whom 87 had the sporadic form, while the other 96 had FHCM. Lechin et al., (1995) found a statistically significant association between *ACE1 DD* genotyped individuals, and left ventricle mass index (LVMI) as well as LVH score, in 108 genetically independent patients (sporadic cases combined and one randomly chosen individual per family), independent of the effect of confounding variables.

Additionally, in 62 patients with HCM, the *DD* genotype of the *ACE1* I/D variant was associated with a greater progression of LVH compared to *II* and *ID* genotypes, independent of age, body mass and resting BP (Doolan et al., 2004).

Perkins and colleagues (2005) illustrated in 389 unrelated patients with HCM that the *ACE1 DD* genotype is associated with increased LVWT in *MYBPC3* carriers, but not in *ACE1 DD* genotyped individuals harbouring the *MYH7* mutation.

Functionality of *ACE* I/D polymorphism

The following subsection will discuss the possible role and mechanism of the *ACE1 I/D* polymorphism in general CVD development.

The genetic localisation of the *ACE1* I/D polymorphism, within intron 16, makes it an unlikely candidate to affect ACE activity and therefore to affect function of the protein. However, it is suggested that alleles at a locus or marker in LD with this *ACE1* I/D polymorphism and located in a functionally conserved region may have a role in direct effects on ACE activity (Tiret et al., 1992; Tiret et al., 1994; Villard et al., 1996). In an attempt to identify possible variants that are in complete LD with the I/D polymorphism, Keavney et al., (1998) analysed nine polymorphisms, including the I/D polymorphism, that spanned 26kb of *ACE1* in Caucasian British families. Measured haplotype analysis revealed that the major variant influencing ACE levels is located 3' of a putative ancestral breakpoint which signifies an ancestral recombination event near position 6435 (Keavney et al., 1998). After refinement of the putative ancestral breakpoint by Farrall et al., (1999), McKenzie and colleagues (2001) examined ten *ACE1* polymorphisms in Afro-Carribean families and compared the results to the study done by Keavney et al., (1998). The results of McKenzie et al., (2001) suggested that the major *ACE1*-linked QTL is likely to lie in close proximity to the 2350*G/A* polymorphism (see page 29). They concluded that further investigations of polymorphisms 5' and 3' of this 2350*G/A* polymorphism were warranted.

The peptide by which increased *AGT* expression and ACE enzyme activity influence cardiac tissue structure and function is through ANGII production (Schunkert et al., 1990; Arnal et al., 1994; Muller et al., 1997). An additional mechanism of action that should be considered is the role of gene-gene interactions in the development of CVD, which is assumed to have a multi-factorial etiology (Wang et al., 2000). The first evidence comes from studies done by Tiret et al., (1994), Alvarez et al., (1998) and Fatini et al., (2000), where they observed a synergistic relationship between the *ACE1* I/D and *AGTR1 A/C*1166 polymorphisms in determining risk for coronary heart disease development. The second line of evidence is from a study that investigated the synergistic effects of the *ACE1* I/D and α-adducin (*αADD*) Gly460Trp polymorphisms on BP responses to sodium loading (Barlassina et al., 2000). Barlassina et al., (2000) found similar BP levels across *ACE1* I/D genotypes and for *αADD* Gly/Gly homozygotes, whereas a positive correlation

and linear association was observed for all *αADD* Trp-allele carriers who also harboured the *ACE1* D-allele.

In summary, several case-control association studies provide evidence for a relationship between the *ACE1* I/D polymorphism and LVM in normotensive and hypertensive groups (Iwai et al., 1994; Schunkert et al., 1994; Perticone et al., 1997). More importantly, LVM measurements indicate a relationship between the *ACE1* I/D polymorphism and LVM (Nakahara et al., 2000).

1.4.4.2. *AGT*

The gene encoding AGT comprises five exons, four introns and a promoter region located on chromosome 1q42-43 (figure 1.7). *AGT* is highly polymorphic with nearly 20 variants indexed in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/). The *AGT* exon 2 variant 704T→C results in a methionine being substituted for a threonine in position 235 of the amino-acid sequence (M235T). It has been suggested that the M235T polymorphism is a marker determining the amount of plasma AGT variation between individuals (Jeunemaitre et al., 1992).

AGT and Blood pressure

Several investigators have shown that the *AGT* M235T variant is associated with EHT in various ethnic groups such as French Caucasians and Japanese (Jeunemaitre et al., 1992; Hata et al., 1994; Tiret et al., 1998). The details of the numbers of subjects studied and their population groups are given in the following sentences. Jeunemaitre et al., (1992) recruited Caucasian hypertensive sibling pairs from America (144 males and 165 females) and 140 controls and from France hypertensive cases (99 males and 91 females) and 98 healthy normotensives for case-control association analysis. The case-control study by Hata and colleagues (1994) enrolled 105 hypertensives (SBP > 160 and DBP > 95mmHg) and 81 normotensives (SBP < 140 and DBP < 90mmHg) from Japan, while Tiret et al., (1998) included French hypertensive cases (453 men and 326 women) (DBP ≥ 100mmHg) and normotensive controls (362 men and 170 women).

However, subsequent studies performed in different cohorts of subjects generated discrepant findings, as no association was found between *AGT* M235T and EHT in Caucasians (Caulfield et al., 1994), Japanese (Kato et al., 1999 and 2000) and Chinese subjects (Cheung et al., 1998) (as discussed in detail below). However, Caulfield and colleagues (1994) found significant linkage between *AGT* and EHT in the 63 European Caucasian families using dinucleotide-repeat markers flanking *AGT*, while no association in the reported population was found between the *AGT* M235T and T174M polymorphisms and EHT. Hypertension was diagnosed when DBP was >95mmHg, while individuals with secondary hypertension due to renal disease, (renovascular hypertension) were excluded from the study (Caulfield et al., 1994). The association studies undertaken in Japanese individuals by Kato et al., (1999) included 1232 individuals stratified into two groups consisting of 254 cases (hypertensives SBP > 160 and DBP > 95mmHg) and 224 normotensive subjects and 463 hypertensive cases and 291 normotensive individuals, respectively. The ensuing case-control study by Kato and colleagues (2000) enrolled 1476 Japanese individuals, 843 hypertensive subjects and 633 normotensive subjects (SBP < 160 and DBP < 95mmHg).

The case-control study by Cheung et al., (1998) consisted of 204 subjects stratified into two groups, cases (101 newly diagnosed hypertensives SBP> 160 and DBP > 90mmHg) and controls (103 healthy subjects without secondary hypertension, heart disease, vascular disease and diabetes). No association between *AGT* M235T and EHT was found in these studies.
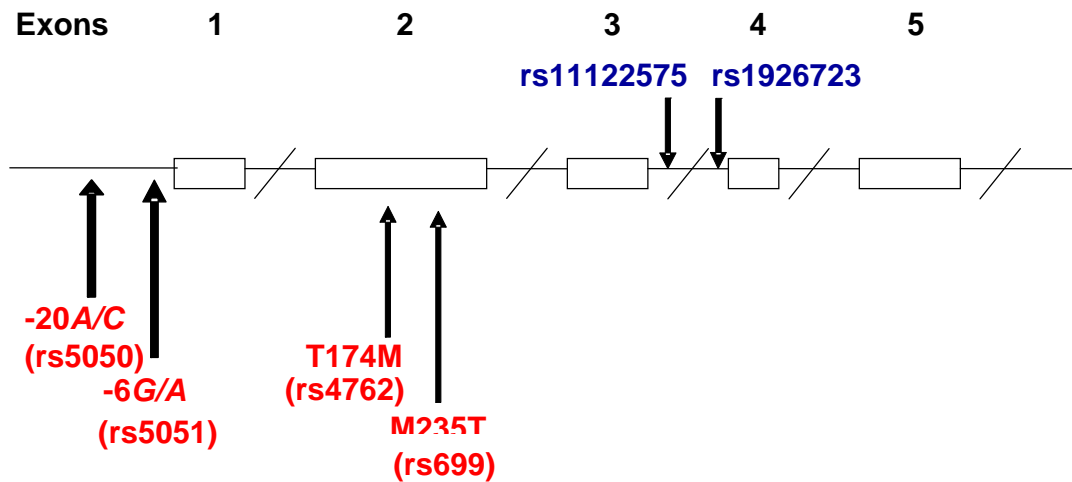
Figure 1.7: Genomic organisation of the human angiotensinogen gene (*AGT*) showing variants investigated in association studies. The locations of various polymorphisms being investigated for possible association with LVH (indicated by vertical black arrows). The markers highlighted in red indicate variants investigated in previous association studies and the blue highlighted markers indicate variants that have not yet been investigated (Table 1.2 section 1.3.2).

Examining the influence of the *AGT* 235T allele in 57 hypertensives (SBP $\geq$ 140 and DBP $\geq$ 90mmHg) and 130 normotensive unrelated subjects of African ancestry failed to confirm any association between *AGT* M235T variant and hypertension, because the allele was non-informative within the ethnic group (Rotimi et al., 1994). Inconsistent results were found for the role of *AGT* in BP (as referred to below) (Hingorani et al., 1996; Kiema et al., 1996; Fardella et al., 1998). Moreover, Hingorani and colleagues (1996) observed a lack of association between *AGT* M235T and BP level in subjects from East Anglia United Kingdom. A total of 223 hypertensives and 187 normotensives were genotyped for the *AGT* M235T variant and statistically analysed with concomitant adjustment for age, sex, BMI and alcohol consumption (Hingorani et al., 1996). In their study, hypertensive subjects were stratified according to SBP $\geq$ 160 and DBP $\geq$ 90mmHg, while controls were healthy individuals matched to hypertensive patients for age, sex and BMI and with no history of CVD or any other disease (Hingorani et al., 1996).

The case-control association study by Kiema and colleagues (1996) carried out with 508 hypertensives and 523 controls from Finland that were matched for age and sex demonstrated no statistically significant association between *ACE1* I/D and *AGT* M235T polymorphisms and EHT. This was a population-based sample of middle-aged randomly

selected individuals from an ethnically homogeneous population with hypertensive subjects being defined as DBP > 105mmHg (Kiema et al., 1996). Similarly, another study examining the genotypes of 64 hypertensives and 62 normotensives matched for age and sex demonstrated no association between the *AGT* T235-allele and EHT in Chilean Hispanic subjects (Fardella et al., 1998). Additionally, no statistical significance was found between *AGT* M235T variant and plasma renin activity and serum aldosterone levels in hypertensives (SBP > 140 and DBP > 90mmHg) and normotensives (SBP < 140 and DBP < 85mmHg) subjects (Fardella et al., 1998). In contrast, another study demonstrated a positive correlation between M235T and severity of human hypertension in 50 untreated subjects (33 men and 17 women) consisting of 19 Caucasians, 24 Hispanics and 7 Asians (Gharavi et al., 1997), whereas yet another study failed to show an association in 343 Caucasian German subjects (Beige et al., 1997).

A series of 477 probands of French Caucasian hypertensive families and 364 control individuals and 92 Japanese hypertensive individuals (DBP $\geq$ 95mmHg) and 122 controls were genotyped for *AGT* M235T (Jeunemaitre et al., 1997). This study showed a positive association between the *AGT* M235T polymorphism, specifically the T235-allele and hypertension in both populations investigated. However, other studies failed to demonstrate an association between *AGT* variants and EHT in 350 hypertensive sibships consisting of 630 European affected sibling pairs (Brand et al., 1998) and 111 Australian Anglo-Celtic Caucasian hypertensive subjects (SBP > 140 and DBP > 90mmHg) (Wang et al., 1999). Furthermore, in a case-control study, no association was found between *AGT* M235T and T174M variants and EHT in 75 patients with EHT (SBP $\geq$ 160 and DBP $\geq$ 100mmHg) and 75 healthy Spanish Caucasian controls (SBP < 130 and DBP < 85mmHg), despite adjustments for confounding variables like age, gender, BMI and alcohol consumption (Fernandez-Llama et al., 1998).

In a population-based study of 9100 Danish men and women matched for age, results showed that the *AGT* M235T polymorphism, particularly the T235-allele, was associated with elevated SBP in women but not in men (Sethi et al., 2001). This association was strengthened for subjects that were homozygous for the T235-allele and the T174-allele.

Moreover, the plasma levels for the double homozygous T235 and T174 correlated with a 10% elevated plasma AGT level in both genders. An association with EHT was evident in 109 hypertensive subjects (SBP > 140 and DBP > 90mmHg) and 86 normotensive individuals (SBP < 140 and DBP < 90mmHg) of Turkish descent (Agachan et al., 2003). In addition, Agachan and colleagues (2003) showed that the frequency of the *AGT* M235T variant (the TT genotype) and the T174M variant (the 174M-allele) were higher in hypertensive than control subjects. A study that recruited 188 Malaysian subjects, 101 hypertensives (SBP ≥ 140 and DBP ≥ 90mmHg) and 87 normotensives (SBP ≤ 140 and DBP ≤ 90mmHg), and recorded BP and BMI measurements found that the TT genotype of the *AGT* M235T polymorphism was significantly associated with EHT (Say et al., 2005).

The influence of various promoter region variants [-20 adenine (*A*)/cytosine*(C)* and -6 guanine *(G)/A*] on AGT basal transcription rates might be used as an indicator of severity of hypertension. Previously a correlation was observed between an increased AGT concentration and variants -20*A/C* and -6*G/A* (Inoue et al., 1997; Ishigami et al., 1997; Zhao et al., 1999); however a non-significant association was observed between the -6 *G/A* variant and BP in 518 normotensive Oji-Cru Canadians (Hegele et al., 1998). Caulfield et al., (1995) established linkage and association between the *AGT* locus and hypertension in 63 affected sibling pairs of African-Caribbean descent using microsatellite dinucleotide (GT) repeats located in the 3' flanking region of *AGT*. The study included individuals with hypertension defined as DBP > 95mmHg and excluded individuals with clinical evidence of secondary hypertension and diabetes mellitus.

Genotyping 556 hypertensives and 547 controls of African ancestry (Nguni, Sotho and Venda Chiefdoms) demonstrated that the *AGT* -20*A/C*, variant particularly the -20*A*-allele markedly modified the relationship between BMI and SBP in hypertensive subjects, while the -20*C*-allele had a protective effect (Tiago et al., 2002). A study by Tsai et al., (2002) recruited 408 hypertensives (229 men and 179 women) and 286 normotensive Taiwanese subjects (151 men and 135 women) and excluded subjects if they had secondary hypertension, caused by primary aldosteronism, renalvascular hypertension,

Cushing syndrome and heart disease. Performing haplotype analysis of the *AGT* locus revealed that the haplotype GGAGCC (which incorporates the -217*G*, -152*G*, -20*A*, -6*G*, from 522C (T174M) and from 704*C* (M235T) alleles) is significantly associated with hypertension in the homogeneous Taiwanese population (Tsai et al., 2003). Additionally, Tsai and colleagues (2003) observed a higher frequency of the haplotype in hypertensive individuals with the *ACE1 II* genotype, suggesting a synergistic interaction between the two genes. Zhu et al., (2005) examined haplotypes produced from rare variants in *AGT* to determine its relationship with BP in 29 Nigerian men with high plasma AGT and 28 with low levels of plasma AGT. From the 24 rare haplotypes produced, 19 haplotypes carried haplotype GCT (consisting of -217*G/A*, 172*C/T* and 2186*G/T*), providing evidence that rare sequence variants might have an influence on variation of BP phenotype. Table 1.3 contains a detailed summary of various case-control association studies discussed in previously written sections.

In summary, various case-control association studies that have been undertaken have yielded discrepant findings as some showed positive association and others show no association with hypertension, however, a large number of these studies reveal no relationship between *AGT* and hypertension.

AGT and LVH *per se*

A study by Jeng et al., (1999) genotyped 91 males and 84 females with hypertension (DBP > 95mmHg) for the M235T variant in AGT, while controlling for confounding factors of hypertrophy such as age, gender and BMI and found an association of the TT-genotype with LVMI. Hence, they suggested that variants within *AGT* might be potential independent mediators of LVM.

Using a study cohort consisting of 80 elite athletes from Finland (50 men and 30 women), Karjalainen et al., (1999) demonstrated that the *AGT* M235T polymorphism significantly associates with LVM independently of BP in both male and female endurance athletes with adaptive cardiac hypertrophy. Genotypes of *AGT* M235T, *ACE1* I/D and *AGTR1*

*A/C*1166 were determined in 83 German male Caucasian endurance athletes to assess if these variants are associated with LVM (Diet et al., 2001). Separate analysis of each polymorphism revealed no association with LVM, while combined analysis confirmed an association in *ACE1 DD* and *AGT* TT genotypes with greater LVM, compared to other genotype combinations. None of the athletes participating in the study had any history of heart disease, hypertension, smoking or kidney disease. Analysis was done adjusting for the influence of covariates such as age, type of sport, number of years active in competitive sport, height, weight, body surface area, SBP and DBP (Diet et al., 2001).

Genetic variation in *AGT* was also modestly associated with LVH in normotensive compared to hypertensive subjects (Tang et al., 2002). This association was independent of the effects of covariates of hypertrophy, such as weight, height, age, sex, SBP, DBP, presence of diabetes and anti-hypertensive medication in 605 participants (302 men and 303).

Kurland et al., (2002) found that the *AGT* T174M variant (heterozygote TM genotype), the T235-allele of M235T and the *AGTR1 A/C*1166 variant (heterozygote *AC* genotype) correlated with a change in LVH during antihypertensive treatment with the *AGTR1* receptor antagonist Irbesartan. In the 84 random Swedish Caucasian subjects analysed, individuals received either Irbesartan or the β-1 adrenergic receptor antagonist atenolol. While only *AGT* and *AGTR1* variants showed association, neither of the *ACE1* I/D nor *CYP11B2* -344*C/T* variants were associated with reduction in LVMI after treatment with either drug (Kurland et al., 2002).

<u>*AGT* and HCM</u>

In HCM, the role of the *AGT* M235T polymorphism is controversial. Numerous investigators have reported both positive and negative findings regarding the relationship between the M235T variant and LVMI. In one Japanese study, a relationship was found between M235T variant and cardiac hypertrophy (Ishanov et al., 1997). A higher occurrence of the T235-allele was observed in 96 sporadic HCM cases compared to 105 unaffected control individuals (Ishanov et al., 1997). However, a meta-analysis in 1999

yielded no significant association between *AGT* M235T polymorphism and HCM or DCM and produced a low odds ratio (Staessen et al., 1999). The *CC* genotype of *AGT* M235T polymorphism showed a trend of association with LVH in a single family of HCM patients (Ortlepp et al., 2002).

Functionality of *AGT* variants

In the following paragraph the role of the *AGT* M235T polymorphism and other promoter region variants in hypertension and CVD development will be discussed.

The localised expression of *AGT* occurs within the myocardium and makes it a good candidate gene to assess its influence on cardiac growth (Dzau et al., 1987; Baker et al., 1992; de Mello and Danser 2000). Several mechanisms of action have been proposed to account for the significant correlation between the *AGT* M235T polymorphism and hypertension. Firstly, Bohlender et al., (1996) showed a significant correlation between this polymorphism and an increased ANGI generation, while the plasma renin concentration did not change. Secondly, in gene titration experiments done in animal models, mice with four copies of *AGT* showed a linear increase in the enzyme concentration compared to zero copy mice (Bohlender et al., 1996; Inoue et al., 1997; Smithies et al., 1997).

It has been demonstrated by *in vitro* experiments that the promoter region variant of *AGT*, the -6*A/G* substitution, affects basal transcription rates of *AGT* (Inoue et al., 1997). Additionally, investigators have identified polymorphisms in this region that are in complete LD with the *AGT* M235T variant in exon 2 (Jeunemaitre et al., 1997; Zhao et al., 1999). A previous linkage study indicated that the *AGT* T174M polymorphism is in complete LD with the *AGT* M235T sequence variant (Jeunemaitre et al., 1992). Jeunemaitre et al., (1992) also found that the *AGT* 174M-allele is present in individuals carrying the 235T-allele. Moreover, it was shown by Jeunemaitre et al., (1992) that the T174M polymorphism is associated with hypertension in French Caucasians.

The variants -20*A/C*, -18*C/T* and -6*G/A* are located in the promoter region of the AGT gene between the TATA box and the transcription initiation site. The *AGT* promoter variants -20*A/C* and -6*G/A* have been found to have a significant association with the plasma AGT concentration and EHT (Ishigami et al., 1997; Jeunemaitre et al., 1997). A previous report describing transfection analysis indicated that human hepatoma cells with -20*C*-allele mutation transcribe a reporter gene at a higher level than the -20 *A*-allele of *AGT* (Zhao et al., 1999). In humans, the transcriptional activity of the -20*A/C* polymorphism is considered to alter the plasma AGT concentrations of the *AGT* mRNA (Ishigami et al., 1999).

Additionally, the -20*A/C* polymorphism is in LD with the *AGT* M235T variant (Sato et al., 1997). The -20 *C*-allele in Japanese population substructures was associated with hypertension (Ishigami et al., 1997), although this finding was not confirmed by other investigators (Jeunemaitre et al., 1992, 1997; Sato et al., 1997). Numerous genetic studies have provided evidence that genetic polymorphisms in general can act independently, or in concert, to elicit a pathological effect in various CVDs (Tiret et al., 1992; Pfeufer et al., 1996; Marian et al., 2002).

Table 1.3 Summary of association studies of *AGT* polymorphisms in hypertension.

AGT Polymorphisms	Reference	Cohorts	Method	Findings
M235T	1	■ 309 American & 109 French Caucasian hypertensive sibling pairs (SBP>160 and DBP>95mmHg) and 140 American & 98 French normotensive controls (SBP<140 and DBP<90mmHg)	Case-control association	positive association with EHT
	2	■ 105 Japanese hypertensives (SBP>160 and DBP>95mmHg) and 81 normotensives (SBP<140 and DBP<90mmHg)	Case-control association	positive association with EHT
	3	■ 779 French hypertensives (DBP≥100mmHg) and normotensive controls	Case-control association	positive association with EHT
	4	■ 63 European Caucasian families (parents and offspring) and 63 index patients and 64 European population-based controls	APM and Case-control	Linkage of *AGT* with EHT negative association with EHT
	5	■ Two Japanese groups: 1) 254 hypertensives (SBP>160 and DBP>95mmHg) and 224 normotensive 2) 463 cases and 291 controls	Case-control association	negative association with EHT
	6	■ 843 Japanese hypertensives and ethnically matched 633 normotensive controls (SBP<160 and DBP<95mmHg)	Case-control association	negative association with EHT
	7	■ 101 newly diagnosed Chinese hypertensives (SBP>160 and DBP>90mmHg) and 103 healthy subjects without heart disease and diabetes	Case-control association	negative association with EHT
	8	■ 57 unrelated African hypertensives (SBP≥140 and DBP≥90mmHg) and 130 unrelated normotensives of African descent	Case-control association	negative association with HT
	9	■ 223 East Anglia United Kingdom hypertensives (SBP≥140 and DBP≥90mmHg) and 187 normotensives matched for age,sex and BMI	Case-control association	no association with BP level
	10	■ 508 hypertensives (DBP>105mmHg) from Oula (homogeneous population) and 523 controls matched for age and sex	Case-control association	negative association with EHT
	11	■ 64 Chilean Hispanic hypertensives (SBP>140 and DBP>90mmHg) and 62 normotensives (SBP<140 and DBP<85mmHg) matched for ethnicity, age and sex	Case-control association	negative association with EHT
	12	■ 477 hypertensive probands and 364 controls (French Caucasian) and 92 Japanese hypertensives (DBP≥95mmHg) and 122 controls	Case-control association	positive association with HT
	13	■ 630 European hypertensive sibling pairs and 111 Australian AngloCeltic Caucasian hypertensives (SBP>140 and DBP>90mmHg)	Affected Sib-pair analysis	negative association with EHT
	14	■ 75 Spanish Caucasian EHT patients (SBP≥140 and DBP≥90mmHg) and 75 healthy controls (SBP<130 and DBP<85mmHg)	Case-control association	negative association with EHT
	15	■ 9100 Danish men and women matched for age	Case-control association	positive association with BP level in women

AGT Polymorphisms	Reference	Cohorts	Method	Findings
M235T	16	■ 109 hypertensives (SBP>140 and DBP>90mmHg) and 86 normotensives (SBP<140 and DBP<90mmHg) of Turkish descent	Case-control association	positive association with EHT
	17	■ 101 Malaysian hypertensives (SBP≥140 and DBP≥90mmHg) and 87 normotensive controls (SBP≤140 and DBP≤90mmHg)	Case-control association	positive association with EHT
-6 *G/A*	18	■ 393 normotensive Oji-Cru Canadians and 125 hypertensives (SBP>140 and DBP>90mmHg)	Case-control association	non-significant association with SBP
-20 *A/C*	19	■ 556 hypertensives and 547 controls of African ancestry	Case-control association	relationship with SBP in HT
-6 *G/A* and M235T	20	■ 408 Taiwanese hypertensives and 286 normotensives	Case-control association	haplotype positively associated with HT

Abbreviations used: APM - affected pedigree member; DBP - diastolic BP; EHT - essential hypertension; Hg - mercury; HT - hypertension; mm - millimeters; SBP - systolic BP. References: 1) Jeunemaitre et al., 1992; 2) Hata et al., 1994; 3) Tiret et al., 1998; 4) Caulfield et al., 1994; 5) Kato et al., 1999; 6) Kato et al., 2000; 7) Cheung et al., 1998; 8) Rotimi et al., 1994; 9) Hingorami et al., 1996; 10) Kiema et al., 1996; 11) Fardella et al., 1998; 12) Jeunemaitre et al., 1997; 13) Brand et al., 1998; 14) Fernandez-Llama et al., 1998; 15) Sethi et al., 2001; 16) Agachan et al., 2003; 17) Say et al., 2005; 18) Hegele et al 1998; 19) Tiago et al., 2002; 20) Tsai et al., 2002).

1.4.4.3. *CYP11B2*

The human *CYP11B2* located on chromosome 8q22 comprises nine exons, eight introns and a promoter region (figure 1.8). Generally, one common variant that has been investigated for association is -344*C/T*. The genetic polymorphism -344*C/T* resides at a locus where a putative binding site occurs for the steroidogenic transcription factor, SF1 (White and Slutsker 1995). It has also been found that the -344 *C*-allele is associated with a four-fold increase in SF-1 binding (White and Slutsker, 1995). Additionally, the -344 *C/T* variant is associated with plasma aldosterone levels and urinary metabolic excretion rates and is therefore considered a potential mediator of EHT through regulatory effects on body fluid balance (Pojoga et al., 1998; Davies et al., 1999; Paillard et al., 1999; Russo et al., 2002).

<u>*CYP11B2* and Blood pressure</u>

Two genetic association studies have observed an association between EHT, in 380 hypertensive and 293 normotensive Caucasian men between the ages of 20-30 years old (Brand et al., 1998) and 73 EHT subjects (29 men and 44 women) and 134 normotensive controls of Japanese ethnicity, (Komiya et al., 2000), and the *CYP11B2* -344*C/T* polymorphism in individuals with low renin levels. The Caucasian subject cohort consisted of healthy young men, with exclusion of individuals with hypertension, diabetes mellitus, hyperlipidaemia and hepatic or renal impairment, while the Japanese patients were stratified as EHT subjects if SBP was > 160mmHg and DBP was >95mmHg, while the control individuals included those with SBP of <140mmHg and DBP <90mmHg.

Staessen et al., (2001) found that three RAAS genes (*ACE*, *α-ADD* and *CYP11B2*) demonstrate epistatic interactions and contribute to the prevalence and incidence of hypertension in unrelated Flemish Caucasians. Their case-control association study included 678 normotensive Caucasian subjects that showed association between the *ACE* I/D, *α-ADD* Gly460Trp and *CYP11B2* -344*C/T* polymorphisms and hypertension. Other studies have undertaken single-gene analyses to demonstrate association between hypertension and *ACE*, *α-ADD* and *CYP11B2* but have produced contradictory results

(Wang et al., 2000). The Staessen group notes a limitation of their study may be differences in genetic background between case and control individuals (Staessen et al., 2001).

Similarly, the result of a later case-control association study performed in a total of 1476 Japanese subjects' generated discrepant findings, where no association was observed between EHT and the *CYP11B2* -344*C/T* polymorphism in a large cohort of non-selected renin status candidates (Kato et al., 2002). The patient and control groups in the Kato et al., (2002) study were stratified as hypertensive individuals if SBP was > 160mmHg and DBP was > 95mmHg and as normotensive individuals if SBP was < 160 and DBP was < 95mmHg).

Tsukada et al., (2002) demonstrated in a case-control study in 250 EHT subjects (154 men and 96 women) and 221 control subjects (144 men and 77 women) that the *CYP11B2* -344*C/T* polymorphism is associated with genetic predisposition to developing EHT in unrelated Japanese subjects.
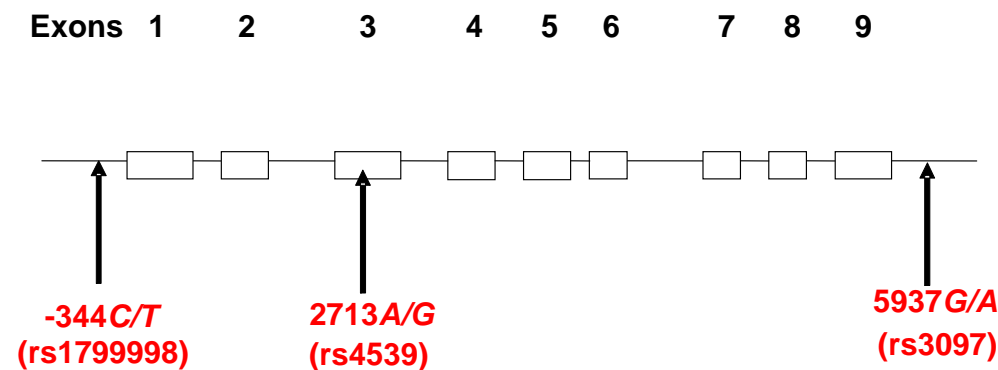
Figure 1.8: Genomic organisation of the human aldosterone synthase gene (*CYP11B2*) showing variants investigated in association studies. The locations of various markers being investigated for possible association with LVH (are indicated by vertical black arrows). The markers highlighted in red indicate three variants investigated in previous association studies (Table 1.2 section 1.3.2).

In the Tsukada et al., (2002) study, the patient and control groups were stratified using the same diagnostic criteria for hypertensive and normotensive subjects as implemented

by Kato et al., 2002. A case-control study using 146 hypertensive and 291 Caucasians controls of Australian Anglo-Celtic descent demonstrated that the *CYP11B2* -344*C/T* and 6547*A/G* polymorphisms showed association with EHT confined particularly to unrelated females, results that were reinforced by haplotype analysis (Kumar et al., 2003). Kumar et al., (2003) studied multiple markers spread across the gene, thereby increasing the statistical power to detect a possible modifying effect of *CYP11B2*.

Tiago et al., (2003) illustrated in 231 newly diagnosed hypertensive patients (SBP ≥ 140 and DBP ≥ 90mmHg) that the -344*C/T* variant of *CYP11B2* is associated with the severity of systolic BP in hypertensive individuals of African ethnicity. Their study included only hypertensive patients of African ethnicity derived from the same gene pool (Nguni, Sotho and Venda chiefdoms) of South Africa to control for population stratification. Another study, in a total of 503 hypertensive subjects (262 males and 241 females) and age-gender and area matched control subjects, the alleles of *CYP11B2* -344*C/T*, Arg173Lys and IC polymorphisms constituted a specific haplotype designated number 4 (-344*T*, 173Lys and IC-conversion alleles), which was shown to be associated with female hypertension (Gu et al., 2004). This association remained after adjustment for covariates such as BMI, serum concentration of glucose, triglyceride (TG) and high-density lipoprotein (HDL) cholesterol, while haplotype number 3 consisting of (-344*T*, Arg173-WT and IC alleles) showed a protective effect in both Northern Han Chinese males and females (Gu et al., 2004).

CYP11B2 and heart size

The -344*C/T* polymorphism of *CYP11B2* has been found to be associated with LV cavity size and function in a study of 84 healthy young Finnish Caucasian adults who had no history of heart disease (Kupari et al., 1998). Similarly, examining this promoter region polymorphism in 120 male Caucasian hypertensive subjects, Delles et al., (2001) found an association between the -344*CC* genotype and LV end-diastolic diameter. Additionally, the *CYP11B2* -344*C/T* polymorphism, particularly the *TC* and *CC* genotypes, was found to be significantly associated with LV volume in 201 Japanese

DCM patients and 183 age-and sex-matched control subjects enrolled in a study by Takai et al., (2002).

The Mayosi et al., (2003) study which employed 995 Caucasians of 229 British families demonstrated that a strong genetic component at the *CYP11B2* locus determines variation in cardiac structure. They analysed six polymorphisms spanning *CYP11B2* and found that only the 5937*G/A* polymorphism was associated with cardiac wall thickness and that both the IC and 5937*G/A* polymorphisms were associated with LV cavity size. Their study included familial data, thereby increasing genotyping accuracy and reducing the effect of population stratification, while concomitantly adjusting for the influence of covariates of hypertrophy, namely, age, sex, SBP, height, weight and body size (Mayosi et al., 2003).

In a population-based sample of 562 hypertensive subjects (SBP $\geq$ 160 mmHg and DBP $\geq$ 95 mmHg) of the Monitoring of trends and determinants in CVD in Augsburg (MONICA) study, no significant association of the *CYP11B2* -344*C/T* polymorphism and LV size and function was detected, even after multivariate analysis to adjust for age, sex, BMI, SBP and antihypertensive drug treatment (Schunkert et al., 1999a). Additionally, the *CYP11B2* -344*C/T* polymorphism's allele status was found not to be a strong risk predictor for MI and did not influence LV remodeling after MI in 609 German patients from the MONICA survey (Hengstenberg et al., 2000).

CYP11B2 and HCM

The *CC* genotype of the *CYP11B2* -344*C/T* polymorphism showed a trend of association with LVH in a study of a single family of HCM patients (Ortlepp et al., 2002). The study by Ortlepp et al., (2002) only looked at a single family harboring the *MYBPC3* mutation in 48 adults (26 were clinically affected and 22 were unaffected) while 100 unrelated healthy patients from the same area as the family served as controls.

Mechanism of *CYP11B2* in LVH

Aldosterone is a major regulator of intravascular volumes and BP and is considered to influence cardiac structure and function (Isaji et al., 2005). Aldosterone synthase

synthesis occurs within the cardiac tissue that contains the molecular machinery needed for *CYP11B2* expression (Lombes et al., 1995; Silvestre et al., 1998). The localisation of expression makes *CYP11B2* an important determinant of LVH through pleiotropic effects on LVM. The cardiac growth effects observed could be mediated through both direct cellular and/or haemodynamic changes on the heart (Bonvalet et al., 1990; Lombes et al., 1995).

1.4.4.4. *AGTR1*

Most of the effects mediated by ANGII are through the human angiotensin II type-1 receptor (AGTR1), which belongs to a family of G-protein coupled receptors. The gene that encodes the AGTR1 receptor is mapped to chromosome 3q21-15 (Curnow et al., 1992). The *AGTR1* spans 47kb and contains five exons and four introns (figure 1.9). *AGTR1* is highly polymorphic with at least 50 different SNPs that have been described to date (Baudin et al., 2005). The AGTR1 receptor is expressed differently in various CVD, it is upregulated in cardiac hypertrophy (Wollert et al., 1997) and downregulated in the failing human ventricular myocardium (Asano et al., 1997; Haywood et al., 1997).

AGTR1 and Blood pressure

The *AGTR1 A/C*1166 polymorphism in the 3'UTR identified by Bonnardeaux et al., (1994) was shown to be associated with hypertension in a case-control association study involving 206 Caucasian hypertensive and 298 normotensive subjects (Bonnardeaux et al., 1994). Additionally, case-control studies in Caucasian hypertensive patients indicated a significant increase in the *C*-allele frequency compared to the normotensive group (*AA* or *AC*) (Bonnardeaux et al., 1994; Wang et al., 1997).

Furthermore, in a study that included 321 Japanese hypertensive subjects and 215 age and sex matched normotensive controls, an association was observed between the *AGTR1* 1166*C*-allele and LVMI in normotensive subjects without HCM, independent of age, sex and BP (Takami et al., 1998).

A case-control study of six promoter region variants of *AGTR1* (-1154*A/T*, -729*T/G*, -535*C/T,* -227*A/C,* -226*G/C* and -166*A/G*) in 149 Japanese hypertensive subjects demonstrated that only the *AGTR1* -535*C/T* polymorphism, particularly the -535 *T*-allele, associated with EHT (SBP ≥ 140 and DBP ≥ 90mmHg) (Takahashi et al., 2000). They also observed that a synergistic interaction between the *AGTR1* -535*C/T* (*T*-allele) and the *ACE1* I/D (the D-allele) polymorphisms determines risk for developing hypertension (see section 1.4.5.1).

However, Ono et al., (2003) reported a lack of association between the *AGTR1 A/C*1166 polymorphism and hypertension in a large cohort of 1492 Japanese hypertensive subjects. Inter-ethnic differences have been suggested to account for the differences in results observed in studies (Jiang et al., 2001; Ono et al., 2003). Furthermore, Zhu et al., (2003) observed an ethnic-related association between two SNPs in *AGTR1* and hypertension in patients of African descent. Moreover, six promoter region variants of *AGTR1* were investigated, although only the -810*A/T* polymorphism demonstrated a relationship with coronary heart disease (CHD) influenced by EHT (Jin et al., 2003).

On the other hand, analysis of the relationship between four RAAS gene polymorphisms (*ACE1* I/D, *AGTR1 A/C*1166, 573*C/T* and *AGT* -6*A/G*) and the response to BP in 206 Caucasian hypertensive patients indicated no association between any of the four RAAS gene polymorphisms and reduction of BP following treatment with an angiotensin II receptor blocker, telmisartan (Redon et al., 2005).

AGTR1 in cardiovascular structure and function

Some studies demonstrated no association between the *AGTR1 CC* genotype and LVH (Castellano et al., 1996; Hamon et al., 1997). Castellano et al., (1996) examined 212 randomly selected individuals from Northern Italy and assessed the relationship between the *AGTR1 A/C*1166 genotype and phenotypes of LVM while adjusting for the influence of confounding variables such as age, sex, height, weight, BP, smoking habits and biochemical parameters. No association was found even after adjusting for these covariates of hypertrophy. The lack of association can possibly be ascribed to a non-ideal

study design and insufficient sample size (Castellano et al., 1996). Similarly, Hamon et al., (1997) recruited 141 Caucasian patients with normal coronary arteries for their study, but did not find an association between the *AGTR1 A/C*1166 genotype and phenotypes of the LV.

<u>*AGTR1* and HCM</u>

In a study done by Osterop et al., (1998), the *AGTR1 A/C*1166 polymorphism, particularly the *C*-allele, was associated with a greater LVM in 104 genetically unrelated subjects with HCM. The Osterop et al., (1998) study determined LVMI and IVS thickness by echocardiographic measurement in a group of severely affected HCM subjects (IVS $\geq$ 15mm) and additionally, quantified the extent of hypertrophy by a point score method (Wigle score).

In contrast, Ishanov et al., (1998) found that the *AGTR1 A/C*1166 polymorphism does not contribute to cardiac hypertrophy development in 53 hypertensive patients with LVH and 96 HCM patients. However, subjects with a family history of HCM who did not manifest the disease had a four-fold increase in *AGTR1 C* and *ACE1* D-allele carrier status compared to subjects without a family history of HCM.

In a subsequent study, the *CC* genotype of the *AGTR1 A/C*1166 polymorphism was shown to have an association with LVH in a single family of HCM patients (Ortlepp et al., 2002). The study included family members with no HCM-causitive mutations to serve as "*built-in*" controls, in order to control for the effect of population stratification (Ortlepp et al., 2002).

<u>Functionality of the *A/C*1166 polymorphism</u>

Various mechanisms have been proposed to explain the *AGTR1 A/C*1166 polymorphism's role in CVD. The first evidence for a mechanistic role comes from several studies that demonstrate an epistatic interaction between the *AGTR1 A/C*1166 polymorphism and *ACE* I/D polymorphism (Tiret et al., 1994; Alvarez et al., 1998). The second mechanism is an increased vasoreactivity of ANGII in the *C*-allele carriers of the

*AGTR1 A/C*1166 polymorphism (van Geel et al., 2000). The evidence for this is a higher BP level in *C*-allele carriers compared to non *C*-allele carriers.
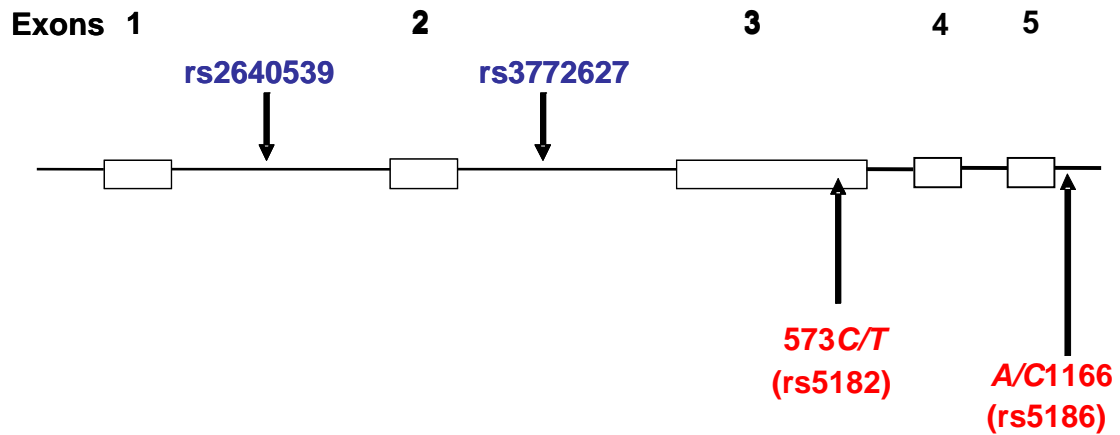
Figure 1.9: Genomic organisation of the human angiotensin II type I receptor gene (*AGTR1*) showing variants investigated in association studies. The locations of various polymorphisms being investigated for possible association with LVH (are indicated by vertical black arrows). The markers highlighted in red indicate variants investigated in previous association studies and the blue highlighted markers indicate variants that have not yet been investigated (Table 1.2 section 1.3.2). Not indicated are the markers used in previous hypertension association studies, these include promoter region variants of *AGTR1* (-1154 *A/T*, -729 *T/G*, -535 *C/T*, -227 *A/C*, -226 *G/C* and -166 *A/G*)

1.4.4.5. *CMA*

The human heart chymase gene (*CMA*) encodes an ANGII-forming serine proteinase expressed in the human LV and blood vessels (Urata et al., 1990). The genomic organisation of *CMA* consists of five exons and four introns that span approximately 3kb of chromosome 14q11.2 (figure 1.10) (Urata 1991). CMA has a high specificity for ANGI, which is cleaved to yield ANGII and Histidine-Leucine (His-Leu) (Urata et al., 1990). In *CMA*, only two polymorphisms (1625*G/A* and -1903*G/A*) have been investigated for association with HCM to date (Pfeufer et al., 1996; He et al., 2005).
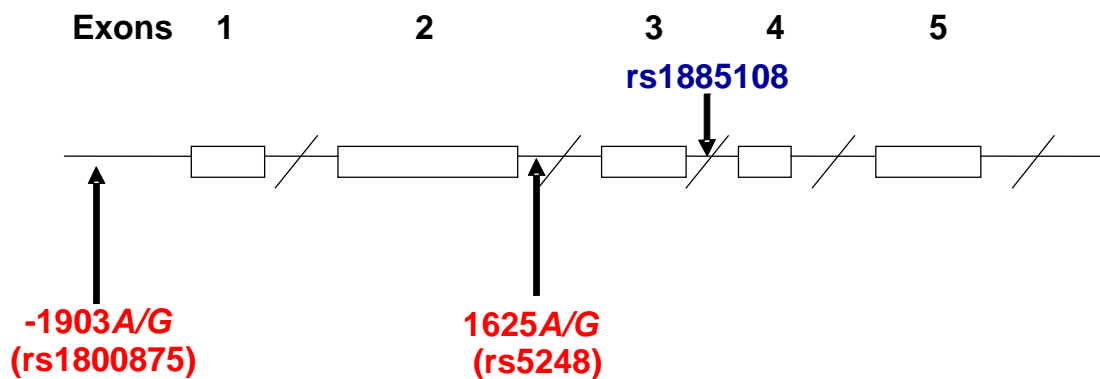
Figure 1.10: Genomic organisation of the human cardiac chymase (*CMA*) gene showing variants investigated in association studies. The locations of various polymorphisms being investigated for possible association with LVH (indicated by vertical black arrows). The markers highlighted in red indicate variants investigated in previous association studies and the blue highlighted marker indicates a variant that has not yet been investigated (Table 1.2 section 1.3.2).

CMA in LVH

A recent drug trial demonstrated that treatment of 157 unrelated Chinese hypertensive patients (85 males and 72 females) that were matched for age with the antihypertensive drug benazepril resulted in a reduction of LVH in *ACE1 DD*-genotyped patients compared to other *ACE1* genotypes (*II* and *ID*) (He et al., 2005).

However, no association was found between the *CMA* -1903G/A variant in the 5' region and regression of LVH when treated with the same drug (He et al., 2005). Additionally, no evidence was found to support a possible association between *ACE1* and *CMA* in regression of LVH (He et al., 2005).

CMA and HCM

A previous family-based association study failed to illustrate an association between *CMA* -1903*G/A* variant and LVH in HCM patients (Ortlepp et al., 2002). However, other case-control association studies were successful in demonstrating a possible interaction between the *CMA AA* and *AG* genotypes of 1625*G/A* polymorphism and *ACE1 DD* genotype in 50 unrelated Caucasian HCM patients and 50 age and gender matched controls (Pfeufer et al., 1996) and in 154 patients with type 2 diabetes (Gumprecht et al.,

2002). He et al., (2005) suggested that their study differed from the Pfeufer et al., (1996) and Gumprecht et al., (2002) studies because of the different population groups used, genetic heterogeneity, different diagnosis criteria and the level of hypertension and LVH.

1.4.4.6. *ACE2*

Recently, a previously unrecognised enzyme, ACE2, has been identified that is involved in the reverse reaction of ANGII formation. ACE2 functions as a counter-regulatory system to ACE1 by degrading ANGII to ANG 1-7 (Lemos et al., 2002; Ren et al., 2002). ACE2 is considered to be involved in regulating cardiac structure and function (Lieb et al., 2006). The ACE2 enzyme is a homologue of ACE1 and is highly expressed in the heart, kidneys and testes (Donoghue et al., 2000; Tipnis et al., 2000). The ACE2 enzyme is encoded by the *ACE2* gene mapped to the X-chromosome. The human form of *ACE2* comprises 18 exons and 17 introns (figure 1.11) and encodes 805 amino-acid residues (Danilczyk et al., 2003). To date, no studies of *ACE2* involvement in HCM have been published.
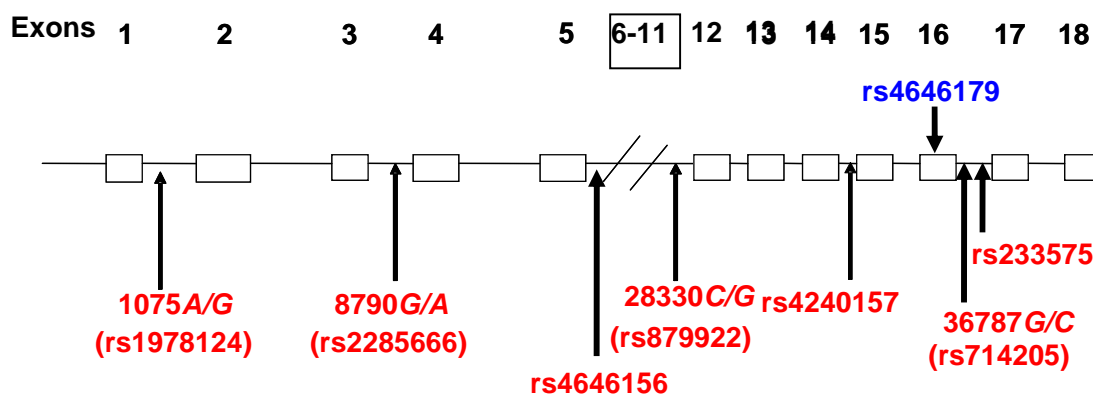
Figure 1.11: Genomic organisation of the human angiotensin-converting enzyme 2 (*ACE2*) gene showing variants investigated in association studies. The locations of various markers (SNPs) being investigated for possible association with LVH (indicated by vertical black arrows). The markers highlighted in red indicate variants investigated in previous association studies and the blue highlighted marker indicates a variant that has not yet been investigated (Table 1.2 section 1.3.2).

ACE2 and blood pressure

It is suggested that *ACE2* is a good candidate for studying hypertension in rats because both *ace2* and hypertension has been genetically mapped to QTL on the X-chromosome in three different rat models (Crackower et al., 2002). Additionally, it was demonstrated that *ACE2* disruption resulted in reduced cardiac contractility and increased ANGII circulating levels in rats (Crackower et al., 2002). However, investigations of human *ACE2*'s role in hypertension showed no association between any of the four intronic SNP's spread across the gene (1075*A/G*, 8790*G/A*, 28330*C/G* and 36787*G/C*) with EHT in an Australian Caucasian population (Benjafield et al., 2004).

ACE2 and LVH

A MONICA echocardiographic sub-study of 1294 German subjects stratified according to gender and age found that polymorphisms of *ACE2* might be associated with LVH. Lieb and colleagues (2006) found a positive correlation between the minor alleles of four variants (*T*-allele of rs4646156, *G*-allele of 28330*C/G*, *G*-allele of rs4240157 and the *C*-allele of rs233575) in men, resulting in a shared common haplotype TGGC for LVM, septal wall thickness (SWT) and LVH. However, women showed no association between any of these minor alleles or any haplotype consisting of these four SNP's investigated and LVH (Lieb et al., 2006). The Lieb et al., (2006) study found gender-specific associations in the general population. The study also adjusted for covariates of hypertrophy, namely, age, body mass index (BMI), systolic BP and antihypertensive medication. Additionally, pair-wise analysis suggested that the four *ACE2* SNP's are in LD (Lieb et al., 2006).

Interestingly, a correlation has been observed between *ACE2* mRNA upregulation in DCM and ischaemic cardiomyopathy (ICM) patients and in both human and rat hearts after MI (Goulter et al., 2004; Burrell et al., 2005).

Summary of the Role of RAAS gene variants in LVH and HCM in general

In summary, some genetic studies provide evidence for the role of genetic variants within the RAAS to be associated with electrocardiographic or echocardiographic determined LVH (Iwai et al., 1994; Schunkert et al., 1994; Delles et al., 2001; Schmieder et al.,

2001). However, contradictory results exist on the role of RAAS gene variants in HCM (Marian et al., 1993; Lechin et al., 1995; Yoneya et al., 1995; Brugada et al., 1997; Ishanov et al., 1997; Tesson et al., 1997; Osterop et al., 1998; Ortlepp et al., 2002), and thus the present study will attempt to elucidate whether there is a role for RAAS gene variants as modifiers of the hypertrophic phenotype in South African families in which HCM-causing founder-mutations segregate (Moolman-Smook et al., 1999).

1.5. Bioinformatics

For the current study, literature on case-control studies performed in various ethnic groups (sections 1.4.5.1 to 1.4.5.6) that demonstrated or failed to show association of RAAS gene variants with LVH and hypertension (mentioned in section 1.3.2 table 1.2) were electronically accessed from the Pubmed (http://www.ncbi.nlm.nih.gov/PubMed) database. Furthermore, the Pubmed database is linked to databases containing all experimentally-derived and computer-predicted biological information on the genes and clinical conditions being investigated. A brief introduction to the "database mining" approach used in the present study is provided below.

The sequencing of the human genome has generated genomic data for use in developing new technologies within biological fields (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001; Baxevanis et al., 2003). This has facilitated the combination of both laboratory techniques and computer programs to form one discipline termed bioinformatics (Baxevanis et al., 2003). This newly developed resource is computationally integrated within various databases to a specific biological segment either DNA, RNA or protein format accessible to users. A total of 968 databases are listed within the January 2007 Nucleic Acid Research (NAR) issue (Galperin et al., 2007) and are accessible via the NAR website at http://www.nar.oupjournals.org.

For the present study, three primary human genome sequence databases, The National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org) and the University of California Santa Cruz (UCSC) database (http://www.genome.ucsc.edu) were used to access published information on the

genes involved in hypertension and HCM. Compiled within these three databases are detailed annotations of the human genome, as well as additional features such as repetitive DNA, CpG islands, expressed-sequence tags (ESTs) and, most importantly for this research, SNPs. The databases include programs that enable certain computational manipulations like data input, retrieval, sequence comparisons and functional prediction.

During 2002, a global initiative (called the International HapMap project) was undertaken to develop a new bioinformatic tool to aid in assessing biological information in a more user friendly way, thereby reducing costs of genotype-phenotype association studies on various disease. The following section will briefly discuss the HapMap project and its goals and applications.

In 2002, the International HapMap project was established to determine common patterns of DNA sequence variation in the human genome and to make this information freely available in the public domain (The International HapMap Consortium, 2003). This international consortium developed haplotype maps of the common DNA patterns across the genome by characterising sequence variants, their frequency and correlations between them, in DNA samples from population groups from African, Asian and European descent. In 2005 the HapMap project deposited data on new "tag" SNPs, allele and genotype frequencies released into the public domain onto the internet at the HapMap data coordination Center (DCC) (http://www.hapmap.org). The availability of this tagged data could provide a new tool for investigating genetic factors that contribute to disease susceptibility, protection against illnesses and to individual drug response (The International HapMap Consortium, 2003).

1.6. The present study: Aim and design

The objectives of this study were to elucidate the possible role of hypertrophy modifier genes by, firstly, selecting genes involved in the RAAS, secondly, looking for variations in these genes, thirdly, prioritising variations for investigation and, fourthly, investigating a role for the selected candidate modifier genes in the clinical phenotype by family-based association studies.

The present study focused on previously postulated hypertrophy-modifier candidate genes, such as those involved in the peptide hormone pathways, specifically the RAAS. The rationale was that variation in, or modulation of, components of these pathways have been shown to have some effect on the extent of hypertrophy that develops in the presence of an HCM-causing mutation, as shown in animal models and HCM patients (Semsarian et al., 2001; Marian et al., 2002). However, previous association studies were inconsistent in design, covariates adjusted for, parameters tested, and in results generated, as discussed earlier, and thus the interpretation of the results remained inconclusive and warranted further investigation.

Using a candidate gene approach and prioritising SNPs that have been used in previous studies allows a more robust opportunity to test their role in the development of hypertrophy and hypertension. Moreover, as previous case-control studies were less than optimally designed for detecting genetic modifiers of hypertrophy, their analysis often consisted of investigating only single SNPs within genes in population groups that were frequently not matched for genetic background or causal mutation (reviewed by Marian et al., 2002 and sections 1.4.5.1 to 1.4.5.6).

The current association study improves on previous studies by investigating a range of SNPs across each gene using a large group of 353 subjects that are of Caucasian and Mixed-Ancestry descent, in which the causal-mutation status was known. Specifically, this study focused on 22 South African HCM families, 12 of which harbour the Ala797Thr founder mutation in *MYH7*, three harbour the Arg403Trp founder mutation, also within *MYH7*, and seven harbour the Arg92Trp founder mutation in *TNNT2*.

Study design:

The Pubmed (http://www.ncbi.nlm.nih.gov/PubMed) publication database was searched for genes involved in the RAAS. The NCBI database was used to retrieve genomic nucleotide sequences for six selected RAAS genes (*ACE1*, *AGT*, *AGTR1*, *CYP11B2*, *CMA* and *ACE2*), which included annotations of previously reported SNPs' positions. At least three intragenic SNPs spread throughout each gene were chosen to be investigated

except in *CMA* where only two SNPs were chosen. SNPs that had been previously investigated in other studies and had a high heterozygosity value (>40%) were given selection priority. Primers were designed to flank regions containing selected SNPs and were used for PCR amplification of 353 individuals of the 22 South African HCM families described above. The PCR products were genotyped using Snapshot primer extension analysis and allele specific restriction enzyme analysis (ASREA). Sequence variants identified by SNaPshot were validated by automated sequence analysis in a representative sample, ~10% of the SA HCM cohort. Thereafter, association was investigated between identified genotypes and the disease phenotype by performing quantitative transmission disequilibrium test (QTDT) analysis (refer to figure 1.12 for a study design summary). However, the X-linked gene *ACE2* was statistically analysed with mixed-effects models using the program R (R Development Core Team., 2007), as at the time of writing, no X-linked family-based association analysis program existed that was capable of handling multi-generation families of various sizes, and also allowed for covariate adjustment.

Bioinformatics tools and published material
(for association status of previously postulated candidate modifier genes)

Six genes involved in the RAAS selected

Panel of 22 HCM families (carriers 195 and non-carriers 158)
(Carriers harboured one of the 3 distinct HCM-causative mutations in *MYH7* or *TNNT2*)

Primers designed for prioritised SNPs

Genotyping via
PCR-based methods

Snapshot primer extension analysis ⟶ Automated Sequencing   ASREA

QTDT analysis   Mixed-effects models

Figure 1.12: Summary of the design of the present study, explained in detail in the text. Abbreviations: SNP- single nucleotide polymorphism, PCR- polymerase chain reaction, ASREA- allele specific restriction enzyme analysis, QTDT- quantitative trait disequilibrium test.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

2. Materials and Methods:

2.1. Study subjects

The University of Stellenbosch (US) Ethics Committee reviewed and granted approval for the present study (N04/03/062). Subjects entered into the study gave written informed consent and blood samples were collected from each subject for molecular genetic testing. During routine mutation screening for HCM-causing mutations, a panel of HCM probands was screened for disease-causing mutations in 11 sarcomeric genes that account for 95% of all HCM. In the process, 22 probands carrying one of three mutations that occur as founder mutations in South Africa, viz. R92W in *TNNT2*, R403W in *MYH7* and A797T in *MYH7* (previously described within a South African population by Moolman-Smook et al., 1999), were identified. Pedigree tracing was performed for these individuals and family members asked to participate in this modifier gene study. These families were of South African Caucasian and of Mixed Ancestry descent (table 2.1). Thus, a panel of 353 individuals that included genetically and clinically affected and unaffected family members was identified and these individuals were screened for the presence of all three founder mutations.

The subjects used in the present study are individuals 18 years and older and were comprehensively clinically characterised by 2D-echo performed by a single cardiologist (Dr Miriam Revera from Pavia University, Italy) who was unaware of the mutation status of each subject. Analysis included echocardiographic recordings in M-mode, 2D and Doppler blood-flow imaging using a 2,5Hz transducer in standard parasternal long-axis and short-axis, apical four-and two-chamber views using a GE Healthcare Vivid7 cardiovascular ultrasound system. To determine the maximal wall thickness (MWT), the heart muscle was divided into three levels, namely, mitral valve, papillary muscle and supra-apex level (figure 2.1). 2D-echo measurements were done in 6 segments of the mitral valve and papillary muscle levels, and in four segments of the smaller supra-apex level, thus in total 16 segments (figure 2.2). The six segments measured in the mitral valve and papillary muscle levels consisted of the anterior interventricular septum (aIVS), posterior interventricular septum (pIVS), anterior wall (AW), lateral wall (LW), inferior

wall (IW) and posterior wall (PW). Evaluation of the supra-apex level consisted of segments IVS, AW, LW and PW as per four chamber view. All these variables were measured according to the recommendation of the American Society of Echocardiography (http://www.asecho.org/guidelines.php) while the atrium volumes were measured using the biplane area-length method (Schiller et al., 1989). Covariates of cardiac structure were noted for each participant; these included systolic BP (SBP), diastolic BP (DBP), age, sex, body surface area (BSA) and heart rate (HR).

Table 2.1: South African HCM-affected families of Caucasian and Mixed Ancestry descent that were analysed in the present study.

	Pedigree	Ethnic group	n	Gene	Mutation	Location
1	131	Caucasian	25	MYH7	A797T	exon 21
2	101	Caucasian	22	MYH7	A797T	exon 21
3	104	Mixed Ancestry	14	MYH7	A797T	exon 21
4	124	Caucasian	4	MYH7	A797T	exon 21
5	123	Mixed Ancestry	16	MYH7	A797T	exon 21
6	138	Caucasian	32	MYH7	A797T	exon 21
7	145	Mixed Ancestry	4	MYH7	A797T	exon 21
8	147	Mixed Ancestry	10	MYH7	A797T	exon 21
9	158	Caucasian	5	MYH7	A797T	exon 21
10	159	Mixed Ancestry	11	MYH7	A797T	exon 21
11	163	Caucasian	9	MYH7	A797T	exon 21
12	172	Caucasian	8	MYH7	A797T	exon 21
13	106	Mixed Ancestry	69	MYH7	R403W	exon 13
14	134	Mixed Ancestry	9	MYH7	R403W	exon 13
15	157	Mixed Ancestry	4	MYH7	R403W	exon 13
16	100	Mixed Ancestry	43	TNNT2	R92W	exon 9
17	103	Mixed Ancestry	5	TNNT2	R92W	exon 9
18	109	Mixed Ancestry	8	TNNT2	R92W	exon 9
19	139	Mixed Ancestry	41	TNNT2	R92W	exon 9
20	137	Mixed Ancestry	7	TNNT2	R92W	exon 9
21	149	Mixed Ancestry	10	TNNT2	R92W	exon 9
22	173	Mixed Ancestry	2	TNNT2	R92W	exon 9

n = number of individuals screened for SNPs in the present study includes mutation and non-mutation carriers, MYH7 -myosin heavy chain gene 7; TNNT2- troponin T gene 2.

Additional hypertrophy parameters, viz. LVM, the Wigle score (Wigle et al., 1985), Spirito-Maron score (Spirito and Maron et al., 1990) and two dimensional left ventricular

hypertrophy score (2-DLVH) (Forissier et al., 2005), were calculated to better describe the extent of hypertrophy. The Wigle score is a semi-quantitative point score method proposed by Wigle et al., (1985) which uses a 10 point scale (refer to section 1.2). The Spirito-Maron score is the sum of greatest wall thickness observed at the mitral valve level in any one of the segments and the greatest wall thickness in any one of the segments at the papillary level, as per definition (Spirito and Maron et al., 1990) (refer to section 1.2), while the 2-DLVH-score is the sum of measurements of MWT in each of the segments at both the mitral valve and papillary level as per definition (Forissier et al., 2005) (refer to section 1.2). Additionally, a new cumulative wall thickness score (CWT) was determined by adding the wall thickness in all three levels of the heart, thus in the 16 segments in total.

The clinical information for each subject was archived in a Microsoft excel file (in M-link format), independently from the primary pathogenic mutations and RAAS genotype status.
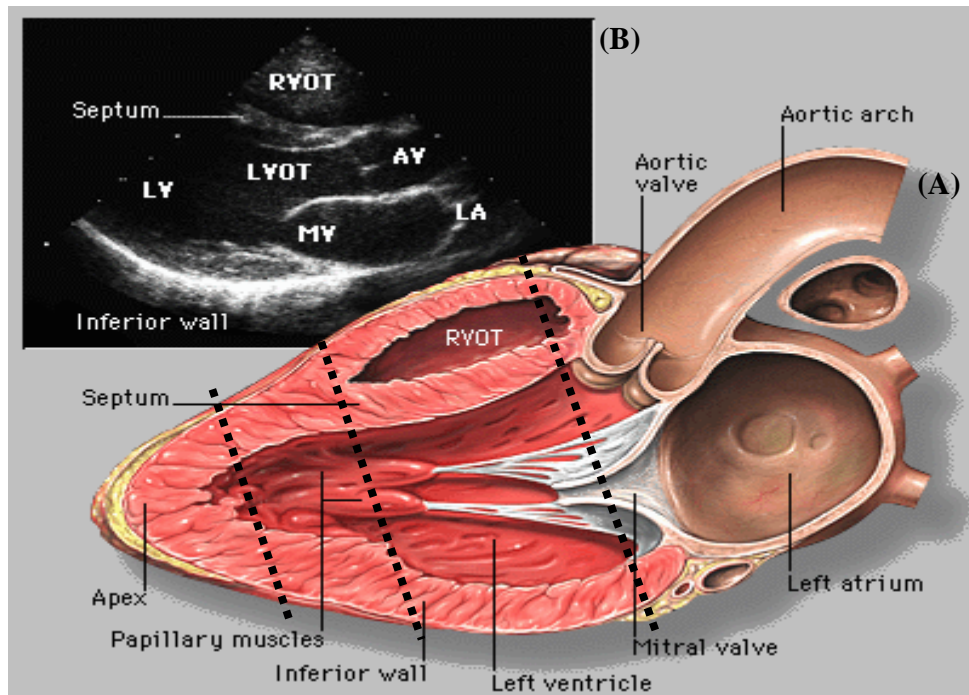
Fig 2.1: Graphical representative example of the heart being divided into 3 levels. A) Long-axis view of left ventricle, taken at level of mitral valve, papillary muscles as well as just above apex (levels indicated by dotted lines). B) An example of a 2D echo ultrasound of the left ventricle. Abbreviations: AV- aortic valve, LA-left atrium, LV-left ventricle, LVOT-left ventricular outflow tract, MV- mitral valve, RVOT-right ventricular outflow tract. Taken from (http://www.med.yale.edu/.../aortic_regurgitation.html) with minor modifications by JC Moolman-Smook.
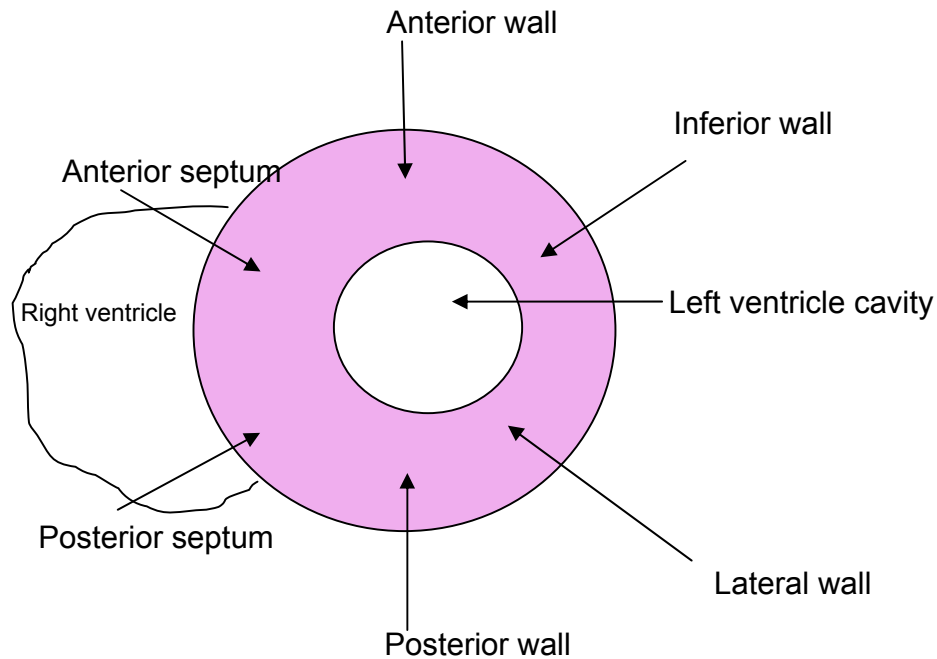
Fig 2.2: Diagrammatic representation of the cardiac wall thickness measurements.
Short-axis view of left ventricle indicating the six segments into which the cardiac walls of the mitral valve and papillary muscles were divided. Not indicated is the apex wall, which was divided into four segments.

2.2. Blood collection

Blood from each individual was collected in 2x 5ml ethylene-diamine-tetra-acetic acid (EDTA) tubes (Vacutainer, RSA) for DNA extraction and in 1x 10ml heparin tube (Vacutainer, RSA) to establish a permanent lymphoblastoid cell lines using the method described by Neitzel (1986). Blood that was drawn from patients at other centres in South Africa was couriered to the research laboratory within 24 hours of sampling.

2.3. DNA extraction

2.3.1 Extracting nuclei from whole blood

DNA was extracted from nucleated blood cells using the method previously described by Corfield et al., (1993) with minor modifications. The DNA extractions were performed by Mrs Ina le Roux. A list of the solutions used for DNA extractions is provided in Appendix I.

2.4. Bioinformatic selection of candidate, RAAS gene sequences

From the literature, six genes known to be involved in the RAAS were selected to be investigated (table 2.2). Publication databases (Pubmed/Medline) (http://www.pubmed.gov) were searched for information on the reported association status of these genes in hypertrophy and hypertension. The National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) was used to retrieve genomic nucleotide sequences which included annotations of previously reported SNPs' positions for the chosen genes. The SNPs selected were those that had been previously investigated in other studies as well as novel SNPs that have a high heterozygosity value ($\geq$0.4), validated by calculated frequency of alleles within African American and European Caucasian ethnic groups (URL: dbSNP). Additionally, polymorphisms that occur in functionally important regions (promoter or exonic) of the gene also received selection priority, as they have the potential to result in protein sequence changes or changes that may affect gene expression levels. Further bioinformatics applications that involved designing primers for both polymerase chain reaction (PCR) and SNaPshot primer extension technique will be discussed in further detail later.

2.5. Polymerase chain reaction (PCR)

The PCR is a technique that enables rapid *in vitro* amplification of a specific DNA fragment which is flanked by unique regions to which oligonucleotide primers are designed (Saiki et al., 1985). Primers bind to these regions and lead to exponential amplification of the target region (figure 2.3). The PCR technique was used to amplify the polymorphic regions of the six chosen RAAS genes.

Table 2.2 Candidate genes: *ACE1, CYP11B2, AGTR1, AGT, CMA* and *ACE2* chosen for investigation.

Gene	Localisation	Size (kb)	Protein function	References (Association)
ACE1	17q23	44.770	converts ANGI to ANGII, a potent vasoconstrictor	1 (LVH)
CYP11B2	8q22	7.284	catalyses conversion of 11-deoxycorticosterone to aldosterone	2 (LVH)
AGTR1	3q21	45.123	receptor for angiotensin II	3 (LVH)
AGT	1q42	11.582	cleaved by renin to yield angiotensin I, leads to angiotensin II formation	4 (LVH)
CMA	14q11	2.759	breaks ANG I down to ANG II	5 (LVH)
ACE2	Xp22	39.981	hydrolyses ANG II → ANG (1-7)	6 (LVH)

Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *ACE2*-angiotensin converting enzyme 2; *AGT*-angiotensinogen; *AGTR1*-angiotensin 2 type-1 receptor; ANGI-angiotensin 1, ANGII-angiotensin 2, *CMA*-cardiac chymase; *CYP11B2*-aldosterone synthase; HCM-hypertrophic cardiomyopathy; kb-kilo-bases; LVH-left ventricular hypertrophy. References for positive and negative association of genes listed with LVH: 1) Lechin et al., 1995 and Tesson et al., 1997; 2) Patel et al., 2000; 3) Osterop et al., 1998; 4) Brugada et al., 1997 and Yamada et al., 1997; 5) Pfeufer et al., 1996; 6) Lieb et al., 2006.

2.5.1. Oligonucleotide primers

Oligonucleotide primers were designed from published gene sequences with the aid of the primer design program DNAMAN version 4.1 (Lynnon Biosoft, USA) (table 2.3). The fragments to be amplified contained more than one SNP, thus primers were selected from DNA sequence 120 base pairs (bp) 5' or 3' to the relevant outermost SNPs. These sequences were copied and pasted into DNAMAN for subsequent primer designing. Primers were designed to have melting temperatures between 50 ^{o}C and 65 ^{o}C and the guanine-cytosine (GC) content of each primer ranged from 40 to 60%. Primers were discarded if possible potential hairpin formations with greater than three intrastrand bonds were detected, meaning a primer would be able to fold upon itself to form a hairpin structure. For each primer the length was ≥ 18bp (table 2.3). In addition, primer sets that contained a GC sequence (clamp) at their 3' ends were always chosen, because of their ability to form three hydrogen bonds, (compared to two by nucleotides AT) with their complimentary base pair. The PCR-amplification primers were custom synthesised at Inqaba Biotec (Pretoria, RSA) or the DNA laboratory, University of Cape Town.

Thereafter, PCR reactions were performed to amplify relevant fragments for each gene as template for SNaPshot analysis (see section 2.7.2.1) or allele-specific restriction enzyme analysis (ASREA) (see section 2.7.3).

2.5.2. SNaPshot interrogation primer design

The oligonucleotide primers designed for SNaPshot primer extension reactions differed between 4-6 nucleotides in length. This was a necessity in order to avoid overlap between the final SNaPshot products, if two or more primers were to be combined in a single SNaPshot reaction termed multiplexing. The primers for the present study were designed only in the forward direction and were designed to bind upstream of the SNP and terminate directly 5' of the SNP site (Makridakis and Reichardt, 2001) (figure 2.4). (table 2.4). SNaPshot interrogation primers were custom synthesised at the DNA Laboratory, Department of Biochemistry, University of Cape Town (UCT), RSA. All primers underwent High Pressure Liquid Chromatography (HPLC) purification (Makridakis and Reichardt, 2001), even though the primers were shorter than 30 nucleotides, in order to prevent failure sequences from possibly extending at incorrect positions.
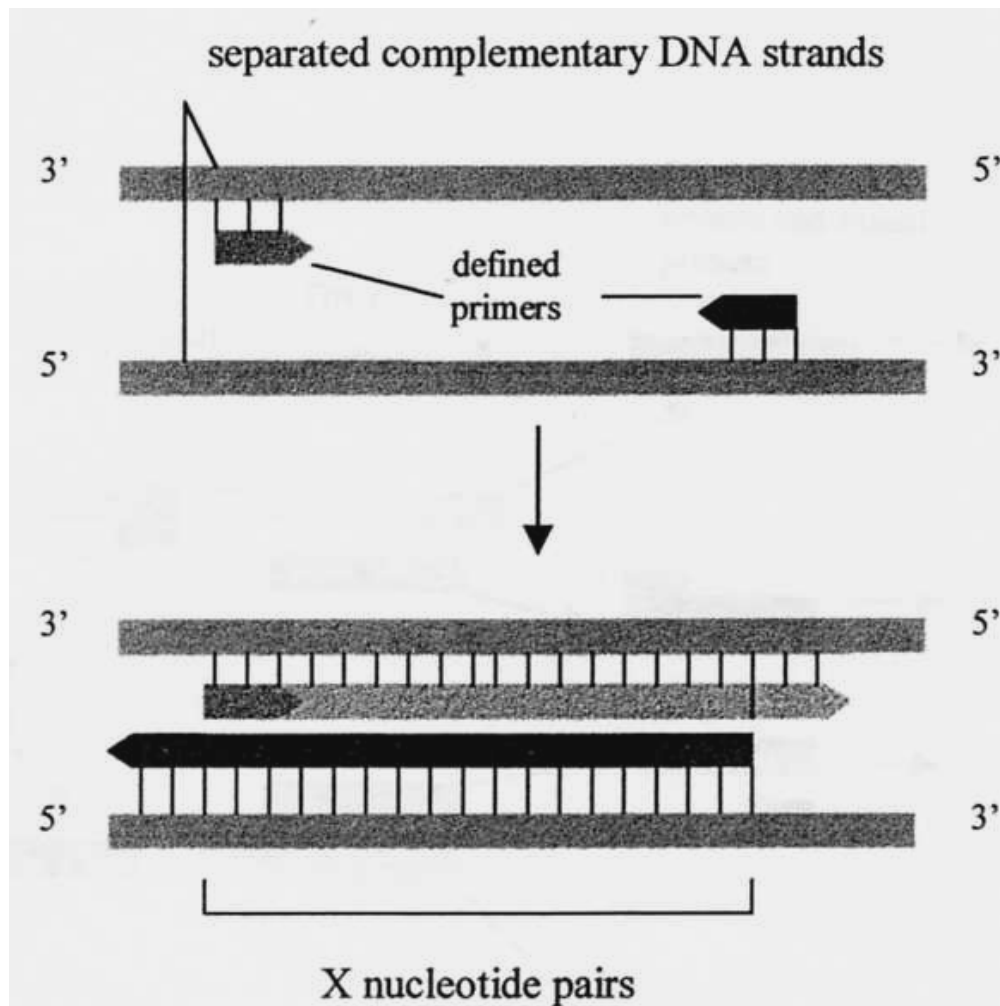
Fig 2.3: Amplification of specific nucleotide sequences using PCR. The double stranded DNA is separated into two strands. The two oligonucleotide primers that are complimentary to each anneal to each separated strand, a DNA polymerase finds each of the primer's binding site and starts the synthesis of the new double stranded DNA molecules. This cycle is repeated for 30 cycles and produces PCR product that doubles in each cycle.
Reproduced and adapted from Molecular Biology of the Cell, 3rd edition.

2.5.3. PCR-amplification conditions

To prepare the amplification reaction mixture, 75µM of each dATP, dCTP, dGTP and dTTP (Promega Corp, Madison Wisconsin USA), 5 microlitres (µl) of a 10X Taq DNA polymerase buffer (Bioline UK Ltd, London, UK), 150ng of each oligonucleotide primer (table 2.3), 0.5 unit (U) Taq DNA polymerase (Bioline UK Ltd, London, UK), 1.5mM $MgCl_2$, and water to a final volume of 50µl were added to individual, labelled 0.5ml

Eppendorf centrifuge tubes. Finally, 200 nanograms (ng) of genomic DNA as template was aliquoted into each tube. For fragments 1 and 3 of *CMA*, fragment 3 of *AGT* and fragment 2 of *ACE1*, 5% formamide (Sigma, RSA) was added to reduce non-specific amplification. For fragments 1 and 2 of *AGT*, fragment 1 of *ACE1*, fragments 1, 2, 3 and 4 of *CYP11B2* and fragments 1, 2, 3, and 4 of *ACE2* no additives were used, while for fragment 3 of *ACE1* and 5% dimethylsulfoxide (DMSO) (Sigma, RSA) was added to the reaction mixtures to reduce non-specific amplification.

Amplification was performed in a Perkin-Elmer thermal cycler (Applied Biosystems Inc, Foster City CA, USA). The cycling parameters and the PCR conditions for each set of oligonucleotide primers are shown in table 2.5. After cycling had been completed, amplification products were analysed on either a 1% or a 2% agarose gel (Hispanger, Spain), depending on the sizes of fragments generated.

Table 2.3: Primer sequences used to amplify polymorphic sites in candidate genes.

		Outer primers					
gene/ protein	FRAGMENTS	forward primer 5'-3'	length bp	reverse primer 5'-3'	length bp	PCR product size, bp
ACE1	1	ctcaccaacatcctggcttcc	21	gcctgcttcttcatgcatcg	20	1274
	2	gactctgtaagccactgctgg	21	ccaacaccacattacctgcc	20	450/737
	3	cagccttgggtcttaacc	18	tggcagtgttagaatggc	18	1198
CYP11B2	1	tcgagattcctcacatgg	18	cttatcgtgagatgagaggg	20	177
	2	gaacccagatgtgctgtcg	19	catagagttggccttgatggc	21	907
	3	cgtttcccttgctgatgacg	20	agattctgtctgccaccaccc	21	244
	4	tggtgagctcagacttggtgc	21	gagttccatttgtgcaggagc	21	1011
	5	atgtgtgcctgcgaatgg	18	agtcttctcctggcctgtgg	20	156
AGTR1	1	tcctagacacgggacatcg	19	tggagggaaccttatggg	18	992
	2	aatacagtgcagacctgg	18	attgctgaggaagattgc	18	1120
	3	ttgtagccaaagtcacctgc	20	aatgtgcagtaccaggtgc	19	1141
AGT	1	catctgtccttctggccagc	20	ccgggctgaatgctaaagg	19	1030
	2	ttccatggagctttgaatcc	20	ttgccttaccttggaagtgg	20	1328
	3	caccttcaatgcagccaacc	20	tccttgcagcaccagttgg	19	332
CMA	1	ggaaatgtgagcagatagtgcagtc	25	aatccggagctggagaactcttgtc	25	185
	2	tccttcaggtcctcattgcc	20	agtctctgaagtggctgcagg	21	333
ACE2	1	tcttcctggctccttctcagc	21	accacaatggcagagaaaggg	21	1044
	2	gtttgtaacccagataatcc	20	gttgaaacacacatatctgc	20	130
	3	ttgtgttaagatcttgtccc	20	aataaactgagctccagc	18	188
	4	tacagggaggaggatgtgcg	20	tgatggcaatacctgtccacg	21	680

Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *ACE2*-angiotensin converting enzyme 2; *AGT*-angiotensinogen; *AGTR1*-angiotensin2 type-1 receptor; bp-base pairs; *CMA*-cardiac chymase; *CYP11B2*-aldosterone synthase. For *ACE1* fragment 2 the two fragment sizes correspond to deletion allele (450bp) if present or insertion allele (737bp) if present.

Table 2.4: SNaPshot primer sequences used in primer extension analysis to detect *ACE1, AGT* and *CMA gene* variants.

			SNaPshot primers			
gene	region/ site	SNP	Sequence (5'- 3')	primer sets	length bp	location
ACE1	rs4298	c/t	tgggagggctggcacaa	1	17	exon
	rs4303	g/t	acctccatgccttcgtccgccgc		23	exon
AGT	rs5051	c/t	agaacaacggcagcttcttcccc	1	23	promoter
	rs4762	c/t	ccagctgctgctgtcca	2	17	promoter
	rs699	c/t	gacaggatggaagactggctgctccctga		29	promoter
	rs11122575	t/c	tcttctatgtccctcacatagtattgcaaatgaca	3	35	intronic
	rs1926723	a/g	tcttctatgtccctcacatagtattgcaaatgaca		23	intronic
CMA	rs1800875	a/g	cctcagccaggcaggtg	1	17	promoter
	rs1885108	g/a	actgtccagagcccatttcgaggactgac	2	29	intronic

Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *AGT*-angiotensinogen; bp-base pairs; *CMA*-cardiac chymase.

Table 2.5: PCR cycling conditions used in amplification of polymorphic sites in *ACE1, CYP11B2, AGTR1, AGT, CMA* and *ACE2* candidate genes.

gene/ protein	region or site	Fragments	TD °C	Time sec	TA °C	Time sec	TE °C	Time min
	rs4298/ rs4303	1	94	30	63 °C	40	72	1
ACE1 ~	rs4342/ rs4343	2	94	30	57°C	40	72	1
	rs4364/ rs4365	3	94	30	62°C	40	72	1
	rs1799998	1	94	30	49°C	30	72	1
CYP11B2 *	rs4539	2	94	30	58°C	30	72	1
	rs5314/ rs3097	4	94	30	60°C	30	72	1
	rs2640539	1	94	30	54°C	30	72	1
AGTR1	rs3772627	2	94	30	58°C	30	72	1
	rs5182/ rs5186	3	94	30	61°C	30	72	1
	rs5051	1	94	30	55°C	30	72	1
AGT	rs699/ rs4762	2	94	30	53°C	30	72	1
	rs11122575/ rs1926723	3	94	30	55°C	30	72	1
CMA	rs1800875	1	94	30	57°C	40	72	1
	rs1885108	2	94	30	57°C	40	72	1
	rs1978124	1	94	30	60°C	30	72	1
ACE2	rs2285666	2	94	30	43°C	30	72	1
	rs879922	3	94	30	48°C	30	72	1
	rs4646179	4	94	30	59°C	30	72	1

Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *ACE2*-angiotensin converting enzyme 2; *AGT*-angiotensinogen; *AGTR1*-angiotensin 2 type-1 receptor; *CMA*-cardiac chymase; *CYP11B2*-aldosterone synthase; °C-degrees Celcius; min-minute; sec-seconds; TA-annealing temperature; TD-denaturing temperature; TE-extension temperature. * *CYP11B2* fragments 3 and 5 were amplified but the SNPs within these fragments were not considered for the present study and thus omitted. ~ *ACE1* fragments 2 and 3 contained the SNPs rs4342, rs4343 and rs4364 which were omitted from the association results because their genotypes did not have a 100% correspondence with the sequencing results.

2.6. Gel electrophoresis

2.6.1. Agarose gel electrophoresis

Verification of PCR-amplification was performed by electrophoresis, allowing visualisation of DNA bands on an agarose gel. The agarose gel was prepared by mixing 1g or 2g of agarose powder (Whitehead Scientific, RSA) with 100ml of 1 X TBE or 1 X di-sodium tetraborate-decahydrate (SB) for a 1% or 2% agarose gel (Appendix I). The mixture was then heated in the microwave oven until the agarose was completely dissolved and thereafter left to cool for 2 minutes. Five microlitres of (10mg/ml) ethidium bromide (Whitehead Scientific, RSA) was added to the agarose solution, which was then poured into a casting tray containing a well-forming sample comb and allowed to solidify at room temperature.

After solidification of the gel, the comb was removed and the gel was placed horizontally into the electrophoresis chamber and covered with buffer (1XTBE or 1XSB) (Appendix I). Electrophoresis was performed as follows: 8μl of each amplification product was mixed with 1μl of bromophenol blue loading dye (Appendix I) and then pipetted into the sample wells. One of two molecular size markers were used to co-electrophorese with PCR products, these included bacteriophage λ DNA (Promega, USA), which had been digested with *Pst* I restriction enzyme (λ Pst) (Promega,USA) and 100bp DNA ladder (Appendix I). Electrophoresis of samples occurred at 200-300V for 15-20 minutes in 1X SB buffer solution or at 100-150V in 1X TBE for 30-45 minutes. Electrophoretically seperated PCR samples were then visualised under ultra violet (UV) light using the Syngene gel documentation G-box HR (Frederick, MD, USA). A permanent photographic record of the gel analysis was obtained again using the Syngene gel documentation G-box HR (Frederick, MD, USA).

2.6.2. 12% non-denaturing polyacrylamide gel electrophoresis (PAGE)

For detection of polymorphic restriction enzyme recognition sites, non-denaturing polyacrylamide gels were used to differentiate between 100bp and smaller fragments. This method involves size separation of restriction enzyme digests using a 12% polyacrylamide solution (Appendix I). The polymorphisms detected using 12%

polyacrylamide gels included, *CYP11B2* rs1799998, rs4539 and rs3097, *AGTR1* rs2640539, rs3772627 and rs5182, *ACE2* rs1978124, rs2285666, rs879922 and rs4646179, and *ACE1* rs4365 (table 2.7).

2.6.2.1. Casting the gel

For a large number of samples, two 400mm x 800mm wide plates, termed the notched (front) and un-notched (back) plates, were washed with Cal-liquid hand soap (Cal-Chem, RSA), rinsed and dried using paper towels. The plates were treated with 70% ethanol sprayed onto the surfaces and wiped clean using paper towels. Gelbond^{TM} PAG polyester film (FMC, Bioproducts, Rocklands, Maine, USA) was attached to the back plates surface by its hydrophobic side, after spraying 70% ethanol between the plate and the gelbond. The exposed hydrophilic side enhances covalent bonding between the gel solution and the gelbond film to create a solid support for subsequent staining of the gel. One millimetre spacers were sprayed with 70% ethanol and placed onto the back plate with the front plate being placed on top of the spacers to create a space for the later insertion of a comb. The plates were clamped firmly together and were sealed at the bottom and the sides using gel-sealing tape (Sigma, Germany). Finally, the gel solution was poured between the two assembled plates and a well-forming comb was inserted between the plates. Thereafter the gel was allowed to polymerise for 30 minutes before use.

2.6.2.2. Electrophoresis

After the gel had polymerised, the gel-sealing tape was removed from the bottom of the plates, and the "well-forming" comb was also removed. The gel was vertically mounted and clamped onto the gel electrophoresis apparatus (Scigen vertical mini apparatus, Whitehead Scientific, R.S.A); if wider plates were used, the larger gel electrophoresis apparatus (Omeg scientific, RSA) was used. The buffer chambers of the apparatus were filled with 1xTBE electrophoresis buffer (Appendix I) and the wells were washed with buffer to remove any acrylamide residue. The digested PCR products (20μl) were mixed with bromophenol blue loading dye (10μl) to give the sample density and aid in visualisation. Thereafter, the total (30μl) of the digested samples, an undigested sample

and a 100bp generuler (Inqaba Biotec, RSA) were loaded into separate wells and electrophoretically separated for 2-3 hours at 150 volts (room temperature).

2.6.2.3. Silver staining

Following completion of electrophoresis of samples, the gel apparatus was dismantled and the gel removed. The gel supported by the Gelbond^{TM} (or the gel itself for smaller gels) was placed into a suitable size tray covered with solution B (0.1% AgNO3) (Appendix I) and agitated on a Labcon shaker (Labdesign, Maraisburg) for 10 minutes. Solution B was then decanted and the gel rinsed with water. Subsequently, solution C (Appendix I) was added to the tray containing the gel and then agitated until stained bands were visible. The gel was viewed on a white light illuminator box (Lauda Thermostat, Germany) and photographed using the Syngene gel documentation G-box HR (Frederick, MD, USA). The gel was sealed in a plastic bag for future reference.

2.7. Genotype analysis

2.7.1. Genotyping *ACE1* I/D polymorphism

PCR-amplification was used to genotype individuals for the insertion/deletion (I/D) polymorphism located within *ACE1*. The primers amplified either 737-bp genomic DNA segment if the insertion (*I*) allele was present or a 450-bp segment if the deletion allele (*D*) was present. Both fragments were amplified in heterozygotes (*ID*). Visualisation of the PCR products was done by electrophoretic separation on a 2% agarose gel stained with ethidium bromide. The results generated on those samples previously included in the study by the Tesson et al., (1997) were compared to present study results.

2.7.2 Genotyping of SNPs

Some of the SNPs localised within *ACE1*, *AGT* and *CMA* were genotyped performing SNaPshot primer extension analysis (table 2.6), while other SNPs localised within *CYP11B2*, *AGTR1*, *ACE2* and *ACE1* were genotyped performing allele specific restriction enzyme analysis (ASREA) (table 2.7).

Table 2.6: Thermal cycling conditions for SNaPshot multiplex primer extension analysis.

gene	region or site	Fragments	TD °C	Time sec	TA °C	Time sec	TE °C	Time min
ACE1	rs4298/rs4303	1	94	30	63 °C	40	72	1
AGT	rs5051	1	94	30	55°C	30	72	1
	rs699/rs4762	2	94	30	53°C	30	72	1
	rs11122575/rs1926723	3	94	30	55°C	30	72	1
CMA	rs1800875	1	94	30	57°C	40	72	1
	rs1885108	2	94	30	57°C	40	72	1

In a single reaction primerset 1, which included both primers designed for *ACE1* variant regions rs4298, rs4303, analysis was done in a single reaction. For *CMA*, primer sets 1 and 2 were added in a single reaction, which included both primers designed for variant regions rs1800875 and rs1885108 in a multiplex reaction. For *AGT*, primer set 1, 2 and 3 each were used in single reactions, which included the primers designed for variant regions rs5051 in a single reaction, rs699 and rs4762 in a single reaction and rs1122575 and rs1926723 in a single reaction. Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *AGT*-angiotensinogen; *CMA*-cardiac chymase; °C-degrees Celcius; min-minute; sec-seconds; TA-annealing temperature; TD-denaturing temperature; TE-extension temperature.

Table 2.7: Details of conditions used for ASREA genotyping of selected gene polymorphisms.

Gene	SNP	Alleles	Enzyme	10 X buffer	Digest time	Digest temperature	PCR product size, bp	Fragment sizes, bp		Type of Gel
								Major allele	**Minor allele**	
	rs1799998	T/C	*Hae*III	NEB 2	overnight	37°C	177	177 (T)	157, 20 (C)	12% PAGE
CYP11B2	rs4539	A/G	*Bsu*36I	NEB 3	4 hours	37°C	907	907 (A)	823, 84 (G)	1.5% Agaose
	rs3097	G/A	*Nsp*I	NEB 2	overnight	37°C	1011	744, 267 (G)	690, 267, 54 (A)	12% PAGE
	rs2640539	G/C	*Mspa*1	NEB 4	4 hours	37°C	992	992 (G)	609, 383 (C)	1.5% Agaose
AGTR1	rs3772627	A/G	*Bsah*1	NEB 4	4 hours	37°C	1120	697, 423 (A)	496, 423, 201 (G)	1.5% Agaose
	rs5182	T/C	*Mnl*1	NEB 2	4 hours	37°C	1141	422, 309, 127, 68 (T)	325, 309, 127, 97, 68 (C)	12% PAGE
	rs1978124	A/G	*Sau*96I	NEB 4	overnight	37°C	1044	1044 (A)	962, 82 (G)	1.5% Agaose
ACE2	rs2285666	G/A	*Alu*I	NEB 2	overnight	37°C	130	130 (G)	78, 52 (A)	12% PAGE
	rs879922	C/G	*Bfa*I	NEB 4	overnight	37°C	188	188 (C)	146, 42 (G)	12% PAGE
	rs4646179	T/C	*HpyCH*4IV	NEB 3	4 hours	37°C	680	402, 257, 21 (T)	402, 180, 77, 21 (C)	2.5% Agaose
ACE1	rs4365	A/G	*Alu*I	NEB 4	overnight	37°C	1198	332, 316, 261, 129, 120 (A)	332, 316, 175, 129, 120, 86 (G)	12% PAGE

Abbreviations used: *ACE1*-angiotensin-1 converting enzyme; *ACE2*-angiotensin converting enzyme 2; *AGTR1*-angiotensin 2 type-1 receptor; bp-base pairs;

CYP11B2-aldosterone; °C-degrees Celcius; NEB-New England Biolabs; PAGE-Polyacrylamide gel electrophoresis

2.7.2.1. SNaPshot primer extension analysis

SNP genotyping was performed using SNaPshot primer extension analysis, a technique used for rapid SNP detection by extending a single base at the target SNP site (Makridakis and Reichardt, 2001) (figure 2.4). This stepwise procedure involves amplifying a PCR fragment containing the SNP of interest. Samples amplified by PCR, along with the relevant oligonucleotide primers (see section 2.5.2) were prepared, in separate Eppendorf tubes, for genotyping analysis via the SNaPshot primer extension technique, according to the manufacturer's instructions (Applied Biosystems, USA). All SNaPshot primer extension analyses were performed at the US Faculty of Health Sciences Department of Medical Biochemistry.

Purification of the PCR DNA template involved removal of unincorporated dinucleotide-triphosphates (dNTPs) and excess amplification primers by treating the PCR products with both shrimp alkaline phosphatase (SAP) (USB Corporation, USA) and exonuclease 1 (Exo1) (New England Biolabs, USA) enzymes. For multiplex reactions, PCR products were combined before enzyme treatment. To achieve cleanup, PCR products were incubated at 37°C for 1 hour, followed by incubation at 75 ^{o}C for 15 minutes (to inactivate enzymes), and stored at -20°C.

The SNaPshot interrogation primer, diluted to a final concentration of 0.5μM, and multiplex reaction mix was added to the combined purified PCR products and subjected to thermal cycling according to manufacturers' instructions in a GeneAmp 9700 (Applied Biosystems Inc, Foster City, CA, USA). Three cycling conditions were set for the single base extension technique and repeated for 27 cycles (see Table 2.6). Following primer extension, each reaction was treated with 1U SAP and incubated for one hour at 37°C to remove unincorporated fluorescently labelled dideoxy-nucleotide-triphosphates (ddNTPs) with subsequent incubation at 72°C for 15 minutes to inactivate SAP.

Overview of the Procedure
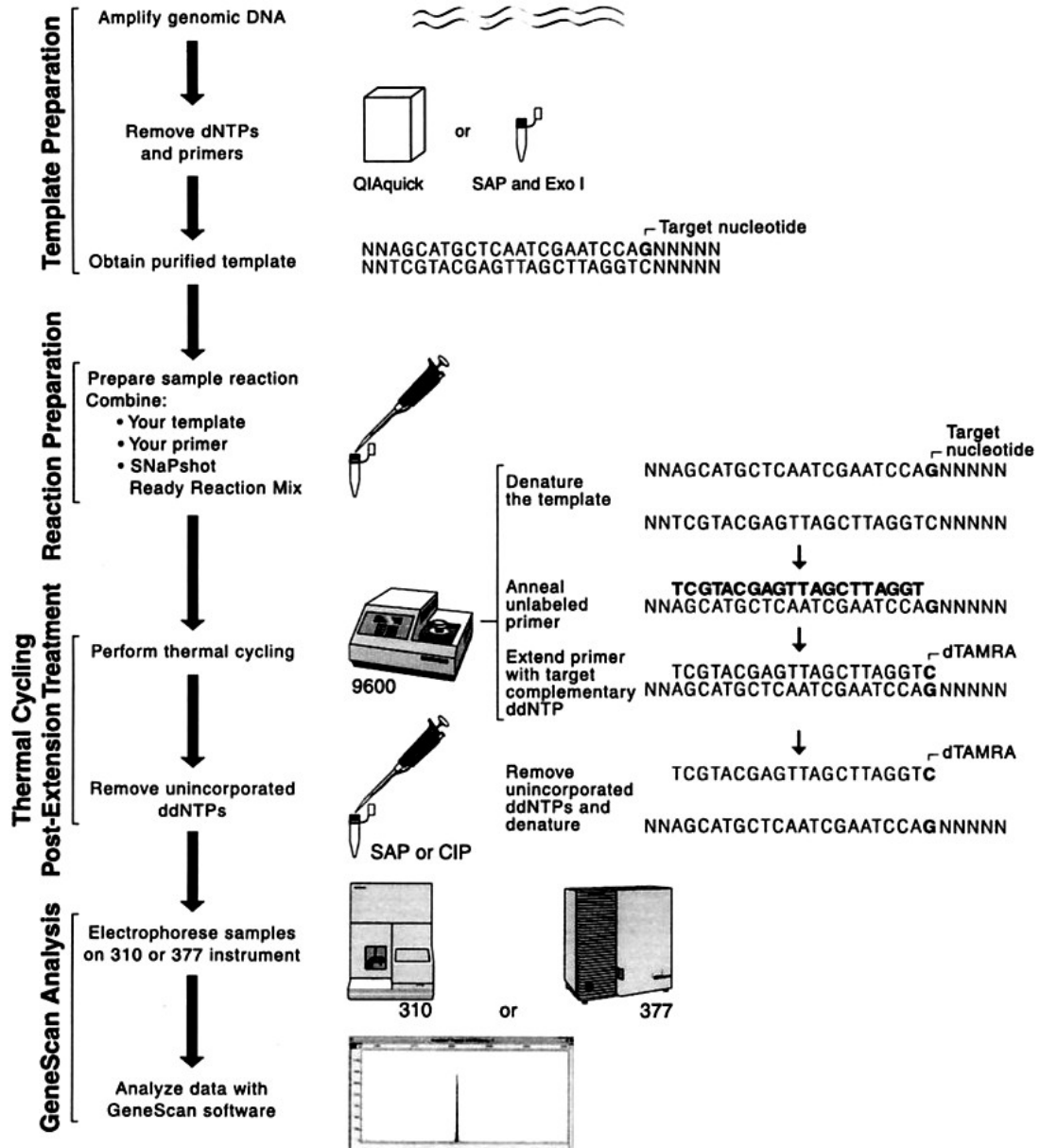
Figure 2.4: A schematic overview of the SNaPshot primer extension technique indicating the different components used in analysing the different fluorescently labelled nucleotides. (Taken from http://www.appliedbiosystems.com/techsupp.)

Samples were denatured in a loading mix containing 1μl product, 0.4μl fluorescently labelled LIZ 120 size standards (Applied Biosystems) and 9μl de-ionised formamide. The denatured samples were electrophoresed on an ABI Prism® 3100 Avant genetic Analyser (Applied Biosystems) and run in filterset E5; on a 50cm capillary with POP6 using 1X TBE as running buffer according to custom-made run module conditions (see Appendix I). The LIZ 120 size standard was included with each run to determine the size of fragments. The data obtained were analysed using Genotyper 3.7 NT software (Applied Biosystems).

2.7.3. Allele-specific restriction enzyme analysis (ASREA) of *CYP11B2*, *AGTR1*, *ACE2* and *ACE1* polymorphisms

All of the polymorphisms chosen underwent DNAMAN restriction mapping to predict whether the polymorphisms affected specific restriction enzyme recognition sequences and to determine expected fragment sizes after ASREA for each amplicon. Only those that resulted in a gain or loss of restriction enzyme recognition sequence were appropriate for investigation using ASREA. ASREA was used to genotype SNPs, localised within *CYP11B2*, *AGTR1*, *ACE2* and *ACE1*.

In *CYP11B2*, three polymorphisms, rs1799998, rs4538 and rs3097, and in *AGTR1* three SNPs, rs2640539, rs3772627 and rs5182, resulted in an alteration of a restriction enzyme recognition site (table 2.7). In *ACE2*, four polymorphisms, rs1978124, rs2285666, rs879922 and rs4646179, also altered a restriction enzyme recognition sequence (table 2.7). In *ACE1*, of the prioritized SNPs, only rs4365 was suitable for ASREA analysis (table 2.7).

As a quality control measure for ASREA genotyping Mendelian inheritance was confirmed within families using the pedigree statistics (PEDSTATS) (Wiggington and Abecasis et al., 2005) program and inconsistencies were resolved by re-examination of the raw data, and re-genotyping where necessary. Additionally, ASREA gels were co-scored by two independent individuals blinded to affectation status, to confirm genotyping accuracy.

2.7.4 Restriction enzyme digestion

PCR-based ASREA was used to screen all mutation and non-mutation carriers in the HCM cohort for the previously mentioned SNPs in the *CYP11B2*, *AGTR1* and *ACE2* genes (table 2.7).

PCR-amplification products were digested using the appropriate enzymes (table 2.7) and buffers. Briefly, 10µl of the relevant amplified PCR product was aliquoted into a 500µl-eppendorf microcentrifuge tube. Thereafter, 2µl of the appropriate 10x restriction enzyme buffer (New England Biolabs, USA), 2-5U of the restriction enzyme (table 2.7) and water to a final volume of 20µl were added to the PCR product. Subsequently, the mixture was incubated at $37^{o}C$ for 2-4 hours or overnight to allow digestion to proceed. Agarose gels (1.5 or 2.5%) or non-denaturing polyacrylamide gels (12%) were used for size separation of PCR-amplified products digested with restriction enzymes (table 2.7) (see sections 2.6.1 and 2.6.2).

2.7.5. Sequence analysis

Sequence analysis was performed to confirm SNaPshot primer extension results for 32 selected individuals ~(10%) of the HCM cohort for each polymorphism analysed by SNaPshot in *ACE1*, *AGT* and *CMA* (table 2.5).

2.7.5.1. Purification of PCR fragment

Prior to sequencing, 37µl of PCR product was purified using the GFX PCR DNA purification kit (Amersham Biosciences, UK). Firstly a GFX column (Amersham Biosciences, UK) was placed in a collection tube for each purification. 500µl Capture buffer (Amersham Biosciences, UK) was added to the GFX column and the PCR product was transferred onto the column. The two solutions were mixed by pipetting and centrifuged at 13000 revolutions per minute (rpm) for 30 seconds (sec) in a Beckman bench top centrifuge (Benchman Instruments Inc, CA, U.S.A). The flow-through was discarded and the GFX column placed back inside the collection tube. A volume of 500µl Wash buffer was added to the GFX column and centrifuged at 13000 rpm for 30 sec.

The flow-through was discarded and the GFX column placed into a fresh 1.5ml micro-centrifuge tube. To elute DNA, 50μl of water was applied directly to the top of the glass fibre matrix in the GFX column. The sample was incubated at room temperature for one minute and was subsequently centrifuged at 13000 rpm for one minute to recover purified DNA.

2.7.5.2. Automated sequencing analysis

The purified PCR products that contained the amplified polymorphic sites were sequenced according to established protocols using BIGDye terminator v3.1 cycle sequencing kit (Applied Biosystems) at the US Department of Biomedical Sciences Tygerberg, South Africa. All samples were analysed using an ABI 3100-Avant genetic analyser.

2.8. Statistical analysis

Genotypic and phenotypic data were captured onto family trees using Cyrillic 2.1 (Cherwell Scientific, UK). This data was converted into an MLINK format, exported and combined with the Excel sheet containing the 2D-echo and covariate data (see section 2.1), to create a pedigree file for statistical analysis.

Family-based association methods, viz. quantitative transmission disequilibrium test (QTDT) and mixed-effects models, were employed to investigate association between polymorphisms and hypertrophic phenotypes, viz. the 16 wall thickness parameters as well as the CWT score (Spielman et al., 1993). QTDT was used for all analyses except *ACE2; t*he reason for this is that ACE2 is an X-linked gene and these can not be analysed by QTDT yet. In mixed-effects models, the variance is split into components for family and not family, whereas the QTDT method further adjusts variance components for specific family and genetic relationships.

2.8.1. Distribution of variables (Summary statistics)

Descriptive statistics were calculated using PEDSTATS (Wiggington and Abecasis et al., 2005). Graphical outputs were generated to summarise the completeness of genotyping,

heterozygosity of markers and their distribution, as well as the distributions and familial correlations for quantitative traits.

2.8.2. Hardy-Weinberg equilibrium (HWE) testing and Linkage disequilibrium (LD) determination

The Haploview v3.6 (Barrett et al., 2005) program was used to assess Hardy-Weinberg equilibrium (HWE) and LD patterns between alleles within our SA HCM cohort. HWE was tested for in unrelated individuals selected from each family by the program.

The Haploview program enables the construction and analysis of haplotypes generated for SNPs within a gene, based on haplotype blocks. Haplotype blocks generated by Haploview are automatically selected using a common block definition from Gabriel et al., (2002). Pairwise LD is analysed in terms of D' values; 95% confidence bounds on D' are generated and based on these, each pair of SNPs is labeled as being in strong LD, uncertain or in strong recombination. If a D' value indicates strong LD, a block would be created.

2.8.3. Quantitative transmission disequilibrium test (QTDT)

QTDT can be used to perform both linkage and association analysis of quantitative traits using families of any size, depending on the model implemented in the program (Abecasis et al., 2000). As the families used in this study ranged from 1-4 generations and between 2-69 individuals, and included individuals and families who were not of the same ethnic origin, the Abecasis test of association was used. This orthogonal model can analyse families of any size, allows for missing parental information and automatically protects against population stratification. The quantitative traits assessed in this analysis are listed in table 2.8. The following covariates were included in the statistical analysis: age, sex, HR, systolic BP, diastolic BP, mutation (yes/no) and (BSA). Additionally, mutation founder groups (A797T, R403W and R92W) were included to adjust for the possible influence of distinct mutations on hypertrophic variability, as well as to adjust for ancestral relatedness within each founder group. The specific relationship between

family members was incorporated into the models using identity by descent (IBD) probabilities estimated with simwalk2 (Sobel et al., 2001).

Table 2.8: Summary of 2D-echo traits measured at three levels and composite scores.

Overall	Mitral valve level	Papillary level	Apex level	Composite scores
LVM	mLVWTmit	mLVWTpap	mLVWTapx	Maron-Spirito score
mIVS	mIVSTmit	mIVSpap	IVSapx	CWTscore
mLVWT	pIVSmit	pIVSpap	AWapx	Wigle score
mPWT	aIVSmit	aIVSpap	LWapx	
	AWmit	AWpap	PWapx	
	LWmit	LWpap		
	IWmit	IWpap		
	PWmit	PWpap		

Abbreviations used for overall, at three levels measures and composite scores see Appendix I.

Building up of variance component model

A variance is a number that indicates the spread of the values of a trait between individuals: for example age, if everybody is the same age the variance is zero. QTDT allows the build-up of a variance component model, where the trait variance can be decomposed into three components, namely, residual environment variance, polygenic variance and additive component of variance.

Procedure of analysis

We firstly performed a heritability test using a variance-component analysis for quantitative traits, which does not require genotypic data. It was suggested by Harshfield et al., (1990), that age and gender of the genotype individuals be used as covariates, because heart size is known to be influenced by age, gender and body mass of individuals. In this study, these covariates as well as HR, systolic BP, diastolic BP, mutation (yes/no), mutation founder groups (A797T, R403W and R92W) and (BSA), were included in heritability analysis.

Subsequently, upon proof of heritable traits, linkage tests were performed by testing the polygenic variance-component, generating p-values for the linkage component. However, QTDT does not provide LOD scores. Instead of only interpreting the linkage

p-values, the results generated from QTDT tests were transformed to logarithm of the odds (LOD) score values by ($x^2/(2\ln(10))$) (Ott et al., 1999) and evidence of significant linkage was considered if the LOD score was ≥ 3 and excluded if the LOD score was below -2, while scores between 3 and -2 were considered ambiguous (Ott et al., 1999).

The association tests were performed with the QTDT programme, using Abecasis's orthogonal model, allowing for additive environmental and genetic effects. The association tests performed assessed association between the 17 markers investigated and the 16 quantitative traits and one composite score, the CWT-score, while adjusting for all covariates mentioned earlier.

Testing linkage in the presence of association

To determine if the investigated marker could be the causal susceptibility variant, orthogonal association was included in the model while testing major gene locus linkage. In such an analysis, a large, i.e. non-significant, p-value would indicate that there is no statistically significant linkage in the presence of association and thus that association accounts for the entire signal (thus that the marker locus could be the true, functional modifier locus). However, a significant p-value indicates that the linkage observed is not explained by the association, suggesting that the marker locus is linked by LD to the modifier locus.

Estimating exact p-values

The significant associations found in the analysis, were retested using Monte-Carlo (1000 times) permutation tests (McIntyre et al., 2000). The empirical p-values derived from 1000 permutations are reported in the result section for these association tests.

2.8.4. Linear mixed-effects models

X-linked *ACE2* analysis

The X-linked gene ACE2 was statistically analysed with mixed-effects models using the package nlme (Pinheiro, et al., 2007) in the programming environment R (R Development Core Team., 2007). The mixed-effects models are simplifications of the

QTDT models with the only difference being that the variance is split into family and not-family, but cannot adjust for IBD probabilities and variance components.

The mixed effects models were, like the QTDT models, adjusted for age, sex, BSA, SBP, DBP, HR, mutation (fixed effects) and for family membership (as a random factor). There are three mutation groups; namely, R403W, R92W and A797T, and each of these are coded as mutation: yes/no. Analysis was done to assess the association between the four X-linked SNPs and the 16 individual traits and CWT-score.

3. RESULTS

3.1. Candidate genes selected

Bioinformatic-based literature searches aided in the identification of six candidate genes which encode key components of the RAAS and have been previously shown to be associated with hypertension and LVH (section 2.4, table 2.2).

3.2 Prioritising SNPs for investigation

This study aimed to target at least three SNPs spread throughout the candidate genes, however, in *CMA* only a single SNP in the 5' regulatory region and one in the middle of the gene were targeted (figure 3.1A-F). The SNPs selected in each of the six chosen genes were prioritised based on the following criteria: those previously reported in other relevant studies of RAAS in CVD (section 2.4, table 2.2) and those with known heterozygosity values $\geq$ 40% and known intragenic locations (table 3.1). Most of the SNPs within *ACE1*, *AGT* and *CMA* were genotyped by SNaPshot analysis, using primers that differed in four to six nucleotides in length, according to each variant, and which could therefore be used in a single multiplex reaction. The SNPs located within *AGTR1*, *CYP11B2*, *ACE2* and one SNP within *ACE1* were chosen for investigation not only because they possessed a high heterozygosity value but also because they could be genotyped by a loss or gain of a restriction enzyme site (referred to as ASREA in this work). An exception was the *ACE1* I/D polymorphism which was genotyped by gel-observation of the different sized PCR amplification products.

A) *ACE1*

Exons

1   2   3   4   5   6 {7 – 15} 16   17 {18 – 23} 24   25

rs4298 **rs4303**

**I/D
(rs4340)**

rs4365

B) *AGT*

Exons   1   2   3   4   5

rs11122575 **rs1926723**

**-6 *G/A*
(rs5051)**

**T174M
(rs4762)**

**M235T
(rs699)**

C). *AGTR1*

Exons 1   2   3   4   5

rs2640539 **rs3772627**

**573*C/T*
(rs5182)**
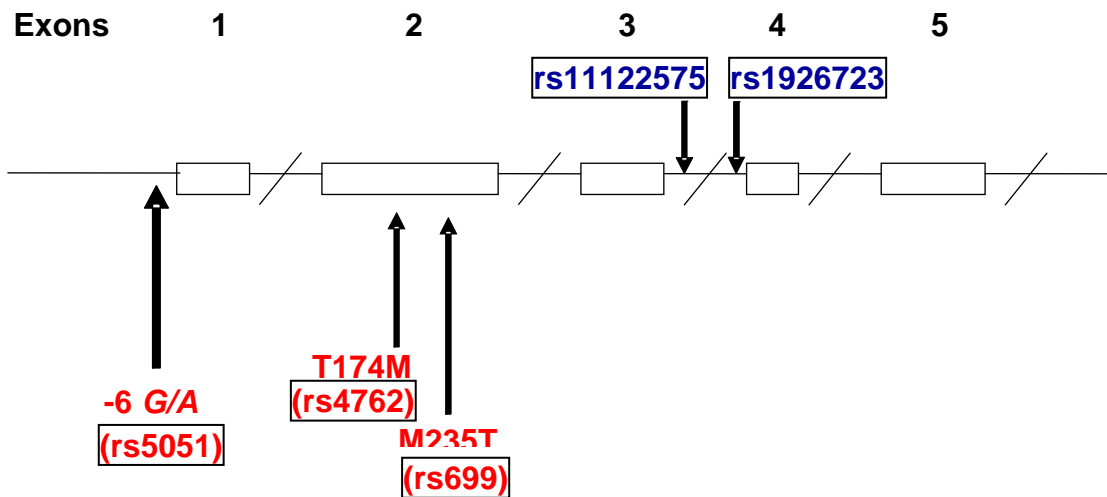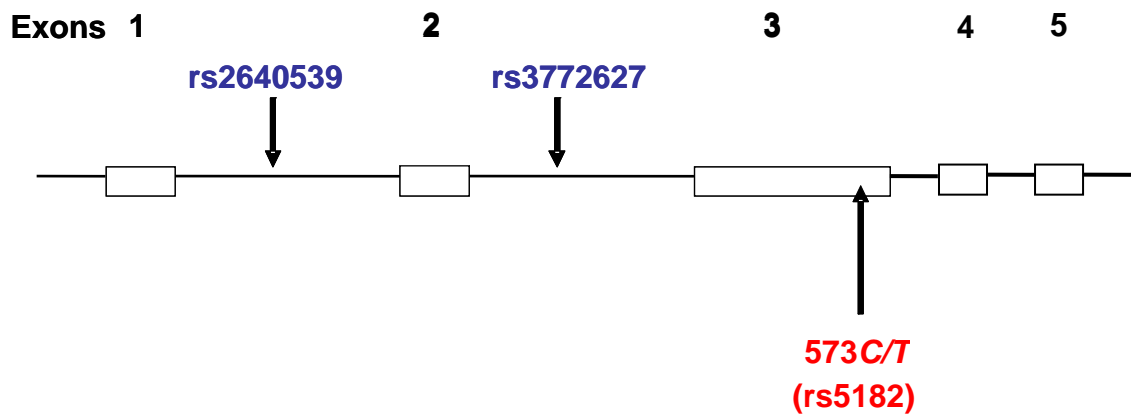
D). *CYP11B2*

E). *CMA*

F). *ACE2*

Figure 3.1: Schematic diagrams showing exon/intron structure of the candidate genes and location of targeted SNPs in A) *ACE1*, B) *AGT*, C) *AGTR1*, D) *CYP11B2*, E) *CMA* and F) *ACE2*. Variants indicated in boxes were investigated by SNaPshot, while the other variants were investigated by ASREA, except for the I/D polymorphism in *ACE1*. The markers highlighted in red indicate variants investigated in previous association studies and the markers highlighted in blue indicate variants that have not yet been investigated (Table 1.2 section 1.3.2).

Table 3.1: SNPs prioritised for investigation.

Gene/ protein	dbSNP rs#cluster id	Heterozygosity	Validation	Intragenic location Ex Syn/ Ex Nsyn (AA)
ACE1	rs4298	0.212	C,F,2	Ex syn (Asn)
	rs4303	0.051	C,F	Ex Nsyn (Ser→Ala)
	rs4340	N.D	N.D	Intron
	rs4365	0.064	C,F	Ex syn (Glu)
CMA	rs1800875	N.A	C,2	-1903 Promoter
	rs1885108	0.491	C,F,2,H	Intron
AGT	rs5051	0.456	C,F	-6 Promoter
	rs4762	0.154	F,H	Ex Nsyn (Met→Thr)
	rs699	0.467	F,2	Ex Nsyn (Thr→Met)
	rs11122575	0.271	C,F	Intron
	rs1926723	0.256	C,F	Intron
CYP11B2	rs1799998	0.436	C,F,2,H	-344 Promoter
	rs4539	0.286	C,F	Ex Nsyn (Arg→Lys)
	rs3097	0.213	F,2,H	3' untranslated
AGTR1	rs2640539	0.189	F,2	Intron
	rs3772627	0.483	C,F	Intron
	rs5182	0.491	F	Ex syn (Leu)
ACE2	rs1978124	0.330	F,2,H	Intron
	rs2285666	0.454	C,F,2	Intron
	rs879922	0.405	F,2,H	Intron
	rs4646179	0.046	C,F,H	Intron

Abbreviations used: Amino acid (AA), Cluster (C), Exon (Ex), Frequency (F), HapMap (H), Non-synonymous (Nsyn), Not available (NA), Not determined (N.D), Synonymous (syn) and Two hit (2).Validation status descriptions: Cluster-validated by multiple independent submissions to the refSNP cluster, Frequency- validated by frequency or genotype data: minor alleles observed in at least two chromosomes, HapMap- genotyped by HapMap project, Two hit- All alleles have been observed in at least two chromosomes a piece.

3.3 Primer design

Primers were designed to regions flanking the chosen SNPs' locations for both PCR amplification of gene fragments and SNaPshot primer extension analyses. A total of 20 outer primer sets for PCR amplification of the target regions and 13 SNaPshot primers were designed (figure 3.2-3.7).

forward primer for PCR

SNaPshot primer for rs4303

SNaPshot primer for rs4298

```
3601 ggtgggagca gtaaggactg gtgcaggctc tggtgaaggc cgttgaagac ttcaacgtgg
3661 aggcctcctc accgaccctg cctgcctgtg tctcagatct caccaacatc ctggcttcct
3721 cgcgaagcta cgccatgctc ctgtttgcct gggagggatg gcacaa C/T gct gcgggcatcc
3781 cgctgaaacc gctgtacgag gatttcactg ccctcagcaa tgaagcctac aagcaggacg
3841 gtgagcaggc ctctccctgt ccaggaacca cgccaggtgt cctctctcag ctgtctcccc
3901 agagtcccag cccagagtca ggcagagcag ctggtatgac aattccagca ggccctgagt
3961 ttcccagaaa gtggaggtgg gaccggcctg cacccagtgt gcctggactt tgctgctggc
4021 ctgccccacg tggccatcct gctgtcactc ctggccctga tgctcctctt tgcctctggg
4081 aacctccagg atctgtttag ctggctgtag ctaattagaa attgtagagt ggcaaccccc
4141 aagccaattt tccagctagc tgcagatcca cgggcctcga gccagtggaa gagccgactt
4201 acagctgaga ggctgaggtc cgagcctttg gcctgagcta catacctcac ccccacgccc
4261 ccaggcttca cagacacggg ggcctactgg cgctcctggt acaactcccc caccttcgag
4321 gacgatctgg aacacctcta ccaacagcta gagcccctct acctgaacct ccatgccttc
4381 gtccgccgc G/T cactgcatcg ccgatacgga gacagataca tcaacctcag gggaccc atc
4441 cctgctcatc tgctgggtaa ggacctggcc tcgcctccac atgagtccca cggaagtgtg
4501 ggtcccgagg taggggtggg ggatgtccag ggtaagggaa ggtgggttgt gaccctcaca
4561 tctcacatgt gtggggcatc atactgtttg cttcacatgc aggagaccat tcgtgttccc
4621 actttacagg tggggaccct gaggcttagg gtcgtgaggg acttagtggt cagagagcta
4681 ggggccaaac caaaggctct ggccctgggt ccagtggggg agccatcagc ctagctcatg
4741 cccaaggaaa caagcactgt ggccctgcct caggattgag tggctggggc ctggcacagc
4801 cagaaatgac agtggcagca tcttgcagcc ccaggacatg tggccctcgg aggagtgtgg
4861 gtgggactga tgtgtgagat ttctggccct aagccaggcc tgcagccctt gagggcccca
4921 gggtacaggt gccggcccca gggtgccact cagcgatgca tgaagaagca ggcacagcca
4981 ggcagggagc caagctgtcc ccttccttcc ttatctagga gacatgtggg cccagagctg
```

reverse primer for PCR

Figure 3.2: *Primer design for the rs4298 and rs4303 sequence variants (in pink) within ACE1 fragment1.*
Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs4298 and rs4303 sequence variants (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue.

forward primer for PCR

**SNaPshot primer for rs5051
on forward strand
(complement)**

```
 841 ggtcccagcg tgagtgtcgc ttctggcatc tgtccttctg gccagcctgt ggtctggcca
 901 agtgatgtaa ccctcctctc cagcctgtgc acaggcagcc tgggaacagc tccatcccca
 961 cccctcagct ataaatagg g catcgtgacc cggcc G/A gggg aagaagctgc cgttgtt c tg
1021 ggtactacag cagaaggtaa gccggggggcc ccctcagctc cttctcggtc ttgtctctct
1081 cagatgtaac tgagctgtgg gctaggagga aaaggccggg aggaggcacg gtgatgactg
1141 aaaaacctct cccctctcat aagaccagtc atccggacgc gggctttccc ccactcggtg
1201 cccacctggg gtcttacagg aggagctgct cctcctcagc aataggacaa gatggtcagg
1261 tcttcctgct tccgctgaga aaagttaggg tcctcaggaa cggagcagac tggtacagga
1321 acagagtcat catggccaag agtccaccgg gtcctcttgc catcaggagg aatagcaggg
1381 cttgtgcagg aattgggggct ggagggaagg gccgggctcg gtcagtctcc agctgggatc
1441 cccagagtgg tcaccctacc cctccctcga gacagactgc ctgactgtgt gtcatcaggc
1501 tggtcaccat ctccctgaac ctcgatttgc tcacctataa aatggaacta ataacgatgc
1561 ctgggctccc tgtctcaggg gctctggtat agctgaagag aactaatata acatgaaagt
1621 gctttctaag ctttgggata agctaaaagg cagattccaa ttttattcga gggcagcgta
1681 gattggtgct tcagctcgtg gatgacagag tcaggggggcc tggttctgag tcctagttct
1741 gtctcttccc agctgtgtga cgttgaacaa gtcactggac ctctctgttc ctctgcaaaa
1801 cagcatgaac caattcatta actacttctc caggatgcag taggtcccag ggactatcct
1861 aggaatgtgg gctgtattag taaacacaac agcgggaacc ctgttccggg gctcacattc
1921 acatcagagc aaacagacaa agacgctgga cagaataagt gcataactac atggtacaga
1981 gggttataag gagggaaaag gggagctgga tgagagagtt gagagtgccc ggtgtggtgg
2041 ggaaagctgc agggtgaaat actgcatcag ggaaacctca gggaaggtga ggactatggt
2101 gaggtcagag gggttgatat gagaacagtg ccctgcaaat ggcaggcacc acaggagcat
2161 gagccgtcat cttca cctt t agcattcagc ccgg gagaag tagggagaca tagaaggggc
2221 aggtgctggc caagagg gag gggcaggaga ggagaaggcg gagggggcact cagggcgagg
2281 gtgtcaggcc cgcc acccca gagcaccatt actcccagga cgcggctgcg tgcagacctg
```

reverse primer for PCR

Figure 3.3: *Primer design for the rs5051 sequence variant (in pink) within AGT fragment 1.* Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs5051 sequence variant (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue. The sequence above is the anti-sense (reverse), the SNaPshot primer is designed for the forward strand.

SNaPshot primer for rs4762

Forward primer for PCR

```
4021 acacgggcat tatggccgtt gggggaggta ggacataatt cagctgatat tggtgcattt
4081 tgcacttgga tcatgtagat attttccatg gagctttgaa tccatttctt ctttttttg
4141 tagacatgaa tggatttatt ctgggctaaa tggtgacagg gaatattgag acaatgaaag
4201 atctggttag atggcactta aaggtcagtt aataaccacc tttcaccctt tgcaaaatga
4261 tatttcaggg tatgcggaag cgagcacccc agtctgagat ggctcctgcc ggtgtgagcc
4321 tgagggccac catcctctgc ctcctggcct gggctggcct ggctgcaggt gaccgggtgt
4381 acatacaccc cttccacctc gtcatccaca atgagagtac ctgtgagcag ctggcaaagg
4441 ccaatgccgg gaagcccaaa gaccccacct tcatacctgc tccaattcag gccaagacat
4501 cccctgtgga tgaaaaggcc ctacaggacc agctggtgct agtcgctgca aaacttgaca
4561 ccgaagacaa gttgagggcc gcaatggtcg ggatgctggc caacttcttg ggcttccgta
4621 tatatggcat gcacagtgag ctatggggcg tggtccatgg ggccaccgtc ctctccccaa
4681 cggctgtctt tggcaccctg gcctctctct atctgggagc cttggaccac acagctgaca
4741 ggctacaggc aatcctgggt gttccttgga aggacaagaa ctgcacctcc cggctggatg
4801 cgcacaaggt cctgtctgcc ctgcaggctg tacaggccct gctagtggcc cagggcaggg
4861 ctgatagcca ggcccagctg ctgctgtcca C/T ggtggtggg cgtgttcaca gccccaggcc
4921 tgcacctgaa gcagccgttt gtgcagggcc tggctctcta taccccgtg gtcctcccac
4981 gctctctgga cttcacagaa ctggatgttg ctgctgagaa gattgacagg ttcatgcagg
5041 ctgtgaatagg atggaagact ggctgctccc tga C/T gggagc cagtgtggac agcaccctgg
5101 ctttcaacac ctacgtccac ttccaaggta aggcaaacct ctctgctggc tctggcccta
5161 ggacttagta tccaatgtgt agctgagatc agccagtcag gccttggaga tgggcagggg
```

SNaPshot primer for rs699

reverse primer for PCR

Figure 3.4: *Primer design for the rs4762 and rs699 sequence variants (in pink) within AGT fragment 2.*
Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs4762 and rs699 sequence variants (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue.

forward primer for PCR

SNaPshot primer for rs1122575

```
10441 agacacagtg cattatttca gagaatagca tttaaaaatt acccaagtaa cacaccttca
10501 atgcagccaa cttaaaaaca gaatgcacca aaggacaccc attcctaggt cctcatcggt
10561 aaatcttcta tgtccctcac atagtattgc aaatgaca T/C gaaggatttt attgtaggtt
10621 ttgctgaaat tttccccaag ggggaggatg acttagttgg gtgatggggg gagcaaacat
10681 ccctgtcgtc agggttgggt gcaaggagca taagcctgcc tggcctctgg gagagccctc
10741 actgtgtggc ctggagcctt cctaactgtg c A/G tcatctcc ccaggaccat ccacctgacc
10801 atgccccaac tggtgctgca aggatcttat gacctgcagg acctgctcgc ccaggctgag
10861 ctgcccgcca ttctgcacac cgagctgaac ctgcaaaaat tgagcaatga ccgcatcagg
```

reverse primer for PCR

SNaPshot primer for rs1926723

Figure 3.5: *Primer design for the rs1122575 and rs1926723 sequence variants (in pink) within AGT fragment 3.*
Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs1122575 and rs1926723 sequence variants (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue.

forward primer for PCR

SNaPshot primer for rs1800875

```
  1 TTAAAATACACAGGTATACATATGACAAAGCAAAGAAGGAAATGTGAGCAGATAGCGCAG  60
 61 TCCTCGTTTCTGAAATTGGTCCCCTGACTGGGGCTATACCTATTCCATTTCCTCACCCTC 120
121 AGCCAGGCAGGTG A/G AGCAAAACTTAAGTCTTGGTGGATCTGAATCTTGATGCTGTGGAGC 180
181 TGTCTTACTAGCCCCAGACTACCTGCCTCTCAATTTCTAATTATATCAGTGAAAGCAAAC 240
241 AGCTTTGATTTGTTTAAGCCTCTGATTTTTTGGTCTAACTGATGTAAGACCACAAGACAA 300
301 GAGTTCTCCAGCTCCGGATTCTCTTCTGTTCTGTTAATGGTGAAATGCCCAGAGAAGAGT 360
361 TGCCAACTTTGGCAAATAAAAAATACAGGATTCCAGTTAAATTCAAATTTTAGATAAACA 420
421 ACAATTTTTTAGTATTAGTGTGTCCCATTCAATATTTGGACATACTTAACTAAAAAATGA 480
```

reverse primer for PCR

Figure 3.6: *Primer design for the rs1800875 sequence variant (in pink) within CMA fragment 1.* Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs1800875 sequence variant (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue.

Forward primer for PCR

SNaPshot primer for rs1885108

```
3721 AGCTTGAGGTTATAAAGCAATTCCGTCATCCAAAATATAACACTTCTACTCTTCACCACG 3780
3781 ATATCATGTTACTAAAGGTGACATCACCTCTCTTCTCCCTTTCCACTTCCCATTCTCCTA 3840
3841 AGCTTCTCCTTCAGGTCCTCATTGCCCTGAATTTTTCTTAGGACTTGGCTATAACATGAA 3900
3901 GCTACTCACCCTGTCCCTCCCTGATCACCTCCAACTGTCCAGAGCCCATTTCGAGGACTG 3960
3961 AC A/G GTCCTTCATTCCCTTCACAGTTGAAGGAGAAAGCCAGCCTGACCCTGGCTGTGGGGA 4020
4021 CACTCCCCTTCCCATCCCAATTCAACTTTGTCCCACCTGGGAGAATGTGCCGGGTGGCTG 4080
4081 GCTGGGGAAGAACAGGTGTGTTGAAGCCGGGCTCAGACACTCTGCAAGAGGTGAAGCTGA 4140
4141 GACTCATGGATCCCCAGGCCTGCAGCCACTTCAGAGACTTTGACCACAATCTTCAGCTGT 4200
4201 GTGTGGGCAATCCCAGGAAGACAAAATCTGCATTTAAGGTGATCCTCCAACTAGGTTTCC 4260
```

Reverse primer for PCR

Figure 3.7: *Primer design for the rs1885108 sequence variant (in pink) within CMA fragment 2.* Forward and reverse primers (highlighted in green) were designed to amplify the genomic region harbouring the rs1885108 sequence variant (in pink font). The SNaPshot interrogation primers used to genotype the variants in subjects from the HCM-affected families are highlighted in blue.

3.4. Polymerase chain reaction (PCR)

3.4.1. Optimising of PCR conditions

PCR conditions were optimised to amplify all the fragments of *ACE1*, *CYP11B2*, *AGTR1*, *AGT*, *CMA* and *ACE2* using the outer primers listed in table 2.3 and using DNA from the same individuals for each optimisation (see figures 3.8 to 3.13).

A) *ACE1* fragment 1

B) *ACE1* fragment 2
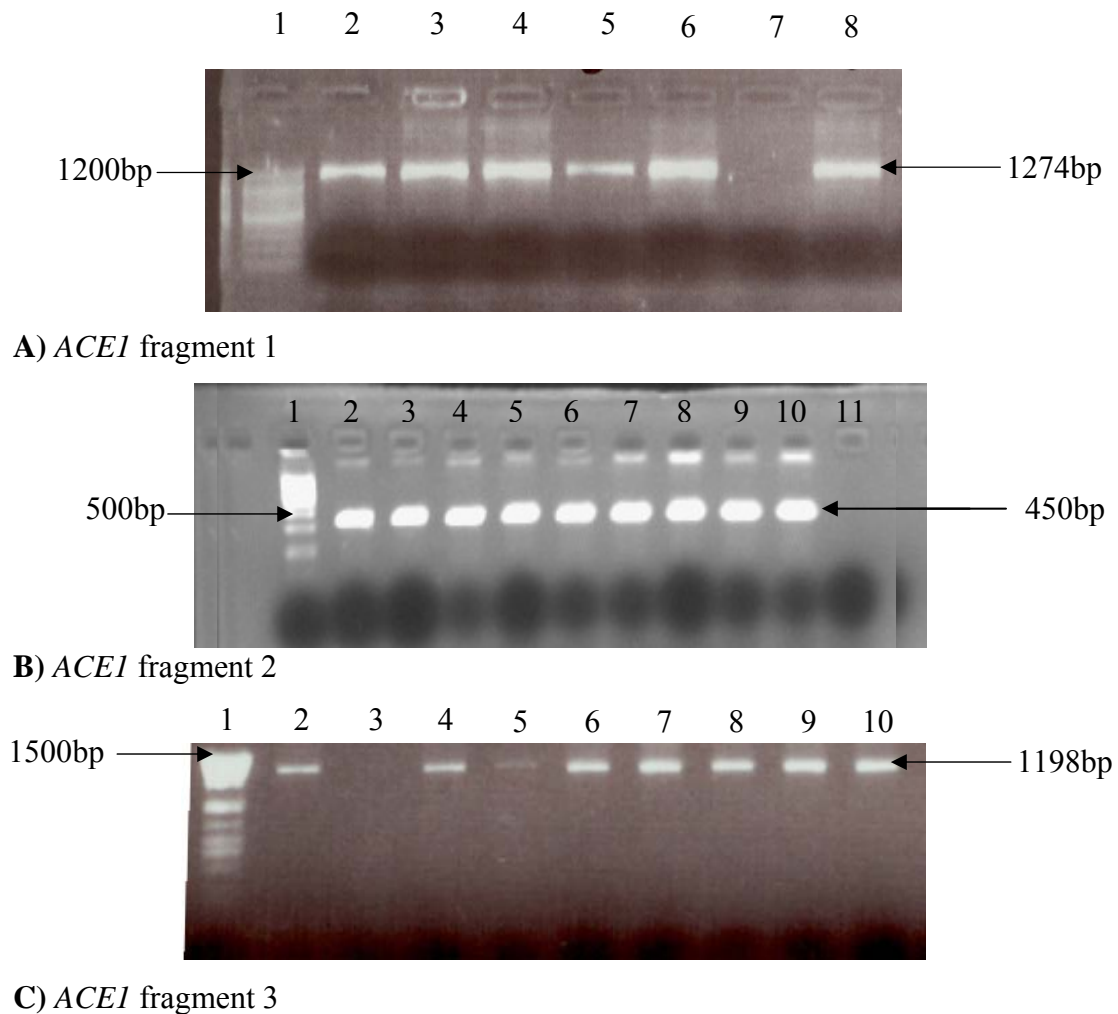
C) *ACE1* fragment 3

Figure 3.8: *Representative 2% agarose gel showing PCR amplified ACE1 fragments 1, 2 and 3.*
A) Lane 1 – molecular weight marker ; lanes 2-6 and 8 – *ACE1* fragment 1 amplicons of 6 selected individuals; Lane 7 – negative control (water blank), B) Lane 1 – molecular weight marker; lanes 2-10 – *ACE1* fragment 2 amplicons of 9 selected individuals; Lane 11 – negative control (water blank) and C) Lane 1 – molecular weight marker; lanes 2, 4-10 – *ACE1* fragment 3 amplicons of 8 selected individuals; Lane 3 – negative control (water blank).
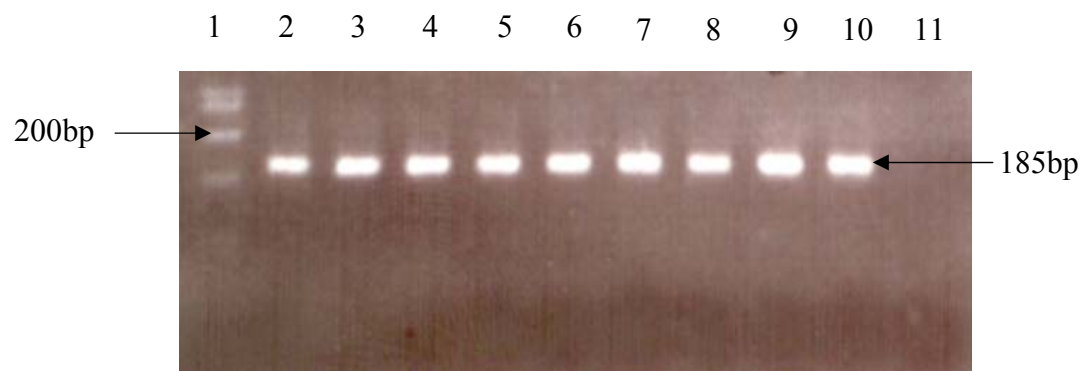
A) *CMA fragment 1*
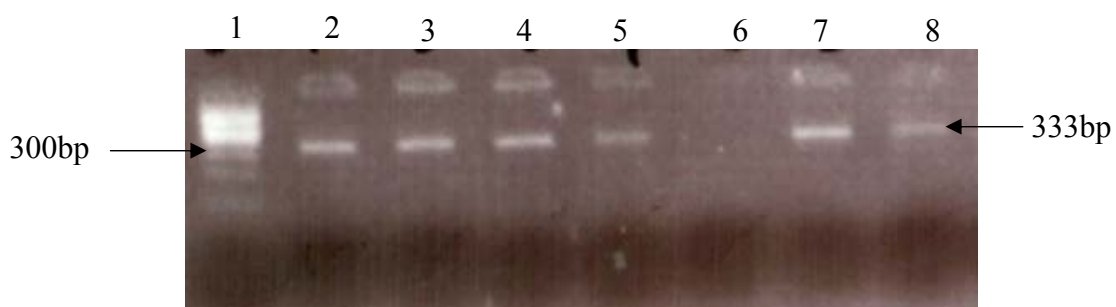
B) *CMA* fragment 2

Figure 3.9: *Representative 2% agarose gel showing PCR amplified CMA fragments 1 and 2.* A) Lane 1 – molecular weight marker; lanes 2-10 – *CMA* fragment 1 amplicons of 9 selected individuals; Lane 11 – negative control (water blank) and B) Lane 1 – molecular weight marker; lanes 2-5, 7 and 8 – *CMA* fragment 2 amplicons of 6 selected individuals; Lane 6 – negative control (water blank).
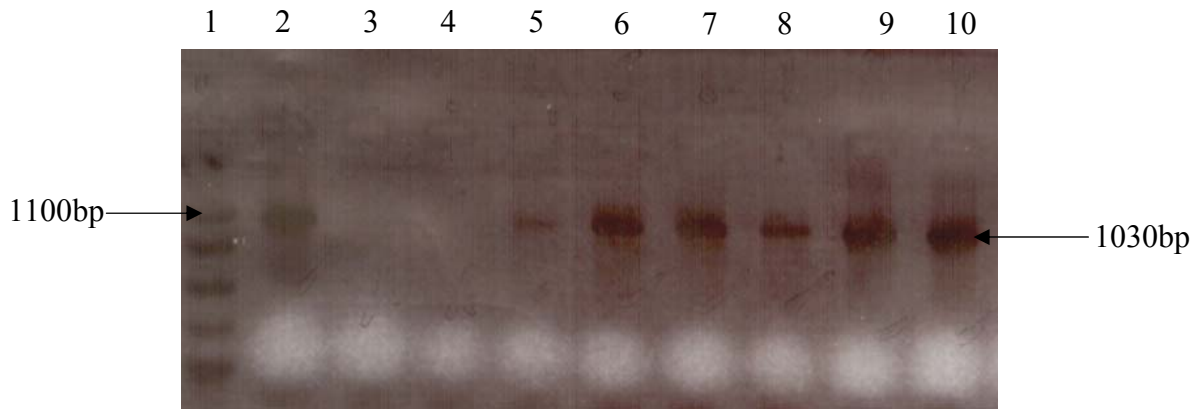
A) *AGT* fragment 1
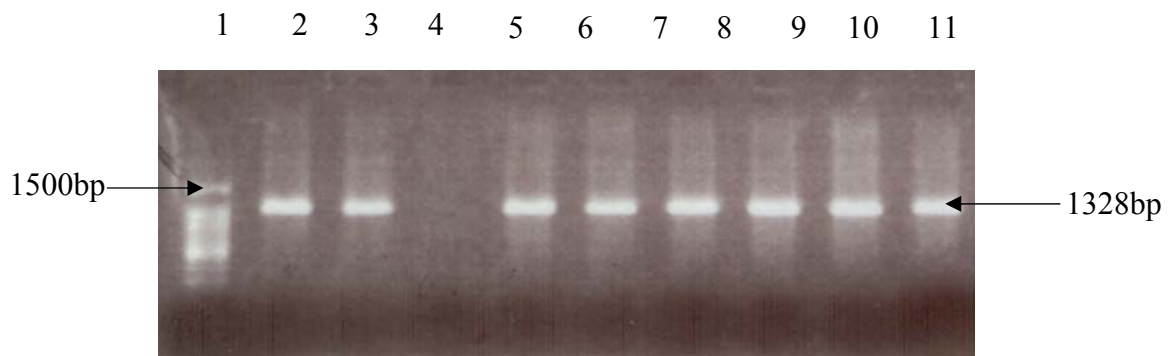
B) *AGT* fragment 2
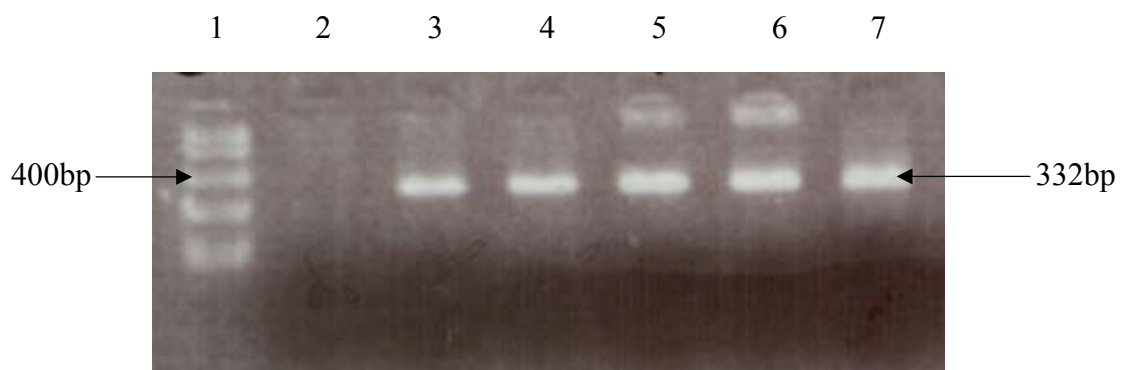
C) *AGT* fragment 3

Figure 3.10: *Representative 2% agarose gel showing PCR amplified AGT fragments 1, 2 and 3.*
A) Lane 1 – molecular weight marker ; lanes 2, 5-10 – *AGT* fragment 1 amplicons of 7 selected individuals; Lane 3 – unsuccessful PCR amplification; Lane 4 – negative control (water blank), B) Lane 1 – molecular weight marker; lanes 2, 3, 5-11 – *AGT* fragment 2 amplicons of 8 selected individuals; Lane 4 – negative control (water blank) and C) Lane 1 – molecular weight marker; lanes 3-7 – *AGT* fragment 3 amplicons of 5 selected individuals; Lane 2 – negative control (water blank).

A) *AGTR1* fragment 1
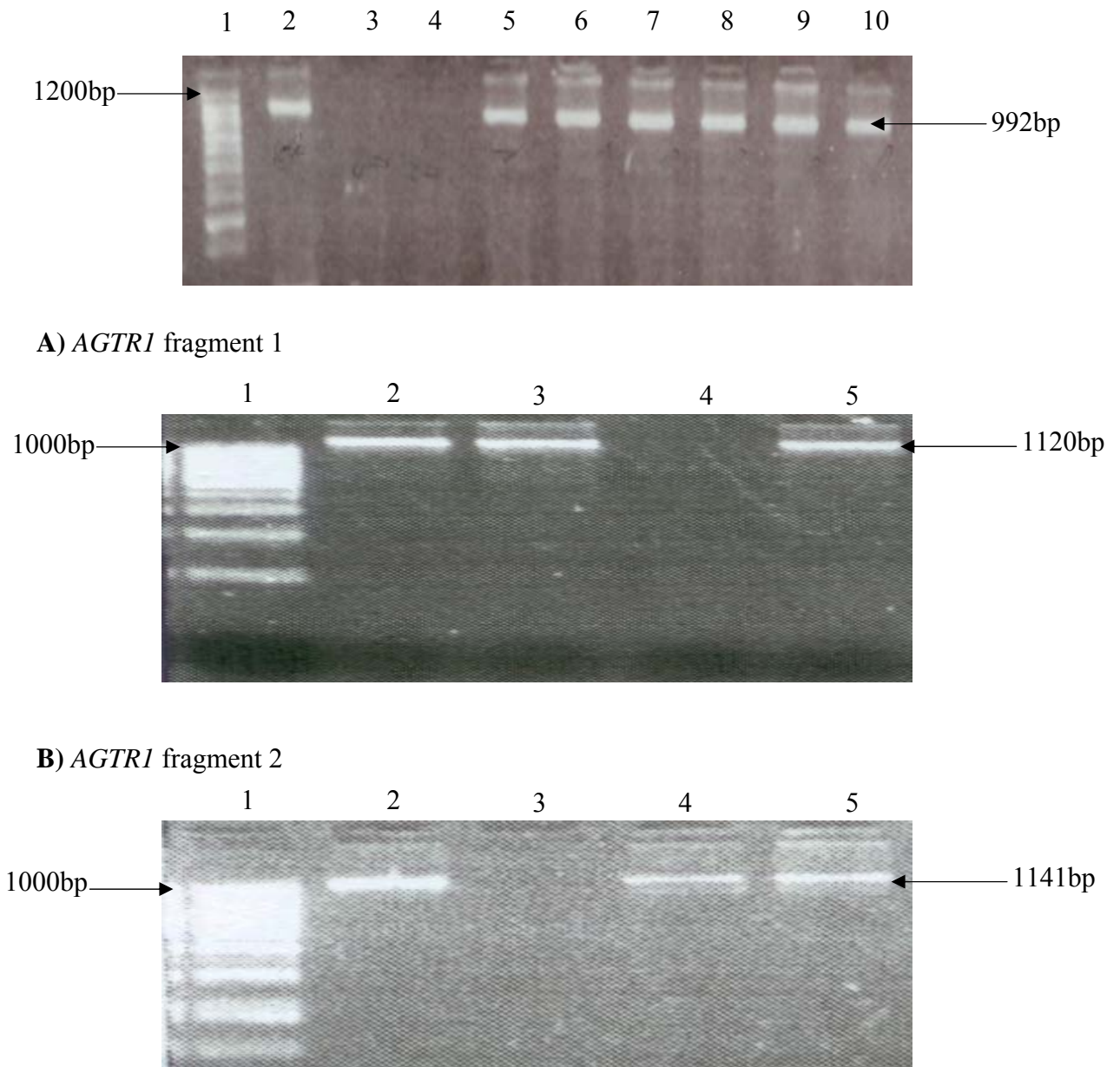
B) *AGTR1* fragment 2

C) *AGTR1* fragment 3

Figure 3.11: *Representative 2% agarose gel showing PCR amplified AGTR1 fragments 1, 2 and 3.*
A) Lane 1 – molecular weight marker ; lanes 2, 5 -10 – *AGTR1* fragment 1 amplicons of 7 selected individuals; Lanes 3 – unsuccessful PCR amplification; Lane 4 – negative control (water blank), B) Lane 1 – molecular weight marker; lanes 2, 3, and 5 – *AGTR1* fragment 2 amplicons of 3 selected individuals; Lane 4 – negative control (water blank) and C) Lane 1 – molecular weight marker; lanes 2, 4 and 5 – *AGTR1* fragment 3 amplicons of 3 selected individuals; Lane 3 – negative control (water blank).
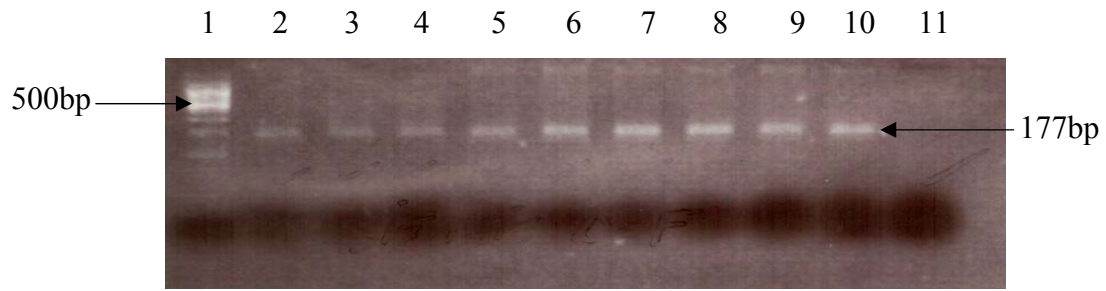
A) *CYP11B2* fragment 1
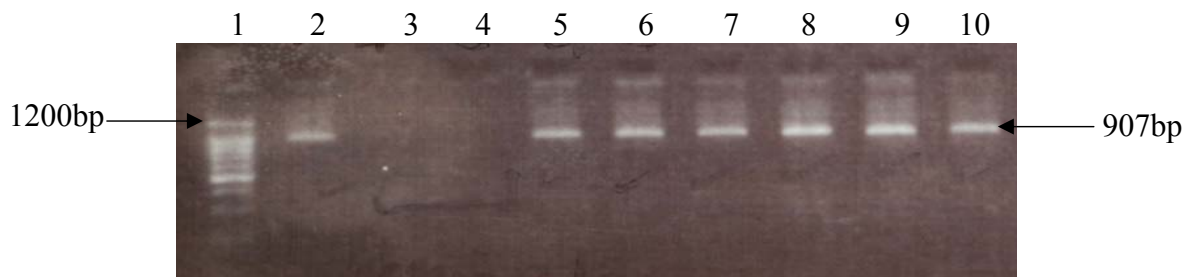
B) *CYP11B2* fragment 2
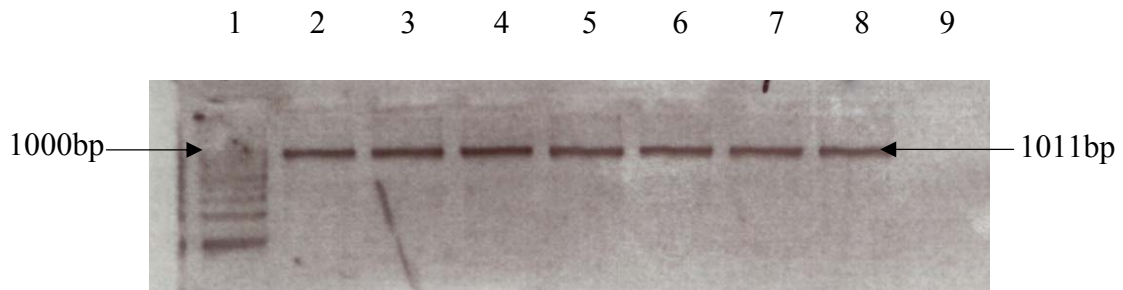
C) *CYP11B2* fragment 4

Figure 3.12: *Representative 2% agarose gel showing PCR amplified CYP11B2 fragments 1, 2, 3 and 4.*
A) Lane 1 – molecular weight marker ; lanes 2-10 – *CYP11B2* fragment 1 amplicons of 9 selected individuals; Lane 11 – negative control (water blank), B) Lane 1 – molecular weight marker; lanes 2, 5-10 – *CYP11B2* fragment 2 amplicons of 7 selected individuals; Lane 3 – no sample loaded; Lane 4 – negative control (water blank) and C) Lane 1 – molecular weight marker; lanes 2-10 – *CYP11B2* fragment 3 amplicons of 9 selected individuals; Lane 11 – negative control (water blank) and D) Lane 1 – molecular weight marker; lanes 2-8 – *CYP11B2* fragment 4 amplicons of 7 selected individuals; Lane 9 – negative control (water blank). Not shown are *CYP11B2* fragments 3 and 5 because the SNPs within these fragments were not considered for the present study.
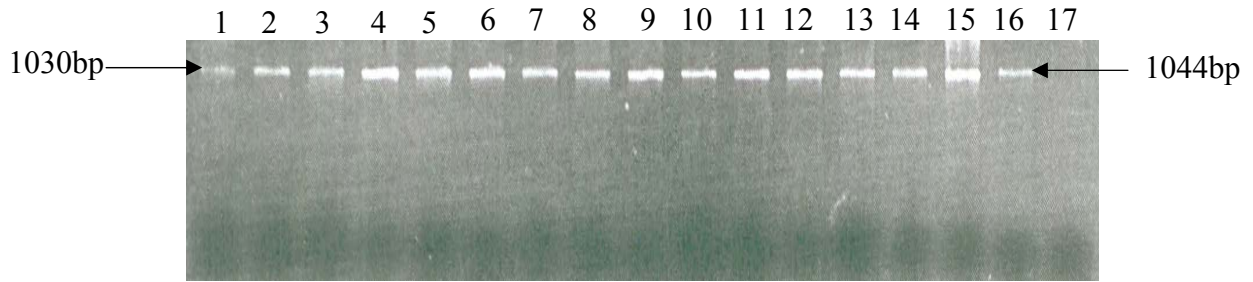
A) *ACE2* fragment 1
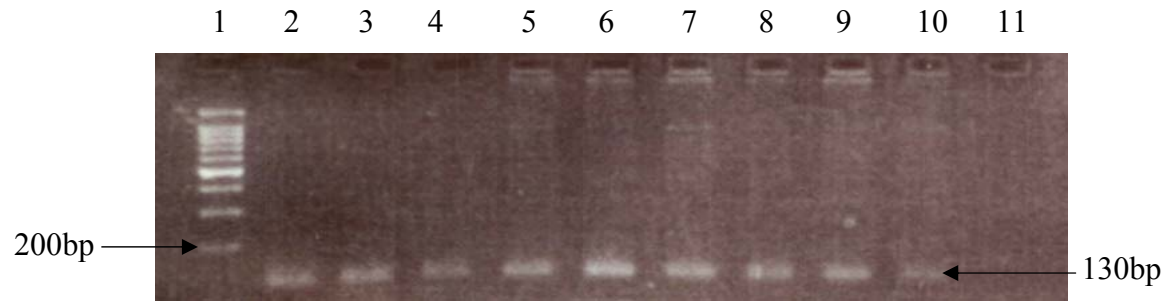
B) *ACE2* fragment 2
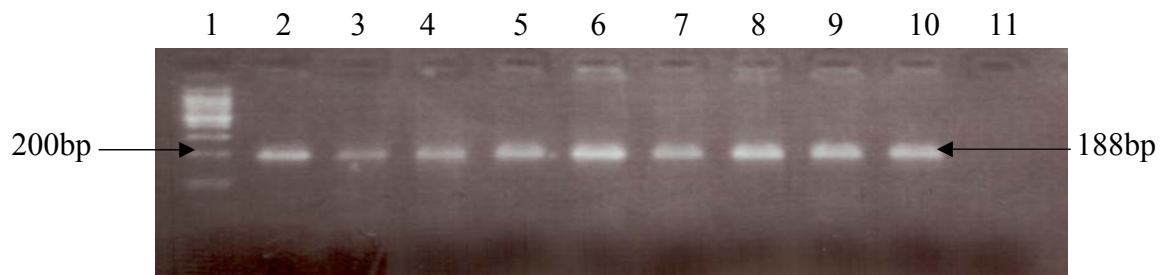
C) *ACE2* fragment 3
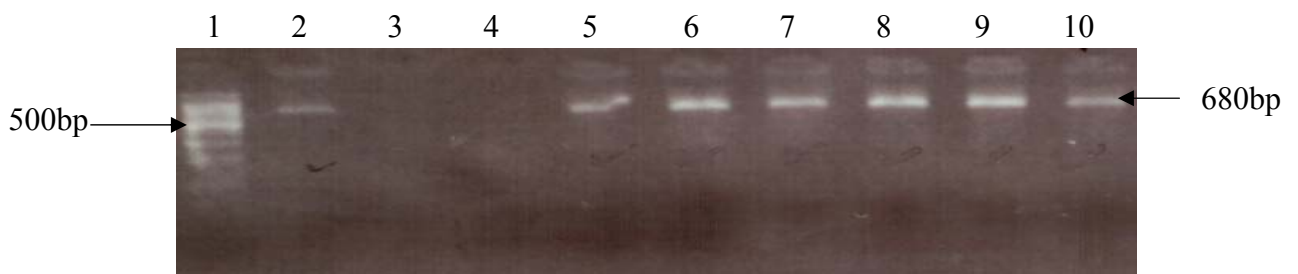
D) *ACE2* fragment 4

Figure 3.13: *Representative 2% agarose gel showing PCR amplified ACE2 fragments 1, 2, 3 and 4.*
A) Lane 1 –PCR amplified marker (fragment of 1030bp in size); lanes 2-16 – *ACE2* fragment 1 amplicons of 15 selected individuals; Lane 17 – negative control (water blank), B) Lane 1 – molecular weight marker; lanes 2-10 – *ACE2* fragment 2 amplicons of 9 selected individuals; Lane 11 – negative control (water blank), C) Lane 1 – molecular weight marker; lanes 2-10 – *ACE2* fragment 3 amplicons of 9 selected individuals; Lane 11 – negative control (water blank) and D) Lane 1 – molecular weight marker; lanes 2, 5-10 – *ACE2* fragment 4 amplicons of 7 selected individuals; Lane 3 – unsuccessful PCR amplification; Lane 4 – negative control (water blank).

3.5. Genotyping of individuals

3.5.1. Genotyping *ACE1* I/D polymorphism by PCR-based method

PCR amplification was used to genotype individuals for the insertion/deletion (I/D) polymorphism located within the *ACE1* gene (figure 3.14). PCR amplification generated a 450bp fragment if the *D*-allele was present or a 737bp fragment if the *I*-allele was present, or both, in the case of heterozygotes.
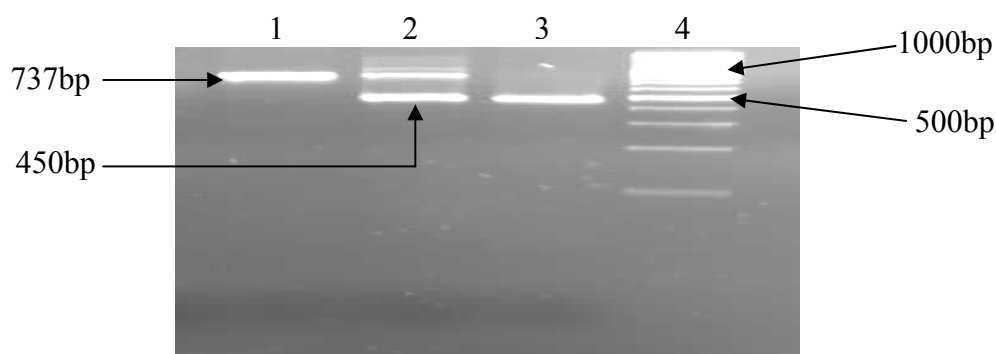
Figure 3.14: *A representative 2% agarose gel of individuals genotyped for ACE1 I/D polymorphism.* Lane 1 = an individual homozygous for the *I*-allele, Lane 2 = an individual heterozygous with both insertion (*I*) and deletion (*D*) alleles present, Lane 3 = an individual homozygous for the *D*-allele and Lane 4 = 100bp Promega marker.

3.5.2. SNaPshot primer extension results

Individuals were genotyped for the two SNPs in *ACE1* fragment 1, one SNP each in *CMA* fragment 1 and fragment 2, one SNP in *AGT* fragment 1 and two SNPs each in *AGT* fragment 2 and fragment 3 using the primer extension technique (Makridakis and Reichardt, 2001) (see section 2.5). The alleles were assigned by Genotyper 3.7 NT software (Applied Biosystems). Figure 3.15 to 3.22 show representative results obtained after single base extension reaction and analysis of *ACE1* rs4298, rs4303, *CMA* rs1800875 and rs1885108, *AGT* rs5051, rs4762, rs699 and rs11122575, respectively, with Genotyper 3.7 NT software (Applied Biosystems). Not shown is a SNaPshot result for *AGT* rs1926723 as a representative electropherogram was unavailable. Only SNPs within *CMA* were multiplexed. Sixteen samples per individual were optimised in single reactions and indicated that different coloured peaks occurred at different positions. For *AGT*, the amplicons containing the investigated SNPs within fragments 1, 2 and 3 were designed for multiplexing but were done as single reactions. The assigned genotypes at each locus are indicated on the figures 3.15 to 3.22.

A   **B**
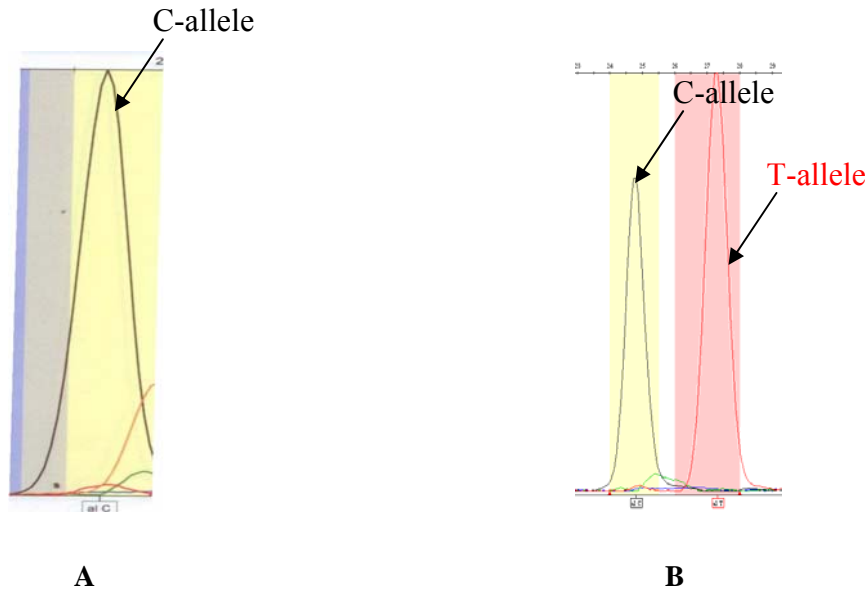
Figure 3.15: *A representative electropherogram of SNP variant analysis of ACE1 rs4298.*
The chromatogram represents analysis of SNaPshot reactions of A) an individual
homozygous for the *C*-allele at rs4298, B) an individual heterozygous for the *C* and *T*-allele at
rs4298. Not indicated an individual homozygous for the *T*-allele at rs4298.

A   **B**
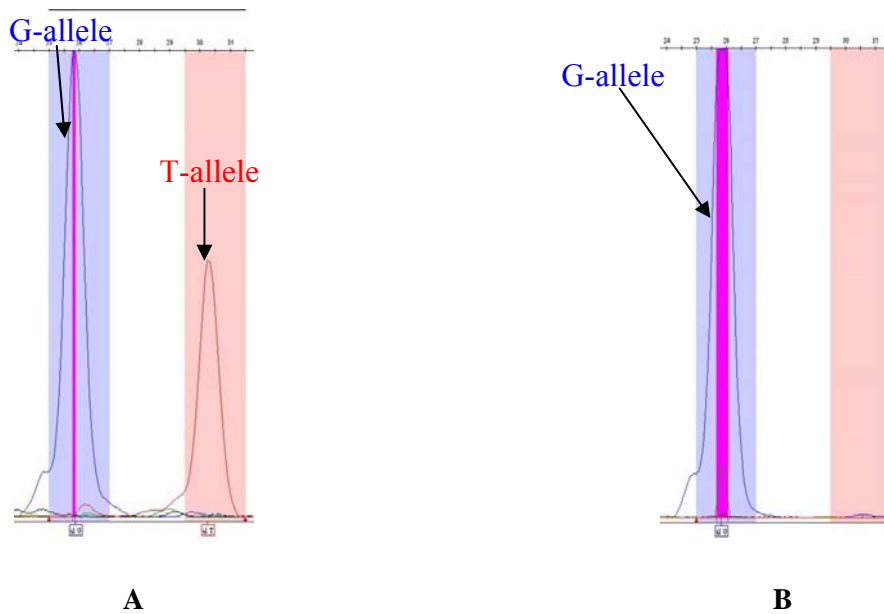
Figure 3.16: *A representative electropherogram of SNP variant analysis of ACE1 rs4303.*
The chromatogram represents analysis of SNaPshot reactions of A) an individual
heterozygous for the *G* and *T*-alleles at rs4303 and B) an individual homozygous for the *G*-
allele at rs4303. Not indicated an individual homozygous for the *T*-allele at rs4303.

A **B** **C**
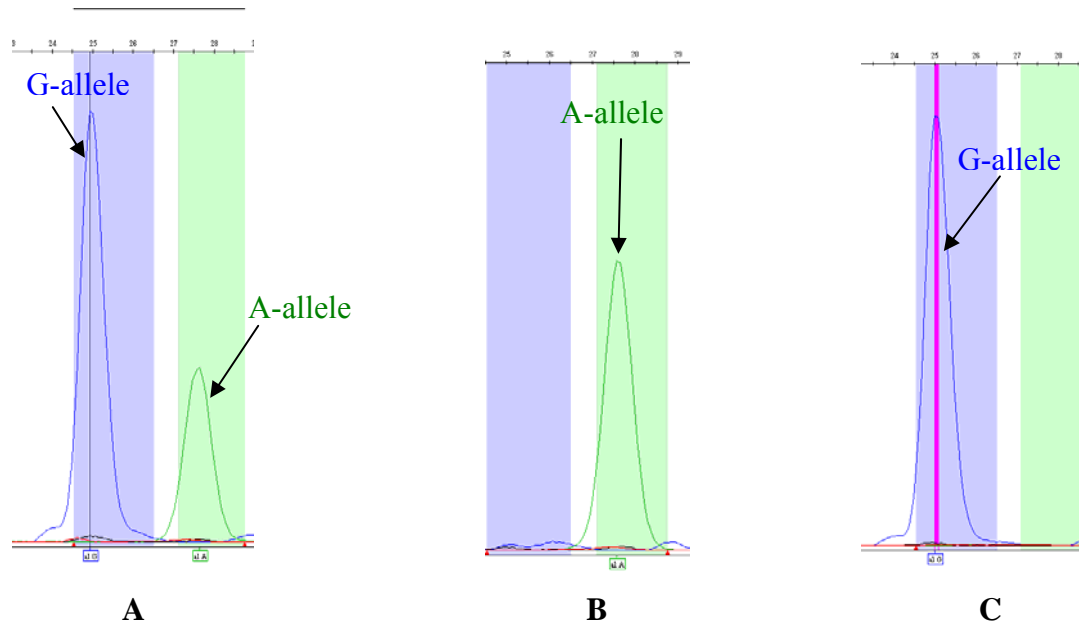
Figure 3.17: *A representative electropherogram of SNP variant analysis of CMA rs1800875.*
The chromatogram represents analysis of SNaPshot reactions of A) an individual
heterozygous for the *G* and *A*-alleles at rs1800875, B) an individual homozygous for the *A*-
alleles at rs1800875 and C) an individual homozygous for the *G*-alleles at rs1800875.

A **B** **C**
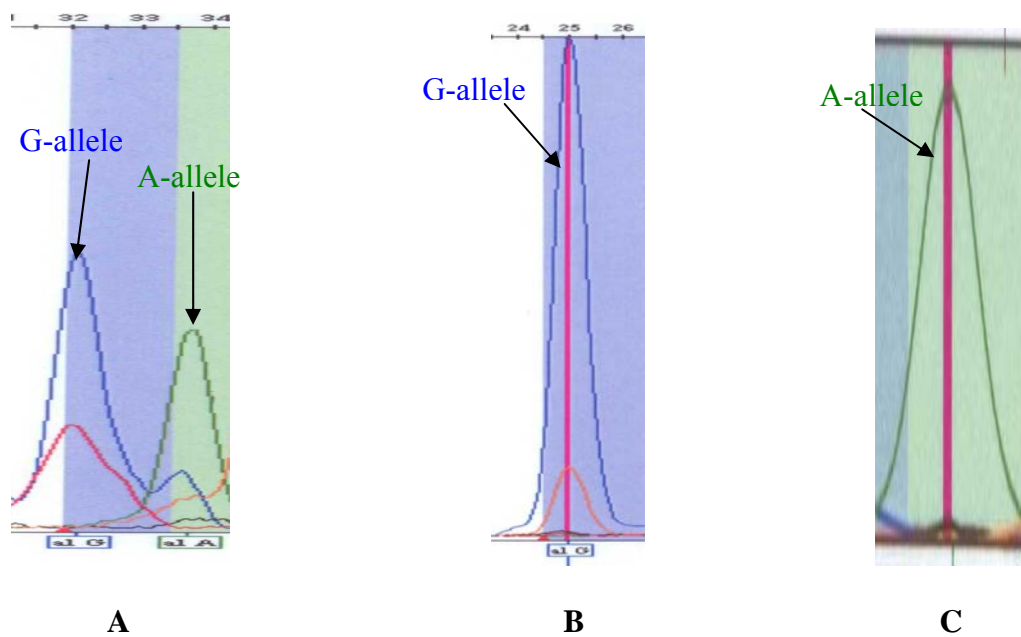
Figure 3.18: *A representative electropherogram of SNP variant analysis of CMA rs1885108.*
The chromatogram represents analysis of SNaPshot reactions of A) an individual
heterozygous for the *G* and *A*-alleles at rs1885108, B) an individual homozygous for the *G*-
alleles at rs1885108 and C) an individual homozygous for the *A*-alleles at rs1885108.
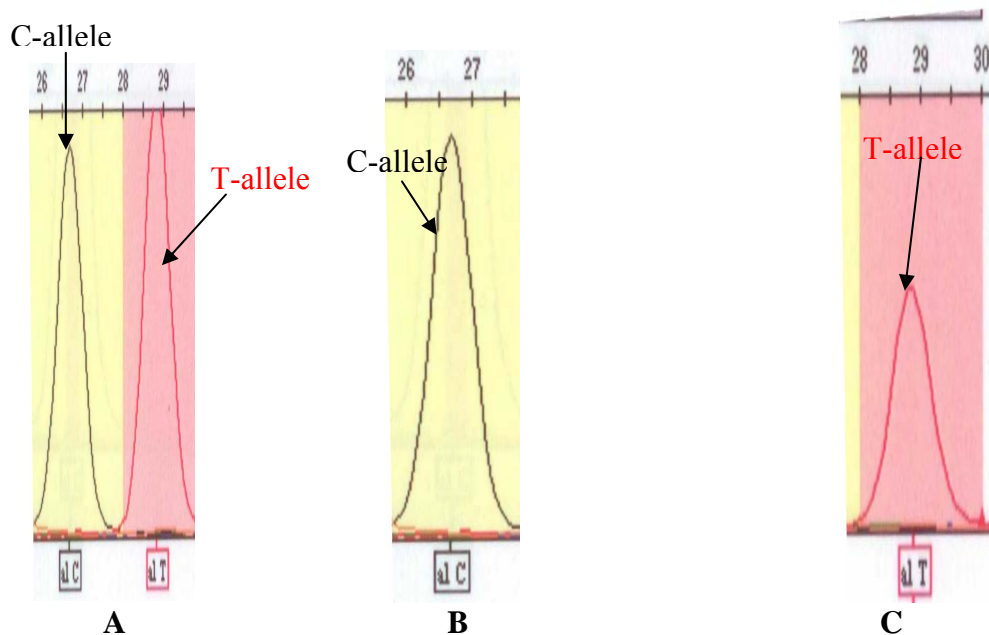
Figure 3.19: *A representative electropherogram of SNP variant analysis of AGT rs5051.* The chromatogram represents analysis of SNaPshot reactions of A) an individual heterozygous for the *C* and *T*-allele at rs5051, B) an individual homozygous for the *C*-allele at rs5051 and C) an individual homozygous for the *T*-allele at rs5051.
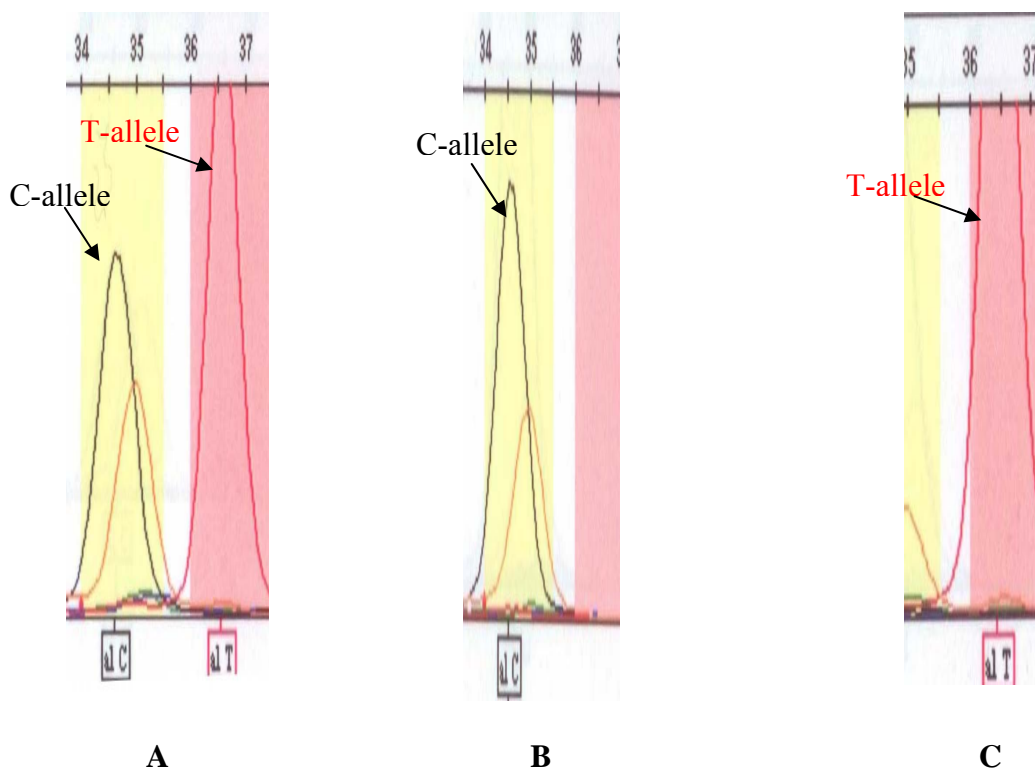
Figure 3.20: *A representative electropherogram of SNP variant analysis of AGT rs699.* The chromatogram represents analysis of SNaPshot reactions of A) an individual heterozygous for the *C* and *T*-allele at rs699, B) an individual homozygous for the *C*-allele at rs699 and C) an individual homozygous for the *T*-allele at rs699.
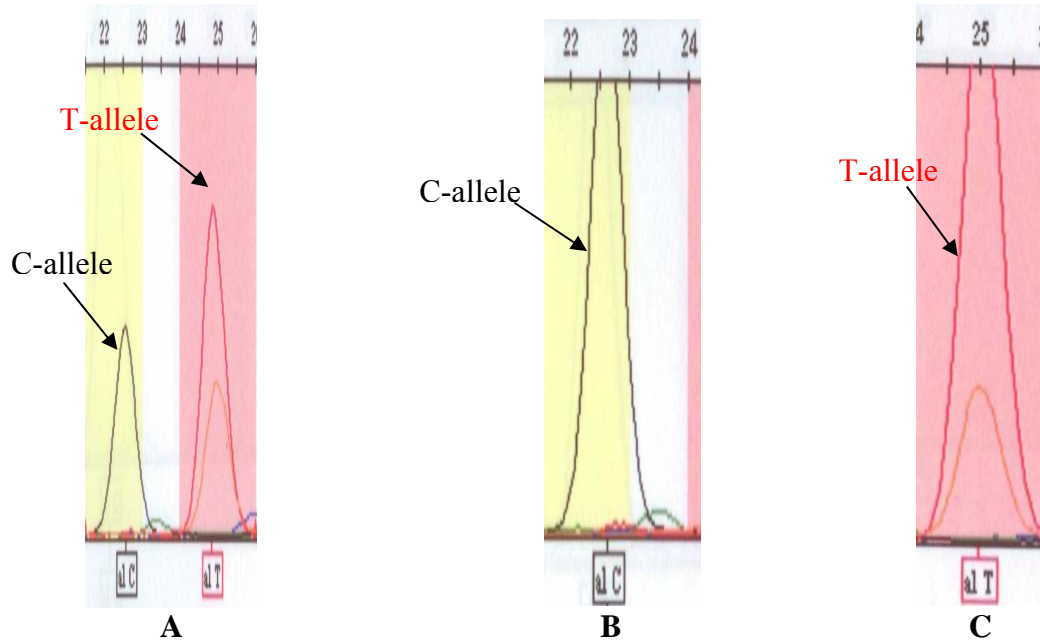
Figure 3.21: *A representative electropherogram of SNP variant analysis of AGT rs4762.* The chromatogram represents analysis of SNaPshot reactions of A) an individual heterozygous for the *C* and *T*-allele at rs4762, B) an individual homozygous for the *C*-allele at rs4762 and C) an individual homozygous for the *T*-allele at rs4762.
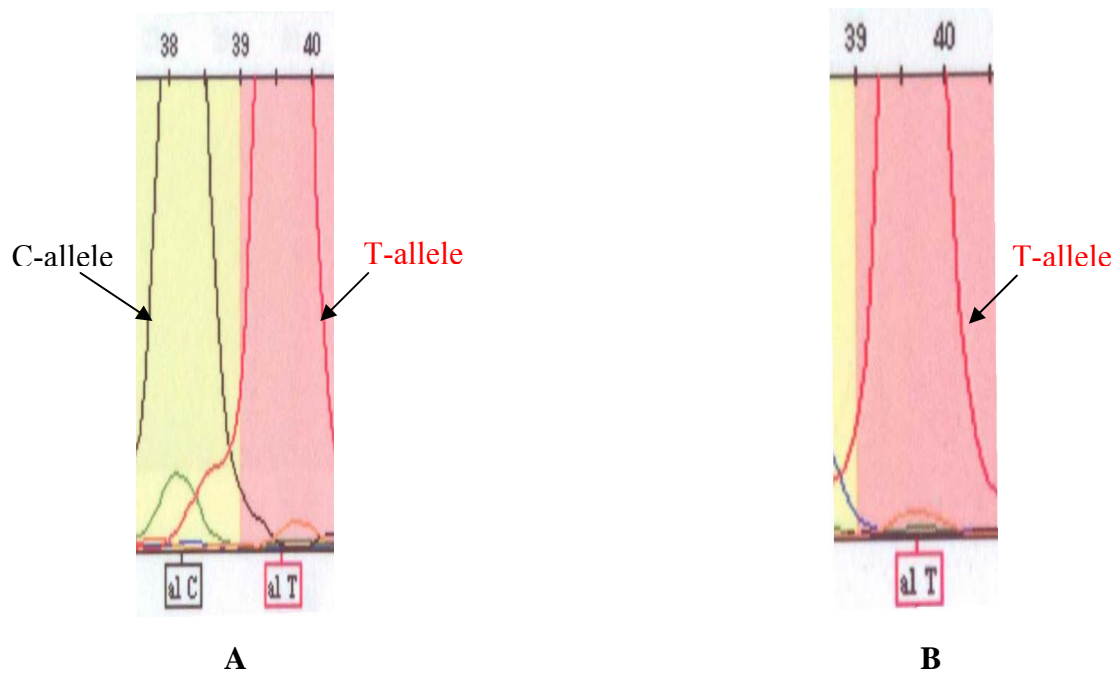
Figure 3.22: *A representative electropherogram of SNP variant analysis of AGT rs11122575.* The chromatogram represents analysis of SNaPshot reactions of A) an individual heterozygous for the *C* and *T*-allele at rs11122575, B) an individual homozygous for the *T*-allele at rs11122575. Not indicated an individual homozygous for the *C*-allele at rs11122575.

3.5.3. Allele Specific restriction enzyme analysis

ACE1/rs4365 polymorphism.

PCR amplification of *ACE1* fragment 3 yielded an 1198bp fragment containing one sequence variant, rs4365 (*A/G*). This fragment contained five invariant *Alu*I restriction sites if the *A*-allele was present and an additional *Alu*I restriction site if the *G*-allele was present. Thus, *Alu*I digestion yielded seven fragments (332bp, 316bp, 175bp, 129bp, 120bp, 86bp and 40bp) for the *G*-allele, while for the *A*-allele six fragments (332bp, 316bp, 261bp, 129bp, 120bp and 40bp) were present (figure 3.23).

Figure 3.23: *ASREA of the ACE1 fragment 3 rs4365 polymorphism.*
A) Representative 12% Polyacrylamide gel showing the fragment sizes generated by *Alu*I restriction enzyme digestion of the 1198bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2 = homozygote *GG*, Lane 3 = heterozygote *AG*, Lane 4 = homozygote *GG*, Lane 5 = uncut. Not indicated is a homozygote AA subject. The small 40bp fragment was not distinguished on this gel. B) Schematic representation of the amplified fragment showing the positions of the *Alu*I restriction sites. * = variable restriction enzyme site. The constant 120bp fragment co-migrated with the 129bp fragment.

CYP11B2/rs1799998 (-344C/T), CYP11B2/rs4539 (2713A/G) and CYP11B2/rs3097 (5937G/A) polymorphisms.

The three sequence variants, rs1799998, rs4539 and rs3097 identified in amplified fragments 1, 2 and 4 of the *CYP11B2* (in the promoter, exonic and 3'UTR region, figure 3.1D), respectively, each alter a restriction enzyme recognition site.

Genotyping the promoter region -344*C/T* variant/rs1799998 of *CYP11B2* was done by digesting the 177bp PCR-amplified fragment 1 containing the polymorphic *Hae*III site. The presence of the *C*-allele creates a *Hae*III restriction enzyme site yielding two fragments of 157bp and 20bp in size after digestion, while the 177bp fragment remained intact in the presence of the *T*-allele (figure 3.24).

PCR amplification of the region containing 2713*A/G* variant/rs4539 yielded a 907bp fragment. The generated amplicon contained only one, polymorphic, *Bsu36I* restriction site. If the *G*-allele is present restriction enzyme digestion with *Bsu*36I yield two fragments of 823bp and 84bp, while the 907bp fragment remained intact in the presence of the *A*-allele (figure 3.25).

PCR amplification of the region where the 5937*G/A* variant/rs3097 resides yielded a 1011bp size fragment containing one invariant *Nsp*I restriction site. For the *G* allele, the fragment was cleaved into two fragments of 744bp and 267bp following *Nsp*I digestion. For the *A* allele, another *Nsp*I restriction site is created and the 744bp fragment was further digested into two fragments of 690bp and 54bp, following restriction enzyme digestion with *Nsp*I (figure 3.26).
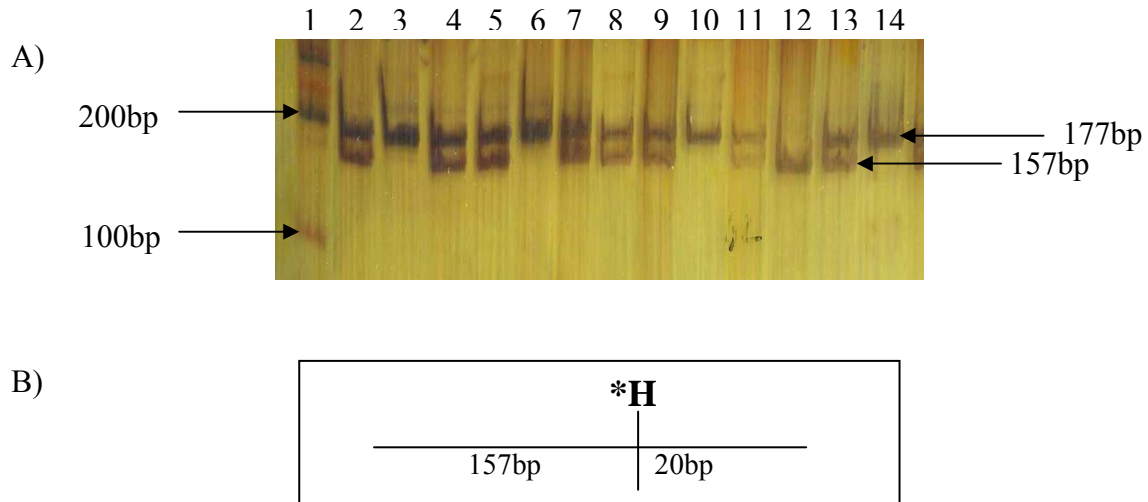
Figure 3.24: *ASREA of the CYP11B2 fragment 1 rs1799998 polymorphism.*
A) Representative 12% polyacrylamide gel showing the fragment sizes generated by *Hae*III restriction enzyme digestion of the 177bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 3, 6, 10 = homozygote *TT*, Lane 2, 4, 5, 7, 8, 9, 11 and 13 = heterozygote *TC*, Lane 12 = homozygote *CC*, Lane 14 = uncut. The small 20bp fragment was not distinguished on this gel. B) Schematic representation of the amplified fragment showing the position of the *Hae*III restriction site. * = variable restriction enzyme site.
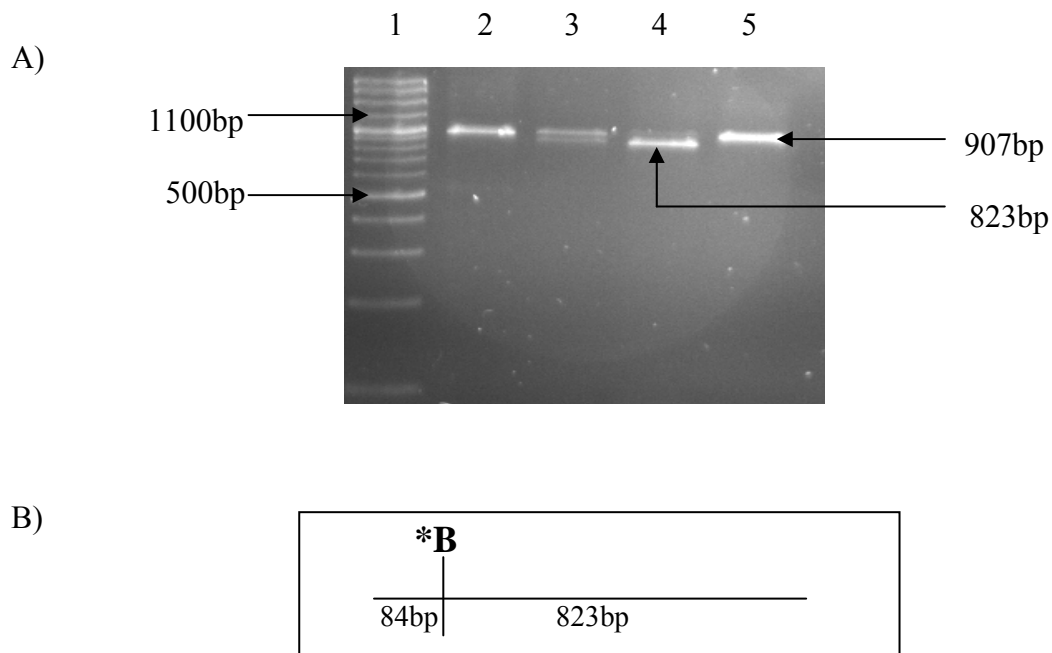
Figure 3.25: *ASREA of the CYP11B2 fragment 2 rs4539 polymorphism.*
A) Representative 1.5% agarose gel showing the fragment sizes generated by *Bsu*36I restriction enzyme digestion of the 907bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2 = homozygote *AA*, Lane 3 = heterozygote *GA*, Lane 4 = homozygote *GG*, Lane 5 = uncut. The small 84bp fragment was not distinguished on this gel. B) Schematic representation of the amplified fragment showing the position of the *Bsu*36I restriction site. * = variable restriction enzyme site.

A)

B)
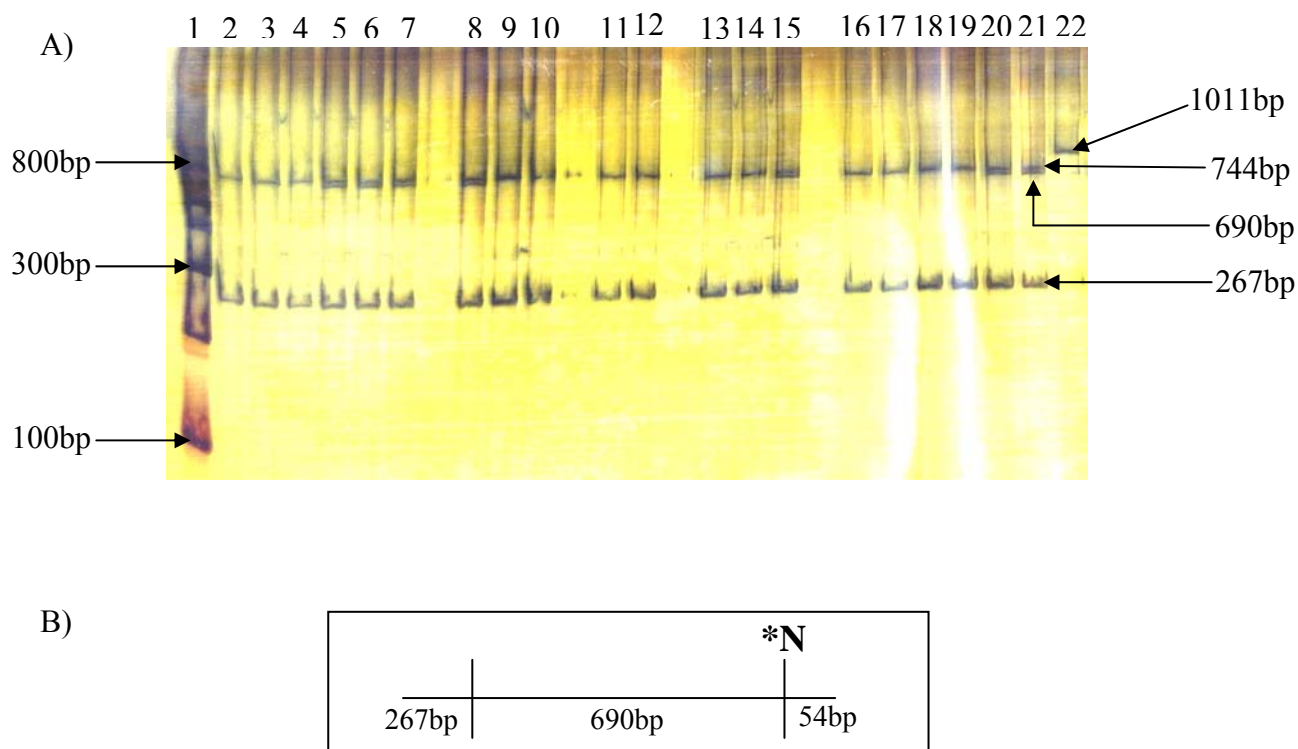
Figure 3.26: *ASREA of the CYP11B2 fragment 4 rs3097 polymorphism.*
A) Representative 12% polyacrylamide gel showing the fragment sizes generated by *Nsp*I restriction enzyme digestion of the 1011bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2, 3, 4, 9, 10 and 13 = homozygote *GG*, Lane 5, 6, 7 and 8 = heterozygote *GA*, Lane 16 and 17 = homozygote *AA*, Lane 22 = uncut. The small 54bp fragment was not distinguished on this gel. B) Schematic representation of the amplified fragment showing the positions of the *Nsp*I restriction sites. * = variable restriction enzyme site.

AGTR1/rs2640539 (C/G), AGTR1/rs3772627 (A/G) and AGTR1/rs5182 (573C/T) polymorphisms.

PCR amplification of the region flanking the rs2640539 polymorphism yielded a 992bp fragment containing a polymorphic *Msp*A1I restriction site. For the *G*-allele, which abolishes the *Msp*A1I site, the 992bp fragment remained undigested, while for the *C*-allele, the creation of an *Msp*A1I site resulted in cleavage of the 992bp fragment into two fragments of 609bp and 383bp in size (figure 3.27).

PCR amplification of the region containing the rs3772627 variant yielded an 1120bp size fragment containing one invariant *Bsah*1I restriction enzyme site. For the *A*-allele, restriction enzyme digestion of the 1120bp fragment 2 by *Bsah1I* yielded two fragments 697bp and 423bp. For the *G*-allele, another *Bsah*1I restriction site is

created, consequently the 697bp fragment was cleaved into two fragments of 496bp and 201bp in size after digestion with *Bsah*1I (figure 3.28).

Amplification of the exonic region of *AGTR1* in which the rs5182 polymorphism resides yielded a 1001bp fragment that contains five invariant *Mnl*I restriction enzyme sites. For the *T*-allele, the 1001bp fragment was cleaved into 422bp, 309bp, 127bp, 68bp, 47bp and 28bp following digestion with *Mnl*I. The presence of the *C*-allele creates an additional restriction site, consequently, the 422bp fragment was digested into two fragments of 325bp and 97bp sizes after digestion with *Mnl*I (figure 3.29).
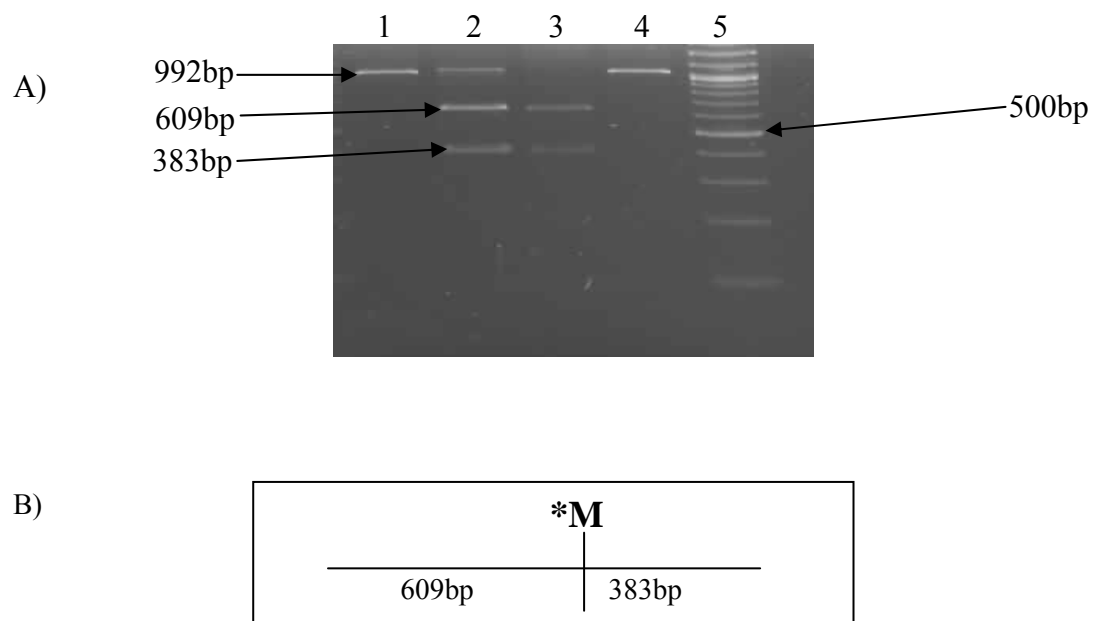
Figure 3.27: *ASREA of the AGTR1 fragment 1 rs2640539 polymorphism.*
A) Representative 1.5% agarose gel showing the fragment sizes generated by *Msp*A1I restriction enzyme digestion of the 992bp PCR-amplified fragment. Lane 1= homozygote *GG*, Lane 2 = heterozygote *CG*, Lane 3 = homozygote *CC*, Lane 4 = uncut, Lane 5 = 100bp marker. B) Schematic representation of the amplified fragment showing the position of the *Msp*A1I restriction site. * = variable restriction enzyme site.
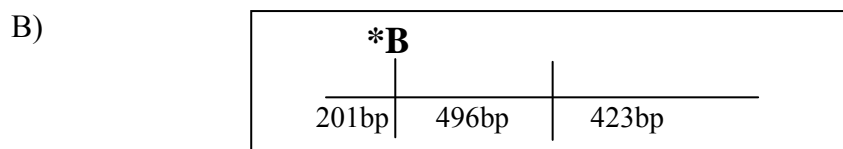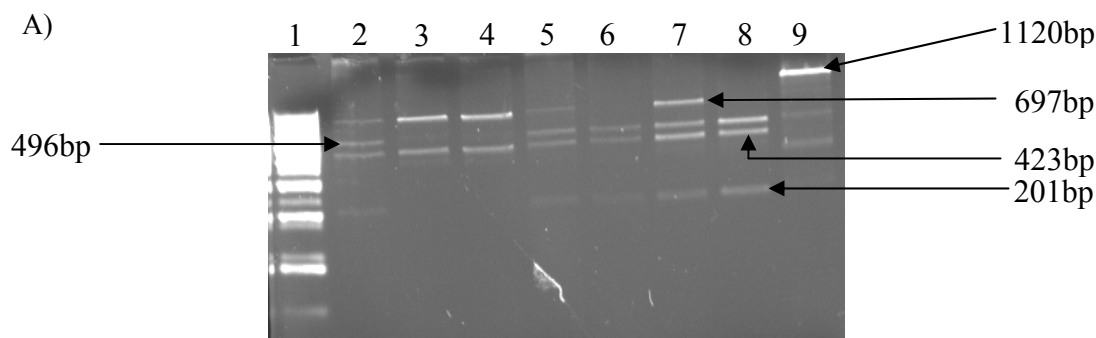
Figure 3.28: *ASREA of the AGTR1 fragment 2 rs3772627 polymorphism.*
A) Representative 1.5% agarose gel showing the fragment sizes generated by *Bsah*1I restriction enzyme digestion of the 1120bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 3 and 4 = homozygote *AA*, Lane 2, 5 and 7 = heterozygote *GA*, Lanes 6 and 8 = homozygote *GG*, Lane 9 = uncut. B) Schematic representation of the amplified fragment showing the positions of the *Bsah*1I restriction sites. * = variable restriction enzyme site.
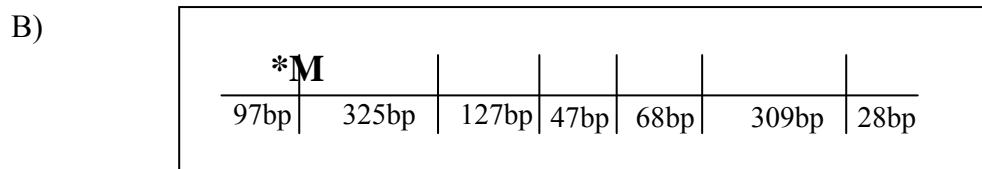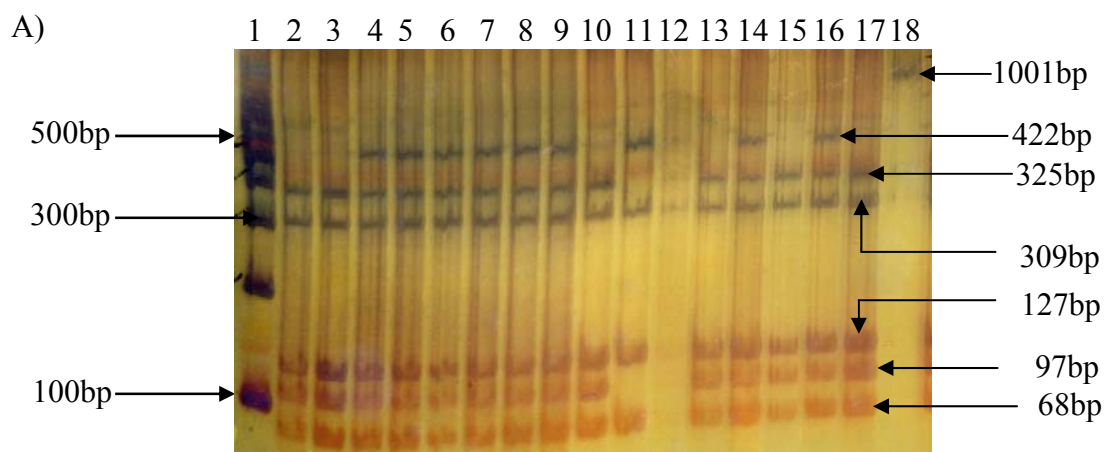
Figure 3.29: *ASREA of the AGTR1 fragment 3 rs5182 polymorphism.*
A) Representative 12% polyacrylamide gel showing the fragment sizes generated by *Mnl*I restriction enzyme digestion of the 1001bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2, 3, 13, 15 and 17 = homozygote *CC*, Lane 4, 5, 6, 7, 8, 9, 14 and 16 = heterozygote *CT*, Lane 11 = homozygote *TT*, Lane 18 = uncut. Lane 10 = partial digest homozygote *CC*. The smaller 47bp and 28bp fragments could not be distinguished on this gel. B) Schematic representation of the amplified fragment showing the positions of the *Mnl*I restriction sites. * = variable restriction enzyme site.

ACE2/rs1978124 (1075A/G), ACE2/rs2285666 (8790G/A), ACE2/rs879922 (28330C/G) and ACE2/rs4646179 (C/T) polymorphisms.

PCR amplification of the various intronic regions of *ACE2* in which the above four polymorphisms reside generated fragment sizes of 1044bp, 130bp, 188bp and 680bp, respectively, which were used to genotype study subjects. The amplicon 1044bp contains one polymorphic *Sau*96I restriction site used for genotyping rs1978124. For the *G*-allele, restriction enzyme digestion with *Sau*96I yields two fragments of 962bp and 82bp, while the 1044bp fragment remains intact in the presence of the *A*-allele (figure 3.30).

The 130bp amplicon contains one polymorphic *AluI* restriction site used for genotyping rs2285666. For the *A*-allele the 130bp fragment is cleaved into two fragments 78bp and 52bp after *AluI* digestion, while the 130bp fragment remains intact in the presence of the *G*-allele (figure 3.31).

The 188bp PCR amplified fragment contained one polymorphic *BfaI* restriction site that was used to genotype rs879922. For *C*-allele carriers, the 188bp fragment remained intact after digestion with *BfaI* as no restriction site is present, while the *G*-allele creates a *BfaI* site resulting in the cleavage of the 188bp fragment into two fragments of 146bp and 42bp (figure 3.32).

The 680bp amplicon in which rs4646179 resides contains two invariant *HpyCH4IV* restriction enzyme sites. For the *T*-allele, the 680bp fragment yielded three fragments (402bp, 257bp and 21bp) after digestion, while the *C*-allele creates an additional restriction enzyme site, that after cleavage yielded four fragments (402bp, 180bp, 77bp and 21bp) (figure 3.33).
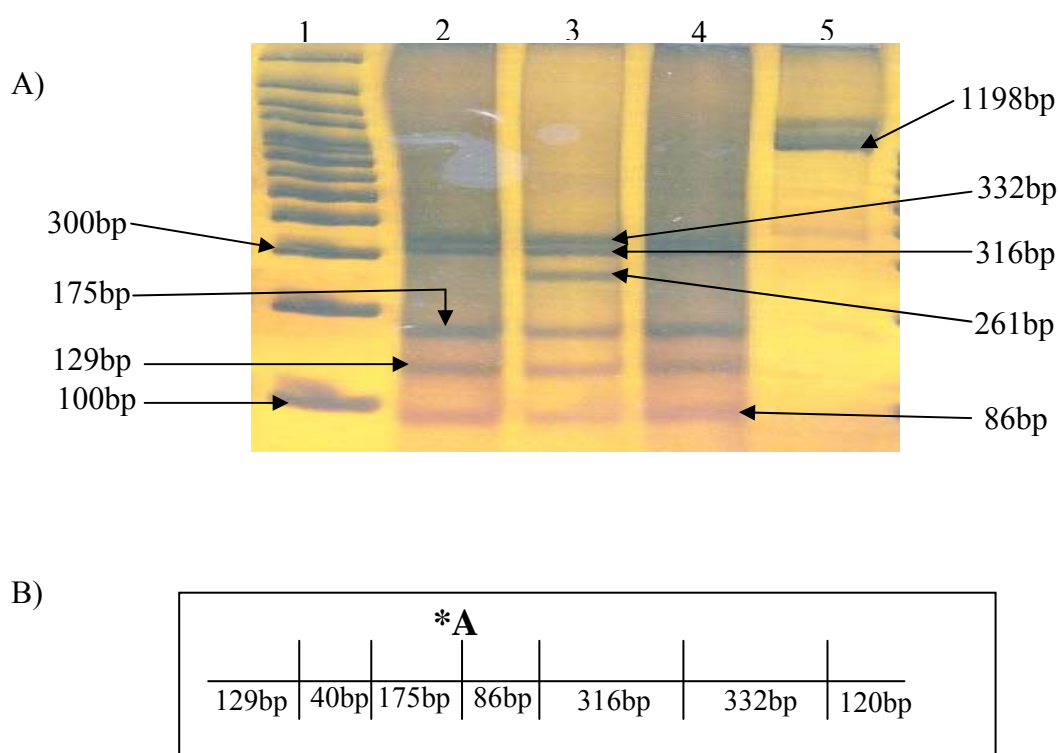
A)

B)
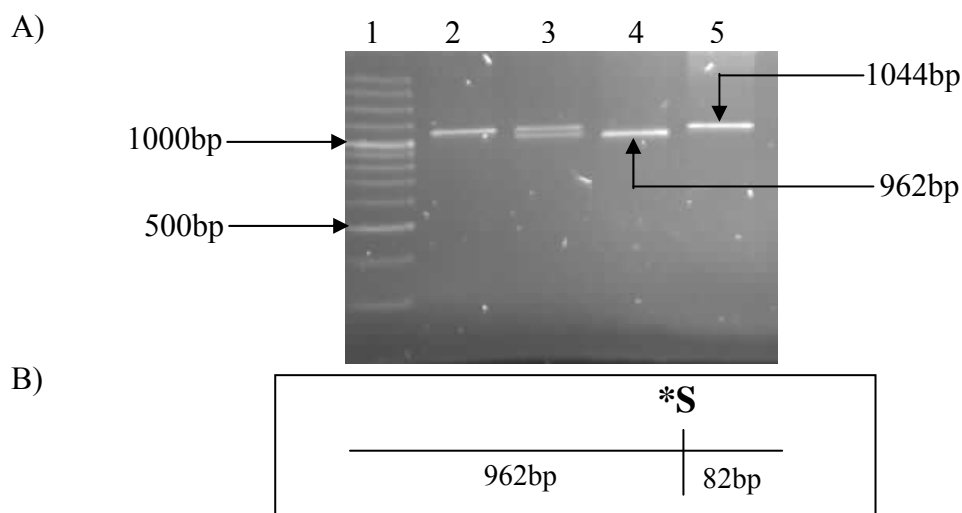
Figure 3.30: *ASREA of the ACE2 fragment 1 rs1978124 polymorphism.*
A) Representative 1.5% agarose gel showing the fragment sizes generated by *Sau*96I restriction enzyme digestion of the 1044bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2 = homozygote *AA*, Lane 3 = heterozygote *GA*, Lane 4 = homozygote *GG*, Lane 5 = uncut. The smaller 82bp fragment could not be distinguished on this gel. B) Schematic representation of the amplified fragment showing the position of the *Sau*96I restriction site. * = variable restriction enzyme site.

A)

B)
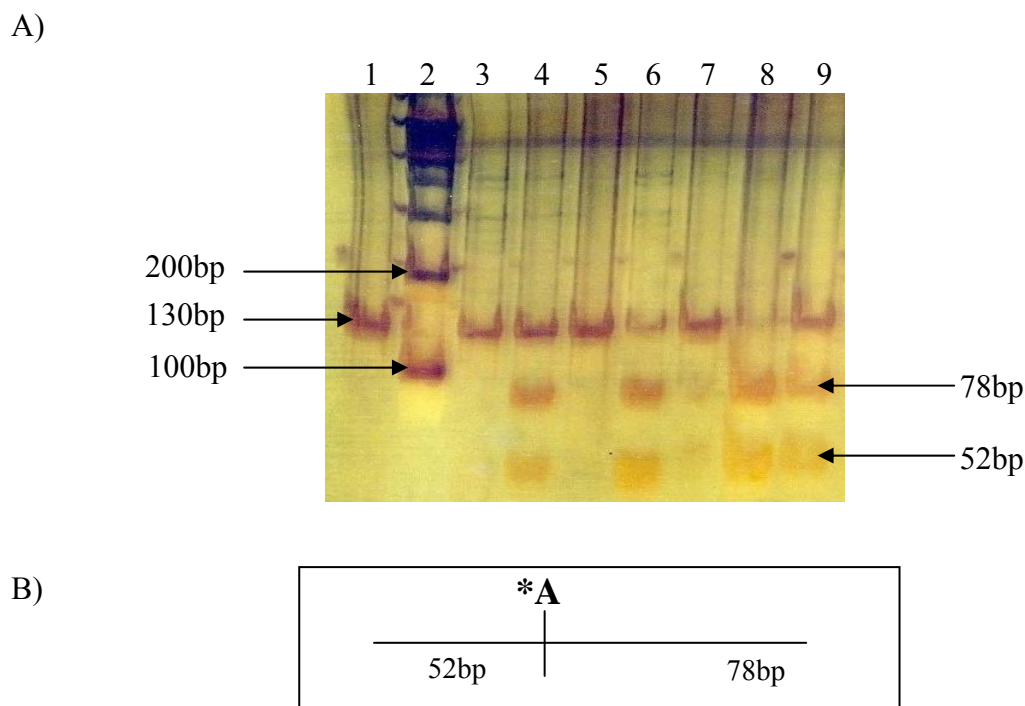
Figure 3.31: *ASREA of the ACE2 fragment 2 rs2285666 polymorphism.*
A) Representative 12% polyacrylamide gel showing the fragment sizes generated by *Alu*I restriction enzyme digestion of the 130bp PCR-amplified fragment. Lane 1 = uncut, Lane 2 = 100bp marker, Lane 3 and 5 = homozygote *GG*, Lane 4, 6 and 9 = heterozygote *GA*, Lane 8 = homozygote *AA*. B) Schematic representation of the amplified fragment showing the position of the *Alu*I restriction site. * = variable restriction enzyme site.

A)

B)
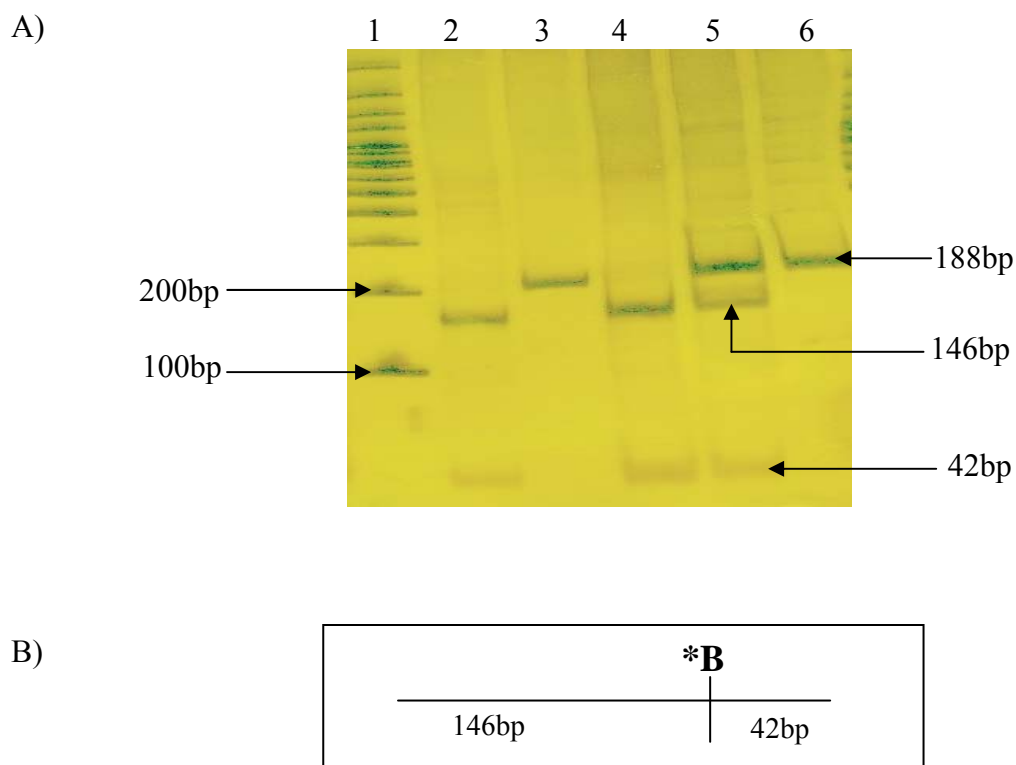
Figure 3.32: *ASREA of the ACE2 fragment 3 rs879922 polymorphism.*
A) Representative 12% polyacrylamide gel showing the fragment sizes generated by *Bfa*I restriction enzyme digestion of the 188bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2 and 4 = homozygote *GG*, Lane 3 = homozygote *CC*, Lane 5 = heterozygote *GC*, Lane 6 = uncut. B) Schematic representation of the amplified fragment showing the position of the *Bfa*I restriction site. * = variable restriction enzyme site.

A)

B)
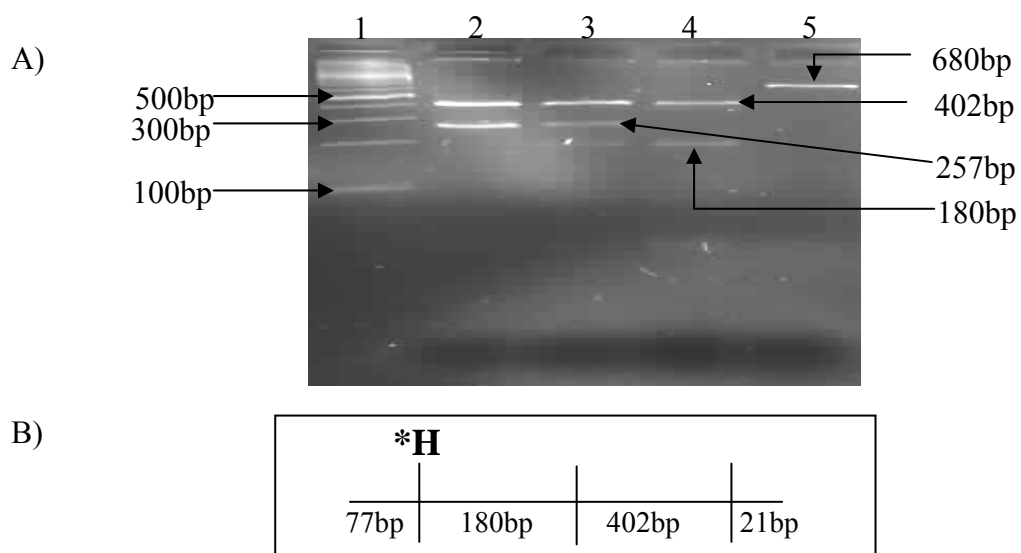
Figure 3.33: *ASREA of the ACE2 fragment 4 rs4646179 polymorphism.*
A) Representative 1.5% agarose gel showing the fragment sizes generated by *HpyCH*4IV restriction enzyme digestion of the 680bp PCR-amplified fragment. Lane 1= 100bp marker, Lane 2 = homozygote *TT*, Lane 3 = heterozygote *CT*, Lane 4 = homozygote *CC*, Lane 5 = uncut. The smaller 77bp and 21bp are not resolved on this gel. B) Schematic representation of the amplified fragment showing the position of the *HpyCH*4IV restriction site. * = variable restriction enzyme site.

3.6. Automated sequence analysis

To assess the accuracy of SNaPshot analysis, two individuals from each of 16 of the 22 pedigrees studied were selected for sequence analysis. Pairs of individuals chosen were never from the same nuclear family. Thus, the sequence of the regions encompassing each SNP genotyped by SNaPshot in *ACE1*, *AGT* and *CMA* were recorded in 32 individuals per SNaPshot SNP (representing about 10% of the total HCM cohort). Shown are representative sequencing results for individuals in the HCM panel for *ACE1* fragments 1, *CMA* fragment 2 and *AGT* fragments 1 and 3 (figures 3.34-3.37). A 100% correlation was found between SNaPshot results for variants used in this study and automated sequencing results.
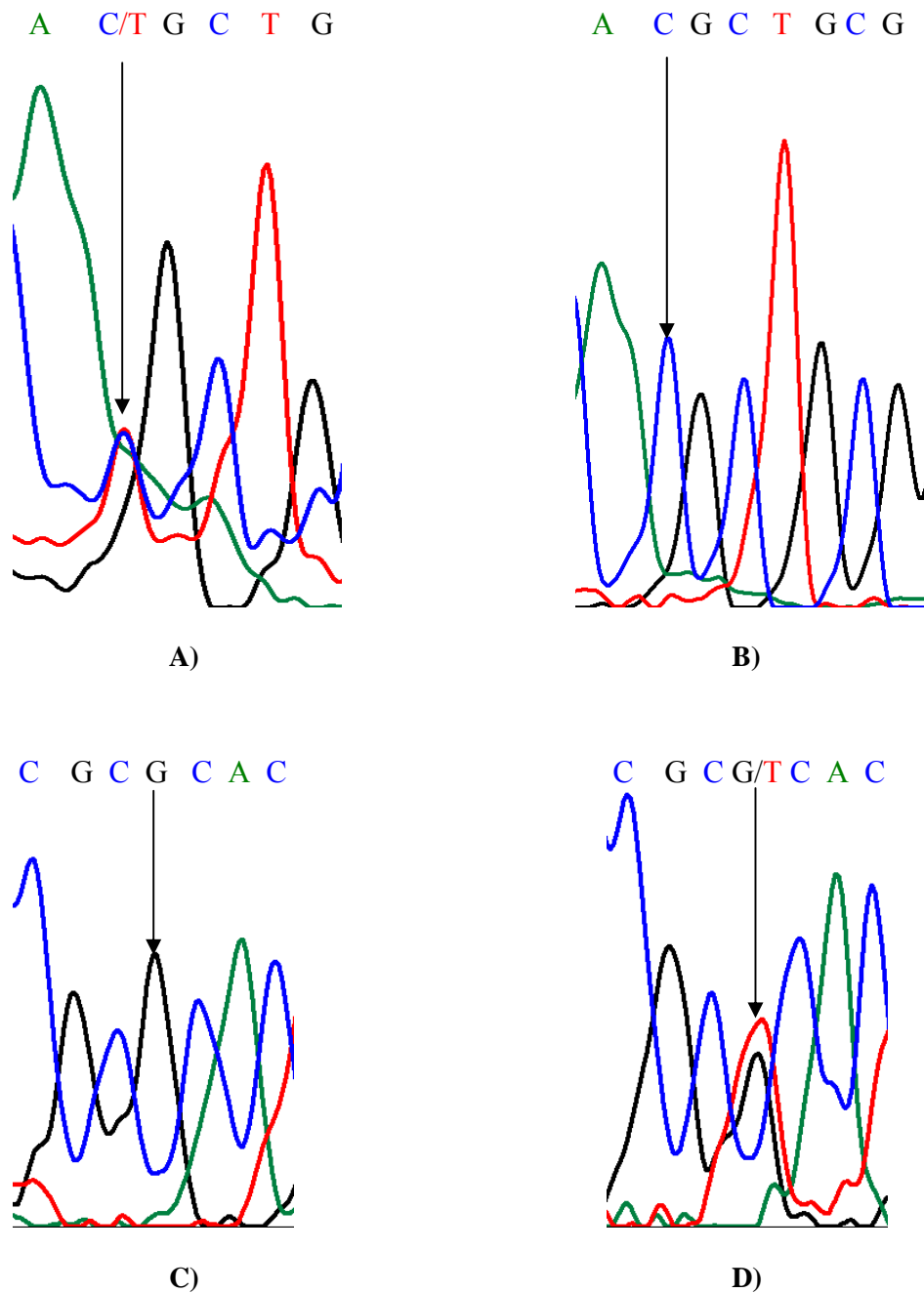
Figure 3.34: *A representative sequence analysis of fragment 1 of ACE1 in the HCM panel.* Chromatogram indicating the partial nucleotide sequences of three individuals in the HCM panel with variant patterns. A) Individual 5202 heterozygous (C/T) at rs4298, B) individual 4778 homozygous (CC) at rs4298, C) individual 4778 homozygous (GG) at rs4303 and D) individual 5143 heterozygous (G/T) at rs4303 with the position of the sequence variation indicated by an arrow.
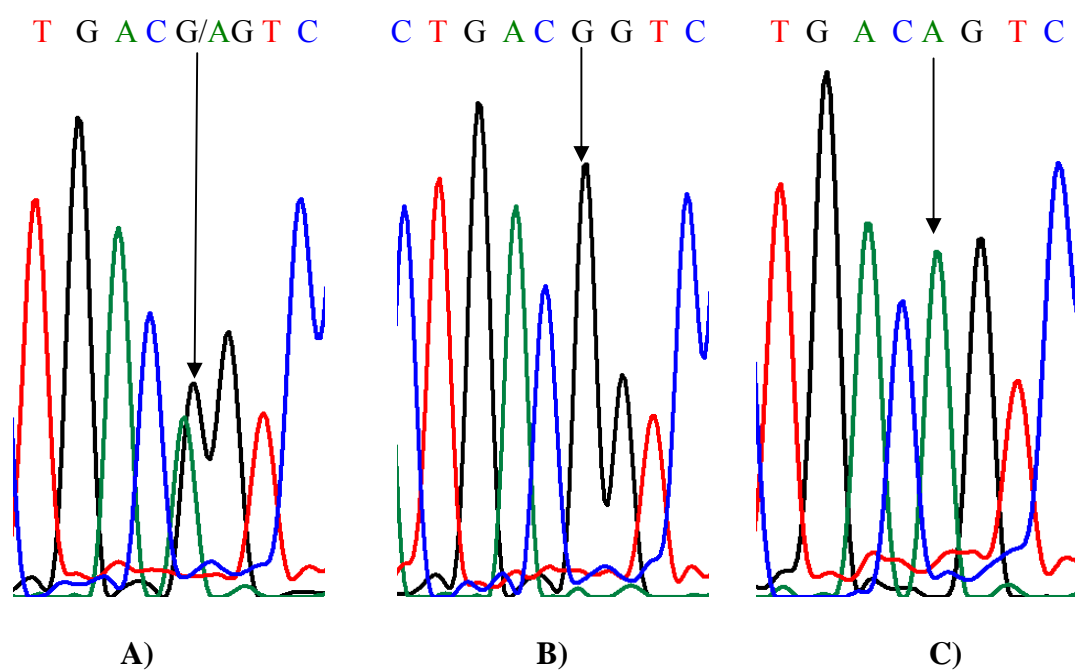
Figure 3.35: *A representative sequence analysis of fragment 2 of CMA in the HCM panel.* Chromatogram indicating the partial nucleotide sequences of three individuals in the HCM panel with variant patterns. A) Individual 5189 heterozygous (G/A) at rs1885108, B) individual 5215 homozygous (GG) at rs1885108 and C) individual 4773 homozygous (AA) at rs1885108 with the position of the sequence variation indicated by an arrow.
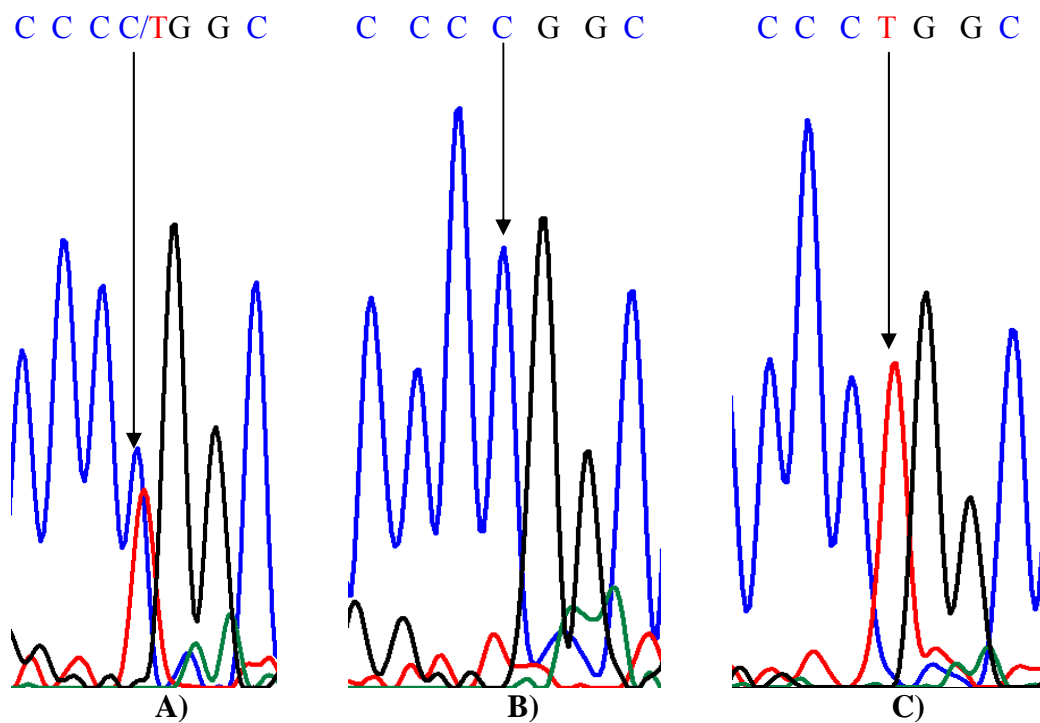
Figure 3.36: *A representative sequence analysis of the reverse strand of AGT fragment 1 in the HCM panel.*
Chromatogram indicating the partial nucleotide sequences of three individuals in the HCM panel with variant patterns. A) Individual 5295 heterozygous (C/T) at rs5051, B) individual 898 homozygous (CC) at rs5051 and C) individual 4367 homozygous (TT) at rs5051 with the position of the sequence variation indicated by an arrow. * The sequence above is in the reverse orientation.
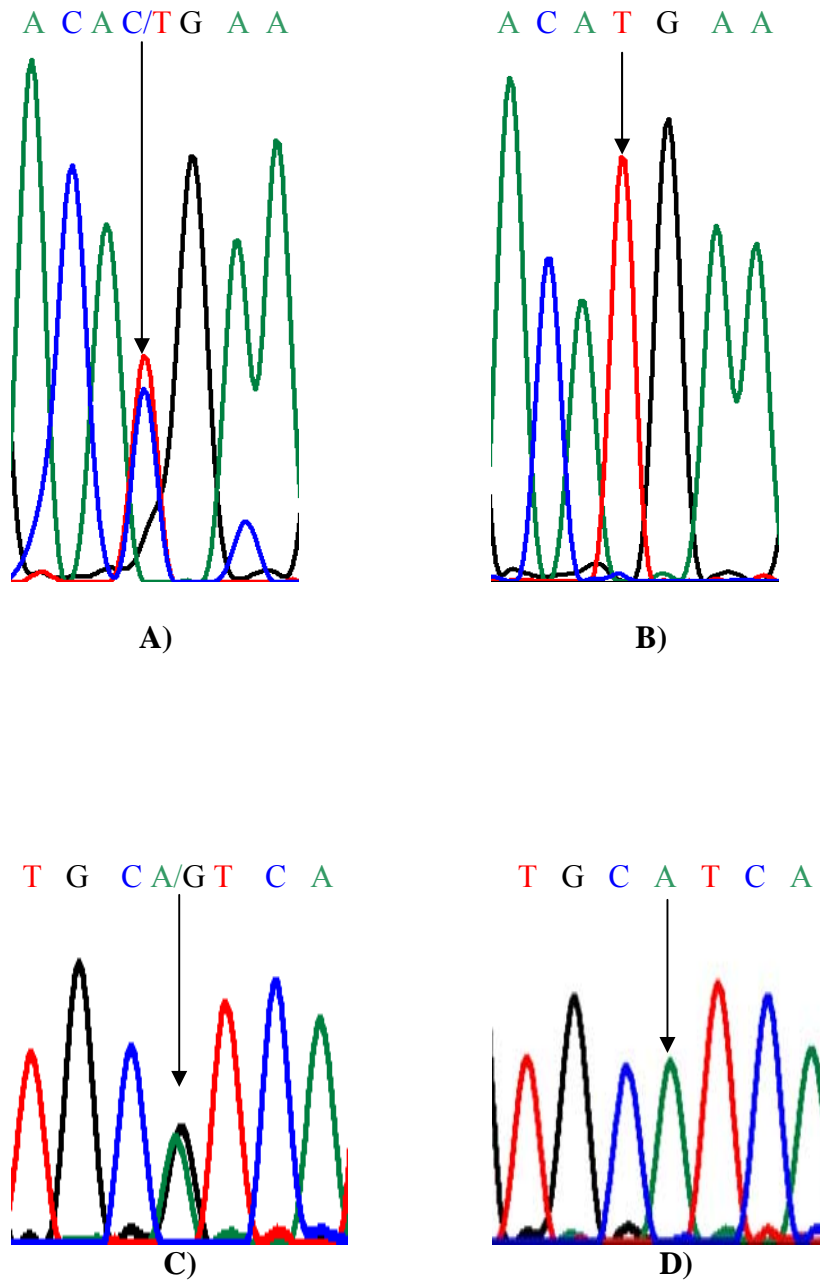
Figure 3.37: *A representative sequence analysis of fragment 3 of AGT in the HCM panel.* Chromatogram indicating the partial nucleotide sequences of three individuals in the HCM panel with variant patterns. A) Individual 4773 heterozygous (C/T) at rs11122575, B) individual 1148 homozygous (TT) at rs11122575 C) individual 5437 heterozygous (G/A) at rs1926723 and D) individual 1148 homozygous (AA) at rs1926723 with the position of the sequence variation indicated by an arrow.

3.7. Statistical analysis

The genotypic and clinical information was used in the context of the family relationships and were analysed statistically by Dr. Lize van der Merwe from the MRC biostatistics unit, except for Hardy-Weinberg equilibrium (HWE) testing and LD determination with Haploview which were analysed by the author.

.

3.7.1. Distribution of variables (Summary statistics)

PEDSTATS (Abecasis et al., 2002) produced descriptive statistics for the six RAAS genes and 25 quantitative traits and three composite scores. The graphical summaries of the initial exploratory statistics (PEDSTATS) have been formatted on compact disk (see back of dissertation). PEDSTATS revealed a mistake in the data; one person with mIVSmit of 110mm instead of 11mm which was corrected before continuing with statistical analysis. Additionally, this analysis revealed slight skewness and curtosis of the data, however, as this could be interpreted to reflect the different groups of individuals within the cohorts, data was not transformed. Furthermore, this analysis included checks for errors of Mendelian inheritance in the genotype data. These genotypes were re-evaluated by revisiting the raw data to monitor database transcription errors. Where inheritance errors were not due to transcription mistakes, re-genotyping using newly generated PCR products was performed not only for the individual in whom the genotype was in question, but for the whole nuclear family involved.

3.7.2. Hardy-Weinberg equilibrium (HWE) testing and LD determination

In pedigree 131 two generations of double first cousins were present, creating pedigree loops. Hence, the whole pedigree was excluded for the purposes of HWE and LD analysis with Haploview vs3.32. The remaining pedigrees were used for HWE testing and LD determination. We did not find any significant deviation from the HWE for any of the SNPs.

Pairwise LD was estimated between the markers of each of the six RAAS genes (figures 3.38 to 3.43). The LOD score is a measure of confidence in the value of D'. A low LOD score corresponds to a low frequency of the polymorphism. In *AGT*, rs1926723 and rs11122575 were in strong LD, and rs699 was in strong LD with rs5051, the latter pair creating a haplotype block of 4kb (figure 3.39). None of the

SNPs investigated within *ACE1*, *AGTR1*, *CYP11B2 and ACE2* were in strong LD with each other (figures 3.38, 3.40, 3.41 and 3.43). The two SNPs in *CMA* were also in strong LD (figure 3.42) as indicated by the red square.
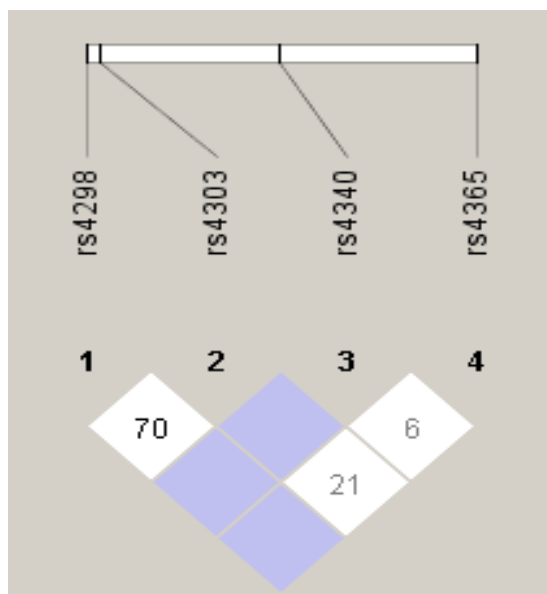
Fig3.38: Plot of LD between *ACE1* markers in the HCM cohort.
D'-values are shown on the squares (%). White squares indicate D'-values < 100% and LOD score<2 and blue squares indicate D' =100% and LOD score <2.
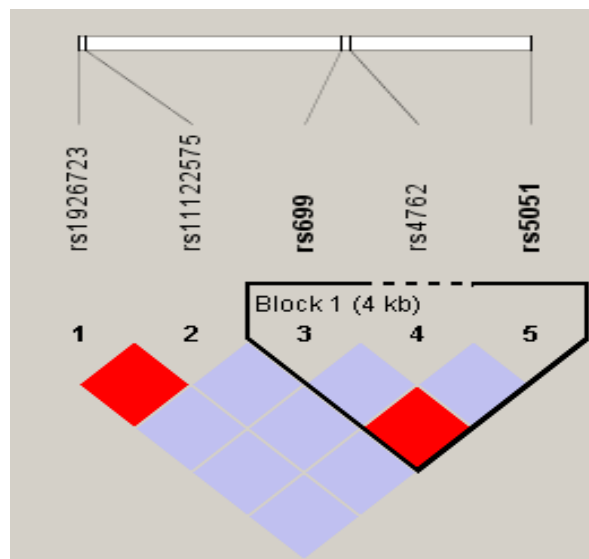
Fig3.39: Plot of LD between *AGT* markers in the HCM cohort.
Blue squares indicate D' =100% and LOD score <2, while red squares indicate D'=100% and LOD score ≥2. The haplotype block extending from rs699 through rs5051, as determined by the confidence intervals method of Gabriels et al. (2002) is indicated in black.
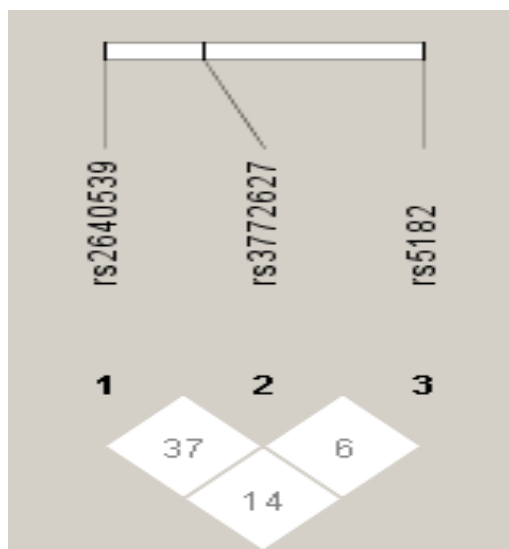
Fig3.40: Plot of LD between *AGTR1* markers in the HCM cohort.
D'-values are shown on the squares (%). White squares indicate the D'-values< 100% and LOD score<2.
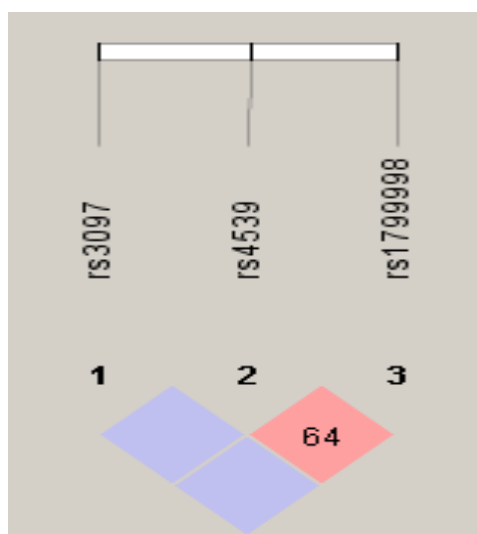
Fig3.41: Plot of LD between *CYP11B2* markers in the HCM cohort.
D'-values are shown on the squares (%). Blue squares indicate D' =100% and LOD score <2, while the pink square shows the D'-values < 100% and LOD score ≥2.
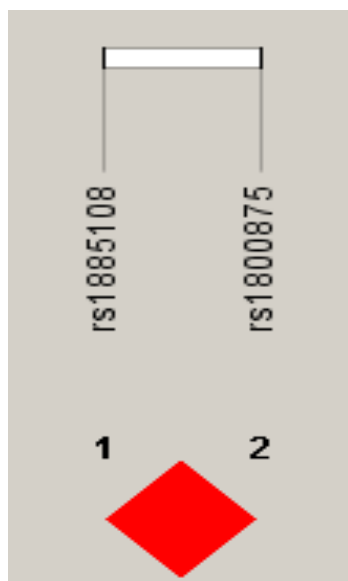
Fig3.42: Plot of LD between *CMA* markers in the HCM cohort.
The red square indicates D'=100% and LOD score ≥2.
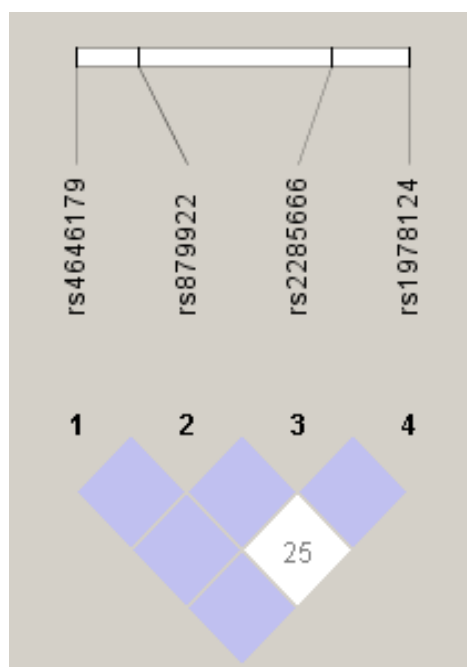
Fig3.43: Plot of LD between *ACE2* markers in the HCM cohort.
D'-values are shown on the squares (100%). The white square indicate D'-values< 100% and LOD
score<2 and the blue squares indicate D'=100% and LOD<2.

3.7.3. Population stratification tests

Table 3.2 contains the p-values for the population stratification tests. Tests were adjusted for all covariates including mutation group. Only three SNPs were non-informative for testing population stratification and were omitted from table 3.2: namely, rs4303 (*ACE1*), rs1112257 (*AGT*) and rs1926723 (*AGT*). The majority of the p-values for the population stratification tests were not statistically significant but for some traits, for a few SNPs, highlighted in bold in table 3.2, statistical significance was reached. Because association was tested using the orthogonal model, which accounts for population stratification, the further results are protected against it.

Table 3.2: The p-values for population stratification test for entire cohort, adjusted for mutation groups and all other covariates.

	ACE1			AGT			AGTR1			CYP11B2			CMA	
Trait	rs4340 I/D	rs4303	rs4356	rs699	rs4762	rs5051	rs2640539	rs3772627	rs5182	rs1799998	rs4539	rs3097	rs1885108	rs1800875
LVM	0.8792	0.9446	0.6400	0.8786	0.1829	0.9004	0.4998	**0.0240**	0.4969	0.9668	0.0494	0.8883	0.6536	0.4122
mIVS	0.6459	0.2610	0.7202	0.3952	0.0735	0.6791	0.1437	**0.0270**	0.5386	0.5322	0.1063	0.7411	0.4451	0.5071
mLVWT	0.6950	0.3243	0.7859	0.2875	0.0789	0.4879	0.1424	**0.0161**	0.4485	0.5368	0.1104	0.9187	0.4248	0.4464
mPWT	0.2239	0.8056	0.2336	0.7711	0.7553	0.6056	0.2660	0.4478	0.6856	0.5969	0.1928	0.0231	0.1439	0.4975
Maron-Spirito	1.0000	0.7883	0.8157	0.4311	0.0944	0.7066	0.1585	**0.0278**	0.4859	0.3381	**0.0377**	0.6201	0.8077	0.4399
CWTscore	0.9349	0.3806	0.4818	0.4145	0.1659	0.7190	0.1874	0.0468	0.3206	0.6414	0.0560	0.3285	0.8945	0.8071
Wigle score	0.4038	NI	NI	NI	NI	NI	NI	**0.0177**	0.1290	NI	NI	NI	NI	NI
mLVWTmit	0.5992	0.3702	0.9545	0.5067	0.1934	0.8454	**0.0170**	0.0694	0.6551	0.6771	0.1617	0.8048	0.4140	0.7649
mIVSTmit	**0.0480**	0.1585	0.4028	0.1807	0.6557	**0.0105**	**0.0128**	0.6273	0.3486	0.5771	0.8966	0.8793	0.7329	0.8404
pIVSmit	0.8496	0.5228	0.3030	0.5704	**0.0281**	0.6926	0.0834	0.1907	0.6886	0.9190	0.1018	0.3340	0.5935	0.5723
aIVSmit	0.3286	0.3038	0.9097	0.4687	0.1361	0.8354	0.0502	**0.0444**	0.7845	0.6570	0.1581	0.7444	0.3951	0.7839
AWmit	0.6601	0.5079	0.9665	0.5266	0.3909	0.8286	**0.0265**	0.0617	0.7126	0.7722	0.0481	0.7054	0.3736	0.7462
LWmit	0.4424	0.3684	0.5993	0.6562	0.6287	0.9058	**0.0168**	0.1880	0.2310	0.9709	0.1235	0.1346	0.6578	0.5855
IWmit	0.4389	0.9494	0.2721	0.3328	0.1549	0.2458	**0.0211**	0.2922	0.4780	0.7974	0.4647	0.6844	0.3672	0.2871
PWmit	0.1766	0.7018	0.0551	0.3268	0.9369	0.2405	0.1152	0.4916	0.7347	0.4927	0.0611	**0.0264**	0.1485	0.7827
mLVWTpap	0.8455	0.4928	0.8417	0.2753	**0.0418**	0.4896	0.1795	**0.0078**	0.2724	0.5098	0.1075	0.5754	0.5151	0.4292
mIVSpap	0.7751	0.4451	0.7408	0.3452	**0.0391**	0.6336	0.1284	**0.0086**	0.3327	0.6728	0.1081	0.4497	0.5728	0.5607
pIVSpap	0.9354	0.9907	0.9294	0.5361	**0.0215**	0.7406	0.3070	**0.0392**	0.4170	0.6890	0.1257	0.4160	0.7112	0.4606
aIVSpap	0.5622	0.3097	0.7470	0.2482	**0.0235**	0.5021	0.1371	**0.0031**	0.4773	0.5348	0.0785	0.4842	0.6691	0.5868
AWpap	0.5364	0.6637	0.8819	0.3538	0.2075	0.4864	0.7998	**0.0277**	0.3908	0.3550	**0.0279**	0.9690	0.6507	0.6259
LWpap	0.3541	0.9524	0.7323	0.9086	0.6830	0.7991	0.1357	0.1538	0.2453	0.7640	0.3356	0.3005	0.6444	0.7952
IWpap	0.0819	0.2879	0.1507	0.4282	0.9952	0.2635	0.1053	0.7406	0.1056	0.0470	**0.0382**	**0.0322**	0.1601	0.3701
PWpap	0.1981	0.5649	0.0949	0.3721	0.9180	0.2194	0.4766	0.2708	0.8499	0.0934	0.0722	**0.0036**	0.0974	0.5910
mLVWTapx	0.8240	0.1745	0.2980	0.7260	0.1139	0.7591	0.2306	**0.0073**	0.1814	0.4392	0.1213	0.1842	0.5723	1.0000
IVSapx	0.9779	0.1369	0.2897	0.6102	0.0570	0.6924	0.3421	**0.0073**	0.2929	0.4741	0.1091	0.1086	0.8663	0.9422
AWapx	0.6021	0.3059	0.6555	0.6166	0.2960	0.6676	0.2348	**0.0090**	0.0361	0.4819	0.0531	0.4723	0.7343	0.7261
LWapx	0.1568	0.4735	0.6170	0.2820	0.4598	0.3474	0.7589	0.6172	0.3349	0.3516	0.4243	0.0944	0.1082	0.6328
PWapx	0.4770	0.8365	0.2503	0.8634	0.6223	0.5896	0.3896	0.4888	0.8793	0.9845	0.6238	0.0616	0.2372	0.5696

NI-not informative

3.7.4. Variance component models: heritability test

The heritability values of *ACE1*, *AGT*, *CMA*, *AGTR1* and *CYP11B2* were estimated for 25 quantitative traits and three composite scores after adjusting for the effects of all the covariates. Table 3.3 contains the estimated percentage variance attributable to environment (E) and genetic factors (G) and p-values for a test of heritability.

Heritability tests after adjusting for SBP, DBP, BSA, age, sex, mutation and HR showed a strong genetic component for determining mIVS, mLVWT, Maron-Spirito score, CWTscore, pIVSmit, AWmit, PWpap, mLVWTpap, pIVSpap, aIVSp, AWpap, and IVSapx (table 3.3).

After heritability was assessed for the 28 parameters, only the 16 individual hypertrophic traits and one composite score, CWT score, were selected for subsequent investigation for association. The rationale for the exclusion of certain parameters is given in the discussion (see section 4.4).

3.7.5. Variance component models: environment, polygenes and additive component

Tables 3.4 to 3.7 contain the estimated variance components (the estimated percentage variance attributable) for environment, (E), polygenes (G) and specific SNP (major gene additive component: A) for the CWT score (table 3.4) and the hypertrophy traits at mitral valve (table 3.5), papillary muscles (table 3.6) and apex (table 3.7) after adjusting for all covariates. Generally, most of the variance for all 17 traits was attributable to environmental effects (between 55 and 100 %). We showed in the previous section, table 3.3, that some of the variance is due to a heritable effect, which is the combined polygene effect (G) and major gene additive effect (A) of tables 3.4 to 3.7.

Table 3.3: Percentage variance attributable to environment, E, and genetic factors, G, and p-values for heritability of 28 echo-measured traits analysed. Tests were adjusted for all covariates.

Trait	E	G	p-value
LVM	70.84	29.16	0.0744
mIVS	68.62	31.38	**0.0094**
mLVWT	69.95	30.05	**0.0148**
mPWT	92.02	7.98	0.7121
Maron-Spirito score	73.08	26.92	0.0534
CWTscore	67.38	32.62	**0.0309**
Wigle score	55.86	44.14	0.9486
mLVWTmit	72.97	27.03	**0.0352**
mIVSTmit	81.76	18.24	0.1803
pIVSmit	54.30	45.70	**0.0009**
aIVSmit	75.33	24.67	0.074
AWmit	77.72	22.28	**0.0458**
LWmit	79.89	20.12	0.1778
IWmit	88.32	11.68	0.5949
PWmit	79.36	20.64	0.1312
mLVWTpap	66.25	33.75	**0.0052**
mIVSpap	67.19	32.81	**0.0068**
pIVSpap	70.18	29.82	**0.0340**
aIVSpap	70.49	29.51	**0.0162**
AWpap	67.99	32.01	**0.0080**
LWpap	83.21	16.79	0.4534
IWpap	93.82	6.18	0.7704
PWpap	65.47	34.53	0.0641
mLVWTapx	69.55	30.45	0.0577
IVSapx	62.72	37.28	**0.0144**
AWapx	77.66	22.34	0.1922
LWapx	82.95	17.05	0.4891
PWapx	97.84	2.16	0.9336

The *ACE1*, *AGT* and *CYP11B2* accounted for between 23 and 31% of the variance of the CWT score, while the SNPs evaluated in *AGTR1* and *CMA* did not contribute to the additive component of variance (table 3.4).

At mitral valve level, the *AGT* and *CYP11B2* accounted for between 15.5 and 21.4% of the variance of the pIVS. Most variants in *AGT* accounted for only a small portion, 1 to 2%, of the variance of the aIVS trait, although rs4762 within *AGT* and variants in the *CMA* and *CYP11B2* account for between 13.6 and 21.3% of the variance of the aIVS trait. For the AWT trait, rs4356 within *ACE1* accounted for 19.9% of the variance, while polymorphisms within *AGTR1* and *CMA* accounted for between 11.9 and 20.9% of variance in this trait. *ACE1*, *AGT*, *AGTR1* and *CYP11B2* all contributed significantly to variance of the LWmit, between 12.1 and 17.7%, while only *ACE1* and *AGT* contributed to variance at the IWmit (between 6.8 and 13.9%). Only variants in *AGT* accounted significantly for variance (~13.5%) at the PWmit (table 3.5).

Table 3.4: Percentage variance attributable to variance components for cumulative wall thickness score (CWT).

Gene	SNP ID	E	G	A
ACE1	rs4340 I/D	72.9	0.0	27.0
	rs4298	72.7	0.0	27.3
	rs4303	72.9	0.0	27.1
	rs4356	72.7	0.0	27.3
AGT	rs699	76.7	0.0	23.3
	rs4762	76.2	0.0	23.8
	rs5051	76.6	0.0	23.3
	rs1112257	76.7	0.0	23.3
	rs1926723	76.6	0.0	23.4
AGTR1	rs2640539	71.6	28.4	0.0
	rs3772627	71.6	28.4	0.0
	rs5182	71.7	27.7	0.6
CYP11B2	rs1799998	69.0	0.0	31.0
	rs4539	71.6	0.0	28.4
	rs3097	71.6	0.0	28.4
CMA	rs1885108	70.4	29.6	0.0
	rs1800875	70.4	29.6	0.0

Abbreviations used: E- Environmental effect, G- Polygenic effect and A- Major gene additive effect

Table 3.5: Percentage variance attributable to variance components at the mitral valve level.

| | | interventricular septum thickness | | | | | | wall thickness | | | | | | | | | | | | | |
| | | posterior | | | anterior | | | anterior | | | lateral | | | interior | | | posterior | | |
Gene	SNP ID	E	G	A	E	G	A	E	G	A	E	G	A	E	G	A	E	G	A
ACE1	rs4340 I/D	55.1	44.9	0	77.5	22.5	0	78.2	21.8	0	83	0	17	86.1	0	13.9	96.9	15.5	3.4
	rs4298	55.1	44.9	0	77.5	22.5	0	78.2	21.8	0	83.2	0	16.8	86.8	0	13.3	83	16.5	0.5
	rs4303	55.1	44.9	0	77.5	22.5	0	78.2	21.8	0	83.2	0	16.8	86.6	0	13.4	83.3	15.3	1.5
	rs4356	55.0	45.0	0	77.5	22.5	0	80.1	0	19.9	82.3	0	17.7	84.4	0	15.6	82.9	16.8	0.3
AGT	rs699	62.8	16.6	20.6	78	20.2	1.8	78.6	17.6	3.8	87.5	0.2	12.3	93.2	0	6.8	86.4	0.1	13.5
	rs4762	61.0	22.4	16.6	81.2	5.2	13.6	79.2	11.5	9.3	87.5	0	12.5	93.2	0	6.8	86.7	0	13.3
	rs5051	62.5	17.8	19.8	77.9	20.6	1.5	78.6	17.6	3.8	87.6	0	12.4	93.2	0	6.8	86.4	0	13.6
	rs1112257	63.1	15.6	21.4	78.2	19.6	2.2	78.6	17.5	3.9	87.5	0.1	12.4	93.2	0	6.8	86.4	0	13.6
	rs1926723	62.9	16.3	20.9	78.2	19.6	2.2	78.6	17.6	3.8	87.5	0.3	12.2	93.2	0	6.8	86.4	0	13.6
AGTR1	rs2640539	57.3	42.7	0	79.3	18.4	2.3	79.1	0	20.9	87.5	0.4	12.1	93.5	6.5	0	81.3	18.7	0
	rs3772627	57.3	42.7	0	78.9	21.1	0	79.5	0	20.5	85.9	0	14.2	93.5	6.5	0	81.4	18.6	0
	rs5182	57.3	42.7	0	78.9	21	0.1	81.2	0.3	18.5	85.9	0	14.1	93.5	6.5	0	81.4	18.6	0
CYP11B2	rs1799998	61.9	20.7	17.4	78.8	0	21.3	78.7	17.7	3.6	84.4	0	15.6	93.5	6.5	0	81.4	18.6	0
	rs4539	62.4	18.5	19.1	79.9	0	20.1	78.6	21.4	0	83.9	0	16.1	93.5	6.5	0	81.3	18.7	0
	rs3097	61.5	23.0	15.5	80	0	20	78.7	21.3	0	83.8	0	16.2	93.5	6.5	0	81.3	18.7	0
CMA	rs1885108	55.1	44.9	0	77.7	5.3	17.1	78	8.9	13.2	86.1	13.9	0	94.4	5.6	0	82.8	17.2	0
	rs1800875	55.1	44.8	0.2	77.7	5.3	17	78	10.2	11.9	86.1	13.9	0	94.5	5.6	0	82.8	17.2	0

Abbreviations used: E- Environmental effect, G- Polygenic effect and A- Major gene additive effect

At papillary muscle level, variants in the *ACE1*, *AGT* and *CYP11B2*, as well as rs1885108 in *CMA*, accounted for between 3.9 and 28.6% of the variance of the pIVSpap trait (table 3.6). The *ACE1, CYP11B2* and *CMA* genes accounted for between 8.1 and 27.9% of the variance of the aIVSpap trait (table 3.6). The *ACE1*, *AGTR1* and *CMA* accounted for between 11.7 and 28.6% of the variance of the aWpap trait (table 3.6). *ACE1*, *AGTR1*, *CYP11B2* and *CMA* accounted for between 8.4 and 22.7% of the variance of the LWpap trait (table 3.6). Only *ACE1* and *CYP11B2* account for between 4.2 and 12.6% of the variance of the IWpap trait but none of the other genes contributed significantly to variance in this trait (table 3.6). *ACE1*, *AGT*, and rs4539 and rs3097 in *CYP11B2* accounted for between 21 and 39.6% of the variance of the PWpap trait (table 3.6).

At the apex level, the *ACE1*, *AGT* and *CYP11B2* accounted for between 27.6 and 35.4% of the variance of the IVSapx trait and between 17.6 and 31.1% of the variance of the AWapx trait (table 3.7). *ACE1*, *AGTR1* and *CYP11B2* variants accounted for between 6.6 and 15.1% of the variance of the LWapx trait (table 3.7). The *ACE1* accounts for a small portion 6-7% of the variance of the LWapx trait (table 3.7). Only *ACE1* and *CYP11B2* contributed significantly to variance of the PWapx trait, accounting for between 10.8 and 17.5% (table 3.7).

Table 3.6: Percentage variance attributable to variance components at the level of the papillary muscles.

<div align="center">Level of Papillary muscles</div>

| Gene | SNP ID | interventricular septum thickness | | | | | | wall thickness | | | | | | | | | | | | | | |
| | | posterior | | | anterior | | | anterior | | | lateral | | | interior | | | posterior | | |
		E	G	A	E	G	A	E	G	A	E	G	A	E	G	A	E	G	A
ACE1	rs4340 I/D	73.5	18.7	7.8	75.8	0	24.2	72	0.1	27.9	78.4	0	21.6	91.9	0	8.1	66.9	0	33.1
	rs4298	73.7	17.5	8.8	75.6	0	24.4	71.4	0	28.6	78.5	0	21.5	92.7	0	7.3	67.8	0	32.2
	rs4303	73.7	18	8.4	75.7	0	24.3	71.8	0	28.2	78.5	0	21.5	92.7	0	7.3	68	0	32.1
	rs4356	72.9	22.2	5	76	0	24	72.6	0	27.4	77.3	0	22.7	87.4	0	12.6	60.4	0	39.6
AGT	rs699	77.7	0	22.3	71.4	28.6	0	68.4	31.6	0	89.3	10.7	0	97.8	2.2	0	73.1	5.9	21
	rs4762	77.8	0	22.3	71.6	28	0.4	68.4	31.6	0	89.3	10.7	0	97.7	0	2.3	69.9	0	30.1
	rs5051	77.7	0	22.3	71.4	28.6	0	68.4	31.6	0	89.4	10.7	0	97.8	2.2	0	73.1	5.6	21.3
	rs1112257	77.7	0	22.4	71.4	28.6	0	68.4	31.6	0	89.3	10.7	0	97.8	2.2	0	73.1	5.7	21.2
	rs1926723	77.6	0	22.4	71.4	28.6	0	68.4	31.6	0	89.3	10.7	0	97.8	2.2	0	73.1	5.8	21.2
AGTR1	rs2640539	73	27	0	72.1	27.9	0	71.1	17.1	11.7	89.1	0	10.9	96.1	3.9	0	69	31	0
	rs3772627	73	27	0	72.1	27.9	0	71.5	13.8	14.7	87	0	13	96.1	3.9	0	68.9	31.1	0
	rs5182	73	27	0	72.4	26.3	1.3	71.9	12.6	15.6	86.2	0	13.8	96.2	3.8	0	68.9	31.1	0
CYP11B2	rs1799998	71.4	0	28.6	72.2	0	27.9	69.1	30.9	0	84.5	0	15.5	95.8	0	4.2	69	31	0
	rs4539	76.1	0	23.9	73.8	0	26.2	69.1	30.9	0	85.9	0	14.2	91.2	0	8.8	71	1.8	27.2
	rs3097	76.3	0	23.7	74.2	0	25.9	69.1	30.9	0	85.6	0	14.4	91.5	0	8.5	71.1	1.8	27.1
CMA	rs1885108	71.5	24.6	3.9	71.4	22.7	5.9	65.3	10.4	24.2	88.6	2.9	8.4	97.8	2.2	0	70.5	29.5	0
	rs1800875	71.5	24.3	0	71.3	20.6	8.1	65.3	9.1	25.7	88.6	1.7	9.7	97.8	2.2	0	70.5	29.5	0

Abbreviations used: E- Environmental effect, G- Polygenic effect and A- Major gene additive effect

Table 3.7: Percentage variance attributable to variance components at the level of the apex.

Gene	SNP ID	Level of Apex											
		interventricular			wall thickness								
		septum thickness			anterior			lateral			posterior		
		E	G	A	E	G	A	E	G	A	E	G	A
ACE1	rs4340 I/D	70.9	0	29.1	69.2	0	30.9	89	4.3	6.7	89	0	11
	rs4298	70.6	0	29.4	68.9	0	31.1	89	4.1	6.9	89.2	0	10.8
	rs4303	70.8	0	29.2	69.1	0	30.9	89	4.7	6.3	89.2	0	10.8
	rs4356	71.1	0	29	70.4	0	29.6	89	4.6	6.4	88.4	0	11.6
AGT	rs699	72.4	0	27.6	82.2	0	17.8	89.6	10.4	0	99.9	0.1	0
	rs4762	72.2	0	27.8	82.3	0	17.7	89.6	10.4	0	99.9	0.1	0
	rs5051	72.4	0	27.6	82.2	0	17.8	89.6	10.4	0	100	0	0
	rs1112257	72.4	0	27.6	82.1	0.3	17.6	89.6	10.4	0	100	0	0
	rs1926723	72.3	0	27.7	82.2	0	17.8	89.6	10.4	0	100	0	0
AGTR1	rs2640539	65.9	34.2	0	81.8	18.3	0	89.5	3	7.5	98.6	1.4	0
	rs3772627	65.9	34.1	0	81.8	18.2	0	84.9	0	15.1	98.7	1.3	0
	rs5182	65.9	34.2	0	81.7	18.3	0	89.8	9.9	0.3	98.7	1.3	0
CYP11B2	rs1799998	64.7	0	35.4	74.4	0	25.6	88.1	0	11.9	82.5	0	17.5
	rs4539	68.8	0	31.2	75.9	0	24.1	90.4	3	6.6	82.6	0	17.4
	rs3097	68.9	0	31.1	76.2	0	23.8	90.5	1.9	7.6	82.8	0	17.3
CMA	rs1885108	64.8	35.2	0	80.4	19.6	0	89.6	10.4	0	99.9	0.1	0
	rs1800875	64.8	35.2	0	80.4	19.6	0	89.6	10.4	0	100	0	0

Abbreviations used: E- Environmental effect, G- Polygenic effect and A- Major gene additive effect

3.7.6. Linkage analysis

The LOD-scores and p-values for testing linkage of the 17 markers of the five RAAS genes to hypertrophy traits are listed in the first two columns of tables 3.8 (for CWT score) and 3.9 to 3.24 (for 16 wall thickness parameters). Significant linkage results (LOD-scores ≥3, and p < 0.05), which represents strong evidence for linkage, are highlighted in bold. The only significant indication for linkage was seen with variants in the *ACE1* (table 3.20). Specifically, several markers (rs4340, rs4303 and rs4356) in *ACE1* showed LOD-scores >3 for trait PWpap (table 3.20), while only rs4340, rs4298 and rs4303 in *ACE1* showed LOD-scores >3 for trait AWapx (table 3.22).

3.7.7. Association tests

Highly significant evidence of association was observed between *AGTR1* rs2640539 and AWmit (p = 0.009) (table 3.11). We estimate that each *G*-allele decreases AWmit by 2.2mm, making it a protective allele.

Evidence of association was observed between *CYP11B2* rs3097 and PWmit trait under an additive model (p = 0.001) (table 3.14), with the *G*-allele representing the protective allele. Each *G*-allele is estimated to reduce PWmit by 1.0mm.

Further, significant association was observed between *AGTR1* rs3772627 and the aIVSpap trait (p = 0.037) (table 3.16). The A-allele of the *AGTR1* rs3772627 polymorphism represents the risk allele with each copy of the *A*-allele estimated to add 1.7mm to aIVSpap thickness. Furthermore, association tests indicated a significant association between *AGTR1* rs5182 and IWpap trait (p = 0.006) (table 3.19). Each T-allele of the *AGTR1* rs5182 polymorphism is estimated to decrease IWpap by 0.6mm making the *T*-allele a protective allele (table 3.19). Additionally, a significant association was observed between *AGTR1* rs5182 and the AWapx trait (p = 0.046) (table 3.23). Again, the T-allele of the *AGTR1* rs5182 polymorphism is acting as a protective allele, and is estimated to decrease AWapx by 1.9mm.

Similarly, a significant association was observed between *CYP11B2* rs3097 and IWpap trait (p = 0.01) (table 3.19). Each *G*-allele was estimated to decrease IWpap by 1.0mm, making the *G*-allele protective for IWpap (table 3.19). A significant association was also found between *CYP11B2* rs3097 and PWpap under an additive

model of association (p = 0.003) (table 3.20), with the *G*-allele representing the protective allele. We estimate that each *G*-allele decreases PWpap by 2.1mm.

3.7.8   Linkage in the presence of association

After modeling linkage and association simultaneously, the p-values generated suggested that for all the SNPs that showed association with one or more hypertrophy traits, viz. rs2640539, rs3772527 and rs5182 in *AGTR1*, and rs3097 in *CYP11B2*, there was no residual evidence for linkage (p > 0.10) (tables 3.11, 3.14, 3.16, 3.19, 3.20, 3.23), suggesting that these variants are in strong LD with the respective functional susceptibility variants in these two genes.

Conversely, evidence for linkage of *ACE1* to hypertrophy parameters (tables 3.20 and 3.22) are not accounted for by association and suggests that the polymorphisms investigated for association are not the functional variants. The results obtained are similar to that generated when modeling only linkage indicated in bold in tables 3.20 and 3.22. The *ACE1* locus could be a QTL for hypertrophy development.

Table 3.8: Linkage and association values for the cumulative wall thickness (CWT) score and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.844	0.161	0.515	0.237
	rs4298	0.844	0.161	NI	NI
	rs4303	0.835	0.161	0.979	0.062
	rs4356	0.810	0.164	0.443	0.230
AGT	rs699	0.110	0.400	0.453	0.647
	rs4762	0.161	0.354	0.953	0.433
	rs5051	0.107	0.402	0.805	0.532
	rs1112257	0.113	0.398	NI	NI
	rs1926723	0.110	0.400	NI	NI
AGTR1	rs2640539	0.000	1.000	0.303	1.000
	rs3772627	0.000	1.000	0.320	0.536
	rs5182	0.000	0.984	0.268	1.000
CYP11B2	rs1799998	1.447	0.108	0.631	0.090
	rs4539	0.571	0.204	0.516	1.000
	rs3097	0.130	0.197	0.296	0.295
CMA	rs1885108	0.000	1.000	0.889	0.996
	rs1800875	0.000	1.000	0.671	0.815

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.9: Linkage and association values for the posterior interventricular septum thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.000	1.000	0.204	1.000
	rs4298	0.000	1.000	NI	NI
	rs4303	0.000	1.000	0.949	1.000
	rs4356	0.000	1.000	0.573	1.000
AGT	rs699	0.032	0.674	0.538	0.733
	rs4762	0.009	0.658	0.610	0.446
	rs5051	0.006	0.684	0.606	0.450
	rs1112257	0.008	0.667	NI	NI
	rs1926723	0.032	0.668	NI	NI
AGTR1	rs2640539	0.000	1.000	0.215	1.000
	rs3772627	0.000	1.000	0.379	1.000
	rs5182	0.000	1.000	0.498	1.000
CYP11B2	rs1799998	0.004	0.711	0.135	1.000
	rs4539	0.007	0.674	0.890	1.000
	rs3097	0.002	0.769	0.577	1.000
CMA	rs1885108	0.000	1.000	0.732	1.000
	rs1800875	0.000	1.000	0.345	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.10: Linkage and association values for the anterior interventricular septum thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.000	1.000	0.226	1.000
	rs4298	0.000	1.000	NI	NI
	rs4303	0.000	1.000	0.858	0.355
	rs4356	0.000	1.000	0.296	1.000
AGT	rs699	0.000	0.963	0.843	1.000
	rs4762	0.006	0.690	0.917	0.874
	rs5051	0.000	0.984	0.657	1.000
	rs1112257	0.000	0.953	NI	NI
	rs1926723	0.000	0.956	NI	NI
AGTR1	rs2640539	0.000	0.924	0.137	1.000
	rs3772627	0.000	1.000	0.288	0.574
	rs5182	0.000	1.000	0.513	0.989
CYP11B2	rs1799998	0.217	0.318	0.672	0.363
	rs4539	0.078	0.437	0.856	1.000
	rs3097	0.089	0.425	0.841	0.883
CMA	rs1885108	0.028	0.550	0.325	1.000
	rs1800875	0.027	0.553	0.636	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.11: Linkage and association values for the anterior wall thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.000	1.000	0.528	1.000
	rs4298	0.000	1.000	NI	NI
	rs4303	0.000	1.000	0.966	0.485
	rs4356	0.035	0.525	0.228	0.657
AGT	rs699	0.000	0.838	0.764	1.000
	rs4762	0.011	0.642	0.802	0.631
	rs5051	0.000	0.838	0.800	1.000
	rs1112257	0.000	0.835	NI	NI
	rs1926723	0.000	0.838	NI	NI
AGTR1	rs2640539	0.414	0.241	**0.009**	0.687
	rs3772627	0.414	0.240	0.225	0.248
	rs5182	0.104	0.408	0.371	0.682
CYP11B2	rs1799998	0.000	0.906	0.576	1.000
	rs4539	0.000	1.000	0.401	1.000
	rs3097	0.000	1.000	0.883	1.000
CMA	rs1885108	0.015	0.607	0.925	1.000
	rs1800875	0.011	0.640	0.705	0.929

Abbreviation used: LPOA-linkage in the presence of association. Bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value.

Table 3.12: Linkage and association values for the lateral wall thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.414	0.241	0.629	0.582
	rs4298	0.340	0.264	NI	NI
	rs4303	0.356	0.258	0.432	0.294
	rs4356	0.658	0.187	0.723	0.448
AGT	rs699	0.003	0.727	0.949	1.000
	rs4762	0.006	0.684	0.434	1.000
	rs5051	0.003	0.728	0.855	1.000
	rs1112257	0.004	0.720	NI	NI
	rs1926723	0.003	0.725	NI	NI
AGTR1	rs2640539	0.010	0.643	0.147	1.000
	rs3772627	0.035	0.528	0.266	0.521
	rs5182	0.040	0.513	0.142	0.687
CYP11B2	rs1799998	0.086	0.428	0.469	0.970
	rs4539	0.110	0.400	0.255	1.000
	rs3097	0.110	0.400	0.144	0.521
CMA	rs1885108	0.000	1.000	0.523	1.000
	rs1800875	0.000	1.000	0.828	0.992

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.13: Linkage and association values for the inferior wall thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.463	0.227	0.973	0.655
	rs4298	0.373	0.252	NI	NI
	rs4303	0.408	0.242	0.709	0.266
	rs4356	0.628	0.193	0.934	0.471
AGT	rs699	0.068	0.453	0.470	0.991
	rs4762	0.068	0.453	0.419	0.755
	rs5051	0.068	0.453	0.362	0.990
	rs1112257	0.068	0.453	NI	NI
	rs1926723	0.068	0.453	NI	NI
AGTR1	rs2640539	0.000	1.000	0.348	1.000
	rs3772627	0.000	1.000	0.724	0.997
	rs5182	0.000	1.000	0.100	1.000
CYP11B2	rs1799998	0.000	1.000	0.680	1.000
	rs4539	0.000	1.000	0.729	1.000
	rs3097	0.000	1.000	0.533	1.000
CMA	rs1885108	0.000	1.000	0.453	1.000
	rs1800875	0.000	1.000	0.889	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

143

Table 3.14: Linkage and association values for the posterior wall thickness at level of mitral valve and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.000	0.919	0.918	1.000
	rs4298	0.000	0.985	NI	NI
	rs4303	0.000	0.960	0.330	0.527
	rs4356	0.000	1.000	0.942	0.976
AGT	rs699	0.059	0.473	0.507	0.938
	rs4762	0.040	0.511	0.522	0.562
	rs5051	0.059	0.473	0.433	1.000
	rs1112257	0.061	0.468	NI	NI
	rs1926723	0.061	0.466	NI	NI
AGTR1	rs2640539	0.000	1.000	0.615	1.000
	rs3772627	0.000	1.000	0.570	1.000
	rs5182	0.000	1.000	0.225	1.000
CYP11B2	rs1799998	0.000	1.000	0.715	1.000
	rs4539	0.000	1.000	0.481	1.000
	rs3097	0.000	1.000	**0.001**	1.000
CMA	rs1885108	0.000	1.000	0.554	1.000
	rs1800875	0.000	1.000	0.673	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.
For association tests, bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value

Table 3.15: Linkage and association values for the posterior interventricular septum thickness at level of papillary muscles and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.001	0.786	0.362	0.429
	rs4298	0.001	0.773	NI	NI
	rs4303	0.001	0.777	0.402	0.124
	rs4356	0.000	0.873	0.357	0.901
AGT	rs699	0.086	0.429	0.550	0.508
	rs4762	0.078	0.439	0.430	0.598
	rs5051	0.084	0.431	0.706	0.370
	rs1112257	0.086	0.427	NI	NI
	rs1926723	0.086	0.429	NI	NI
AGTR1	rs2640539	0.000	1.000	0.456	1.000
	rs3772627	0.000	1.000	0.226	0.966
	rs5182	0.000	1.000	0.721	1.000
CYP11B2	rs1799998	0.801	0.166	0.643	0.140
	rs4539	0.061	0.466	0.925	1.000
	rs3097	0.040	0.514	0.778	1.000
CMA	rs1885108	0.000	0.894	0.788	1.000
	rs1800875	0.000	0.885	0.573	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.16: Linkage and association values for the anterior interventricular septum thickness at level of papillary muscles and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.217	0.317	0.336	0.304
	rs4298	0.226	0.312	NI	NI
	rs4303	0.222	0.315	0.965	**0.044**
	rs4356	0.180	0.341	0.554	0.390
AGT	rs699	0.000	1.000	0.342	0.968
	rs4762	0.000	1.000	0.353	0.785
	rs5051	0.000	1.000	0.713	0.874
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.000	1.000	0.254	1.000
	rs3772627	0.000	1.000	**0.037**	0.828
	rs5182	0.000	1.000	0.253	0.976
CYP11B2	rs1799998	0.905	0.153	0.809	0.172
	rs4539	0.351	0.259	0.626	1.000
	rs3097	0.334	0.265	0.405	0.581
CMA	rs1885108	0.000	0.847	0.701	1.000
	rs1800875	0.000	0.793	0.574	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.
For the asociation test, bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value.

Table 3.17: Linkage and association values for the anterior wall thickness at level of papillary muscles and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.249	0.301	0.525	0.271
	rs4298	0.263	0.295	NI	NI
	rs4303	0.244	0.303	0.754	0.173
	rs4356	0.254	0.300	0.535	0.319
AGT	rs699	0.000	1.000	0.633	1.000
	rs4762	0.000	1.000	0.310	1.000
	rs5051	0.000	1.000	0.943	1.000
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.009	0.653	0.461	0.992
	rs3772627	0.027	0.556	0.258	0.849
	rs5182	0.015	0.607	0.225	0.961
CYP11B2	rs1799998	0.000	1.000	0.931	1.000
	rs4539	0.000	1.000	0.407	1.000
	rs3097	0.000	1.000	0.707	0.994
CMA	rs1885108	0.126	0.384	0.545	0.355
	rs1800875	0.150	0.364	0.634	0.523

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.18: Linkage and association values for the lateral wall thickness at level of papillary muscles and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	1.120	0.132	0.473	0.563
	rs4298	1.020	0.141	NI	NI
	rs4303	1.040	0.139	0.369	0.167
	rs4356	1.160	0.129	0.623	0.299
AGT	rs699	0.000	1.000	0.436	0.995
	rs4762	0.000	1.000	0.087	1.000
	rs5051	0.000	1.000	0.294	0.996
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.021	0.579	0.216	0.989
	rs3772627	0.063	0.464	0.852	0.990
	rs5182	0.081	0.436	0.338	0.951
CYP11B2	rs1799998	0.153	0.359	0.621	0.555
	rs4539	0.084	0.430	0.858	1.000
	rs3097	0.092	0.420	0.337	1.000
CMA	rs1885108	0.002	0.770	0.505	0.898
	rs1800875	0.003	0.741	0.215	0.989

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.19: Linkage and association values for the inferior wall thickness at level of papillary muscles and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.061	0.468	0.171	1.000
	rs4298	0.040	0.510	NI	NI
	rs4303	0.044	0.502	0.505	0.988
	rs4356	0.254	0.299	0.723	0.431
AGT	rs699	0.000	1.000	0.808	0.988
	rs4762	0.000	0.871	0.399	0.364
	rs5051	0.000	1.000	0.617	0.999
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.000	1.000	0.744	0.996
	rs3772627	0.000	1.000	0.950	1.000
	rs5182	0.000	1.000	**0.006**	1.000
CYP11B2	rs1799998	0.001	0.777	0.434	0.945
	rs4539	0.063	0.462	0.634	1.000
	rs3097	0.052	0.483	**0.010**	0.722
CMA	rs1885108	0.000	1.000	0.081	0.994
	rs1800875	0.000	1.000	0.681	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.
For the asociation test, bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value.

Table 3.20: Linkage and association values for the posterior wall thickness at level of papillary muscles and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	**3.780**	**0.041**	0.458	0.078
	rs4298	**3.156**	0.051	NI	NI
	rs4303	**3.290**	**0.049**	0.211	0.131
	rs4356	**7.465**	**0.016**	0.517	**0.015**
AGT	rs699	0.089	0.423	0.501	1.000
	rs4762	0.628	0.192	0.365	0.240
	rs5051	0.092	0.419	0.274	1.000
	rs1112257	0.092	0.420	NI	NI
	rs1926723	0.089	0.423	NI	NI
AGTR1	rs2640539	0.000	1.000	0.980	1.000
	rs3772627	0.000	1.000	0.808	1.000
	rs5182	0.000	1.000	0.260	1.000
CYP11B2	rs1799998	0.000	1.000	0.902	1.000
	rs4539	0.217	0.318	0.852	1.000
	rs3097	0.209	0.323	**0.003**	0.584
CMA	rs1885108	0.000	1.000	0.190	1.000
	rs1800875	0.000	1.000	0.639	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.
For association tests, bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value.

Table 3.21: Linkage and association values for the interventricular septum thickness at level of apex and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.599	0.197	0.565	0.102
	rs4298	0.578	0.201	NI	NI
	rs4303	0.578	0.201	0.698	0.112
	rs4356	0.463	0.227	0.948	0.243
AGT	rs699	0.042	0.509	0.689	0.708
	rs4762	0.052	0.486	0.546	0.714
	rs5051	0.040	0.512	0.797	0.460
	rs1112257	0.044	0.505	NI	NI
	rs1926723	0.042	0.506	NI	NI
AGTR1	rs2640539	0.000	0.995	0.373	1.000
	rs3772627	0.000	0.997	0.144	1.000
	rs5182	0.000	1.000	0.234	1.000
CYP11B2	rs1799998	2.062	0.079	0.830	0.097
	rs4539	0.445	0.232	0.895	1.000
	rs3097	0.470	0.226	0.148	0.692
CMA	rs1885108	0.000	1.000	0.663	1.000
	rs1800875	0.000	1.000	0.683	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.22: Linkage and association values for the anterior wall thickness at level of apex and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	**3.256**	**0.049**	0.783	**0.034**
	rs4298	**3.139**	0.051	NI	NI
	rs4303	**3.172**	0.051	0.391	0.059
	rs4356	2.325	0.071	0.490	**0.042**
AGT	rs699	0.002	0.758	0.856	0.523
	rs4762	0.002	0.763	0.742	0.603
	rs5051	0.002	0.756	0.856	0.523
	rs1112257	0.002	0.750	NI	NI
	rs1926723	0.002	0.748	NI	NI
AGTR1	rs2640539	0.000	1.000	0.284	1.000
	rs3772627	0.000	1.000	0.150	1.000
	rs5182	0.000	1.000	**0.046**	1.000
CYP11B2	rs1799998	2.198	0.075	0.870	0.139
	rs4539	1.403	0.111	0.405	1.000
	rs3097	1.359	0.114	0.300	0.215
CMA	rs1885108	0.000	1.000	0.802	1.000
	rs1800875	0.000	1.000	0.171	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.
For association tests, bold highlighting indicates a Markov Chain Monte Carlo (MCMC) exact p-value

Table 3.23: Linkage and association values for the lateral wall thickness at level of apex and each SNP analysed.

Gene	SNP ID	Linkage		Association	LPOA
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.001	0.783	0.381	1.000
	rs4298	0.001	0.784	NI	NI
	rs4303	0.001	0.796	0.864	1.000
	rs4356	0.000	0.808	0.181	0.995
AGT	rs699	0.000	1.000	0.321	1.000
	rs4762	0.000	1.000	0.717	0.995
	rs5051	0.000	1.000	0.489	0.978
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.003	0.726	0.951	0.983
	rs3772627	0.213	0.319	0.743	1.000
	rs5182	0.000	0.993	0.615	1.000
CYP11B2	rs1799998	0.071	0.449	0.960	0.993
	rs4539	0.001	0.791	0.834	1.000
	rs3097	0.002	0.760	0.201	1.000
CMA	rs1885108	0.000	1.000	0.223	0.996
	rs1800875	0.000	1.000	0.286	0.994

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

Table 3.24: Linkage and association values for the posterior wall thickness at level of apex and each SNP analysed.

| Gene | SNP ID | Linkage | | Association | LPOA |
		LOD-score	p-value	Additive model p-value	p-value
ACE1	rs4340 I/D	0.091	0.422	0.831	0.978
	rs4298	0.081	0.436	NI	NI
	rs4303	0.081	0.430	0.964	1.000
	rs4356	0.098	0.413	0.966	0.487
AGT	rs699	0.000	1.000	0.909	1.000
	rs4762	0.000	1.000	0.988	1.000
	rs5051	0.000	1.000	0.701	0.981
	rs1112257	0.000	1.000	NI	NI
	rs1926723	0.000	1.000	NI	NI
AGTR1	rs2640539	0.000	1.000	0.565	1.000
	rs3772627	0.000	1.000	0.926	1.000
	rs5182	0.000	1.000	0.950	1.000
CYP11B2	rs1799998	0.844	0.161	0.350	0.068
	rs4539	0.571	0.204	0.462	1.000
	rs3097	0.557	0.206	0.061	0.205
CMA	rs1885108	0.000	1.000	0.621	0.992
	rs1800875	0.000	1.000	0.915	1.000

Abbreviation used: LPOA-linkage in the presence of association. NI-not informative.

3.7.9. X-linked gene analysis

Table 3.25 gives the association p-values for the X-linked *ACE2* gene. Four markers in *ACE2* were tested for association and linkage to 16 wall thickness parameters and CWT-score. One marker, rs879922, was significantly associated with both pIVSmit (p = 0.0047) and LWmit (p = 0.027). None of the other markers investigated in *ACE2* showed significance with any of the 16 quantitative traits and CWT-score investigated (p > 0.05) (table 3.25).

The estimated difference in pIVSmit between each rs879922 group and the male *C* hemizygote was: 1.5mm for male *G*; and for females: 0.2mm for CC, 2.1mm for GC and 7.1mm for *GG* homozygotes. The estimated difference in LWmit between each rs879922 group and the male *C* hemizygote was: 0.6mm for male *G*; and for females: 0.1mm for *CC*, 0.5mm for *CG* and 5.6mm for *GG* homozygotes.

Table 3.25: Association analysis p-values for each SNP of *ACE2*.

	rs1978124	rs2285666	rs879922	rs4646179
CWT	0.4739	0.5334	0.3891	0.3184
pIVSmit	0.6255	0.3772	**0.0047**	0.2120
aIVSmit	0.6052	0.7689	0.0934	0.1937
AWmit	0.1570	0.7324	0.3958	0.6777
LWmit	0.0792	0.7372	**0.0270**	0.9524
IWmit	0.1214	0.2967	0.3950	0.8405
PWmit	0.6081	0.5182	0.3696	0.4845
pIVSpap	0.7983	0.6542	0.5393	0.4819
aIVSpap	0.5186	0.5397	0.2008	0.1541
AWpap	0.1445	0.4242	0.4443	0.7666
LWpap	0.4301	0.6901	0.5601	0.7773
IWpap	0.2090	0.8147	0.8819	0.2672
PWpap	0.5450	0.6092	0.5658	0.7331
IVSapx	0.5347	0.3393	0.1843	0.4517
AWapx	0.6151	0.4769	0.2767	0.2996
LWapx	0.8692	0.6777	0.7703	0.7507
PWapx	0.3755	0.5370	0.0864	0.3618

CHAPTER 4
DISCUSSION

4.1. Previous HCM hypertrophy modifier studies

Most early studies of hypertrophy modifiers in HCM selected genes involved in cell growth and BP control (see section 1.3.1), including genes that encode key-components of the RAAS (Marian et al., 2002). Most of these studies were performed in unrelated individuals, rather than members of families, and furthermore did not control for differences in ethnic background or primary HCM-causing mutations in the cohorts. Additionally, many of these studies did not make adjustments for hypertrophy covariates, such as age, sex, body size and blood pressure. These factors alone would contribute greatly to hypertrophic variability and would obscure the effects of any genetic modifiers. However, in 2002, Ortlepp and colleagues investigated the role of RAAS gene variants as modifiers of hypertrophy within individuals of a single family carrying the same HCM-causative mutation. Although, this study controlled for age, sex and the presence of hypertension, it did not index LVM to BSA, and showed that genetic polymorphisms in the genes encoding components of the RAAS had an influence on the degree of LVH.

4.2. Choice of candidate genes and SNPs

The present family-based association study implemented a candidate gene approach in which an *"a priori-based"* hypothesis was employed to assess association between a genotype and a quantitative trait: genes were chosen because they encode key-components of the RAAS pathway known to be involved in control of BP and cell growth. Additionally, the genomic structure and location of these genes were known, as was the function of the encoded products. Wherever possible the SNPs selected for investigation were those that had been investigated in previous studies and included those with and those without functional data. However, as this study aimed to investigate a spread of SNPs throughout the candidate genes, some additional SNPs, not previously investigated, were also targeted. These newly chosen polymorphisms had to possess a heterozygosity value of 40% or higher to be included in the investigation, to optimize the chance of meeting the requirement of the QTDT program for a threshold number of informative matings. This approach was used to re-investigate the role of RAAS genes in LVH (discussed in sections 1.4.4.1 to 1.4.4.6), attempting to test their role in a more

robust manner by addressing the caveats present, but largely/often ignored, in previous studies, as described above.

As discussed, the genes that encode components of the RAAS represent attractive candidates for susceptibility to LVH development because of their regulatory role in control of BP and cell growth. This is further supported by their association with LVH (section 4.1). Although the data are intriguing, association studies are subject to several shortcomings such as population stratification, phenotypic heterogeneity, numbers of markers tested, sample size and statistical power. The following sections will discuss the relevance of these pitfalls in more detail.

4.3. Caveats in association studies

As association studies aim to be susceptibility marker studies to infer risk and/or even causality associated with a specific genotype, their reproducibility, or lack thereof, is a confounding factor in determining the degree to which results can be used for risk stratification because of the occurrence of false positives or negatives. Below, some of the causes of false positive and negative findings are discussed.

Population stratification

Discrepant associations obtained in different case-control studies are often ascribed to allelic differences between populations because of divergent genetic background, which may arise due to geographical locations of population groups (Cardon and Palmer, 2003). Should cryptic substructure exist in a case and/or control group, population stratification may result in either a type I error (finding an association when it does not exist) or a type II error (finding no association when it does in fact exist) (see section 1.4.3.2 for examples).

Statistical power

The power of a genetic association study is the probability that a true association is detected by the test. It reflects the ability of the study to detect true effects and to differentiate between the absence of an effect and an inconclusive result. The power for a

family-based association test depends on the following factors- the minimum size of the effect regarded as clinically or biologically meaningful (a difference might be statistically significant, but not scientifically meaningful at all), the variation in the trait being tested, the correlations in the trait variables between the members of families, the allele frequencies of the polymorphism being tested, the number of familes, the total number of individuals and the significance level selected for the test. Generally, the following will increase the power of a specific test: more families, more members per family, less trait correlation between family members, detecting a larger effect, less variation in the trait being tested and increasing the significance level. The relationship between allele frequencies and power is too complicated to generalise. The extent of the effect of these factors is interdependent. The only way to calculate the power for a specific test is by simulating the distribution of the test statistic (bootstrapping). This is a complicated, lengthy and time-consuming process (Diao and Lin et al., 2006; Abecasis et al., 2001). Markers that do not show association in pilot studies should not necessarily be ruled out but could be retested in follow-up studies using larger sample size, thus with improved power, to determine if these markers can truly be excluded as playing a role in disease pathogenesis. Meta-analysis is another statistical method employed to overcome small sample size in genetic association studies and so to increase study power (Egger et al., 2002). In meta-analyses, genotypic and phenotypic data from several studies are pooled to identify a common main effect, while controlling for study-dependent factors (Glass et al., 1976). However, it still has its limitations due to between-study heterogeneity, as a result of genotypic, phenotypic and methodological confounders (Gambaro et al., 2000).

Number of markers tested

Single SNP studies have limitations, as they may fail to capture all the available LD information in flanking SNPs - unless they are tag SNPs for the true functional variant (or happen to be the functional variant) (Niu et al., 2002). Investigation of all tag SNPs in a given gene, and/or haplotype analysis of multiple SNPs spread throughout a gene can resolve this problem by combining information of multiple adjacent SNPs into relatively more informative multilocus haplotypes. Haplotypes are more informative as they capture all the regional LD information, and are therefore considered to be a powerful and robust

method for detecting association in complex disorders (Akey et al., 2001; Daly et al., 2001).

Additional confounding factors in association studies

Another limitation of association studies is publication bias against negative results (Easterbrook et al., 1991). Additionally, an explanation for the lack of a consistent relationship between marker and trait could also be that the independent effect of a single allele/gene is too small or variable to detect within the subjects presenting with the disease. Furthermore, there could also be epistatic effects of one gene with other genes within specific individuals that produce a clinical phenotype that is measurable in some, but not in other, subjects studied.

4.4. Controlling for confounding factors in the present study

A number of confounding factors that typically plague association studies were controlled for in the present study. Individuals belonging to three cohorts in which founder mutations segregated were investigated. Due to the ancestral relatedness within each cohort, genetic background noise should be reduced because individuals were more closely related, compared to cohorts consisting of the general unrelated population. The fact that three distinct cohorts were used was adjusted for by including the mutation groups as covariates in the analyses.

Population stratification

Population stratification was a concern in the present study, because families were either of Mixed Ancestry or Caucasian descent. The possible effect of population stratification was controlled for throughout the association analyses by using family-based controls and by implementing Abecasis's orthogonal model which protects against population stratification (Iles M., 2002).

Power

We did not estimate the power of any of the tests performed in this study, as it was considered to be beyond the scope of this thesis.

Phenotype

In HCM, formal quantification of the degree, and extent, of hypertrophy is difficult because of the regional and individual nature of increases in wall thickness. Accordingly, a number of parameters were measured, which included composite scores and absolute measurements.

Because HCM subjects usually, but not always, present with asymmetrical hypertrophy, it is difficult to quantify the extent of hypertrophy for all individuals in a cohort by using only one score (Reichek et al., 1983). However, it is not clear which composite score is appropriate. Although LVM has often been used as the phenotypic parameter in HCM genetic modifier studies (Perkins et al., 2005; Marian et al., 2002), assessment of LVM by echocardiography, which assumes that the left ventricle takes on an even geometric shape, may not be accurate due to the variable and non-uniform nature of hypertrophy in HCM. The Wigle score captures the distribution and extent of hypertrophy on a more global scale, but the score is semi-quantitative. On the other hand, the Maron score, although quantitative, does not capture the full extent of hypertrophy in all segments of the heart.

Thus, in the absence of more accurate calculation of LVM, such as by MRI, it may be more appropriate to focus on measurements of those heritable regions of the ventricular wall frequently affected by hypertrophy. Such measurements could be used as a range of individual wall thickness values, or as composite scores derived from them, in quantitative trait analyses. Thus, in statistical data analysis the only composite score used was the in-house derived CWT score (see section 2.1), as it is a global, quantitative estimate that attempts to capture the total amount of hypertrophy observed in all 16 segments of the heart muscle.

Compensating for multiple testing

Performing any association test may result in a false positive finding (Berry et al., 1998). If the significance level is 5%, the probability of a false positive finding is 5%. If multiple tests are done, all at 5% significance levels, the probability of a false positive finding

increases with the number of tests done. Thus some published papers have applied a Bonferroni adjustment in an attempt to correct for type I errors (false positive findings) introduced by such multiple testing. Bonferroni assumes that tests are statistically independent and multiplies each p-value with the number of tests done. So it will only produce a significant p-value in circumstances where the p-values were already very significant (Bland et al., 1995; Perneger et al., 1998) and therefore put a constraint on the statistical power of the study. Perneger et al., (1998) argued that the Bonferroni correction is too conservative, because closely-linked markers are not independent and genotypes are correlated when familial data is being used. The present study used QTDT to calculate empirical p-values for the within-family component of association using a Monte-Carlo permutation framework, because it provides a global p-value with a built-in adjustment for multiple testing. It is valid for traits with normal or non-normal distributions (McIntyre et al., 2001).

4.5. Heritability tests for quantitative traits and composite scores

Although there are many definitions of heritability, in the present study, heritability was calculated by means of variance components analysis, as the proportion of variance in a trait attributable to common genes. This provides a broad measure and, as with all estimation, in some cases one might estimate significant heritability while the trait might not be heritable, while in other instances, the detection of heritability might be obscured in a small sample (Elston et al., 2000). This method does not distinguish between a single major gene, a few moderately strong genes or numerous polygenes with small effects (Thomas D., 2004). Other authors have defined heritability solely for a specific locus (Ziegler A and König IR., 2006).

Heritability and evidence of strong familial aggregation in LVM have been shown within different population groups, namely, Caucasians (Bielen et al., 1991; Garner et al., 2000; Mayosi et al., 2002), African Americans (Kotchen et al., 2000), American Indians (Bella et al., 2004) and Caribbean Hispanic families (Juo et al., 2005) and Taiwanese (Chien et al., 2006). In the latter study, the authors demonstrated that heritability and major gene effects accounted in part for significant familial correlations observed in this population

(Chien et al., 2006). Chien and colleagues (2006) provided several reasons for the high heritability estimates observed in African Americans in the Kotchen et al., (2000) study, suggesting that this might be due to sample selection criteria, for example, ascertaining hypertensive siblings, genetic background of these subjects and also expression of a range of complex multiple traits in comparison to unmeasured factors. Obviously, these factors may also apply to studies performed in other populations groups.

The present study also investigated the heritability of LVM, shown in familial studies to have a strong genetic component, but focused on heritability of LVM after adjustment for a variety of covariate factors that may influence heart size and structure. Interestingly, the findings of the present study are in contrast to previous reports because LVM was not significantly heritable in the SA HCM families, although other traits were (table 3.3). This may be because previous studies did not adjust for the covariates used in this study; alternatively, heritability may have been masked by factors not taken into account in this study.

In the present study, strong association p-values were found that did not correspond to heritability p-values (tables 3.3, 3.14, 3.16, 3.19, 3.20, 3.23). It was decided not to exclude traits that do not show heritability, as it has been suggested by Elston et al., (2000) that rare disease traits may have a strong genetic component, and yet give rise to a small overall familial correlation.

It should be pointed out that in this study, we analysed raw, untransformed, trait values, as the association would subsequently be tested with Markov Chain Monte Carlo permutations, which does not require distributional assumptions. Further, there are many different approaches to adjusting or normalising trait values for family-based variance component analysis and it was not clear which, if any, would be most appropriate for this study (Ziegler A and König I., 2006).

4.6. Estimating and testing heritability

The heritability of the CWT score was accounted for, in part, by variants in *ACE1*, *AGT* and *CYP11B2* (table 3.4). The genetic component of the pIVSmit trait was accounted for by variants in *AGTR1*, *CMA*, *AGT* and *CYP11B2,* with those in *AGT* and *CYP11B2* appearing to have stronger effects than those in *ACE1*, *AGTR1* and *CMA*. The variants in the latter three genes did not convert any of the polygenic variance to an additive variance component which the two stronger effect genes did (table 3.5). The aIVSmit trait's heritability was contributed to by variants in *AGT*, *CMA* and *CYP11B2,* with variants in *CMA* and *CYP11B2* appearing to play a much larger role in the heritability of the aIVSmit trait than those in *AGT* (table 3.5). Variants in *CMA* and *AGTR1* contributed to the heritability of the AWmit trait (table 3.5), while heritability of the LWmit trait was in part accounted for by variants in *ACE1*, *AGT*, *AGTR1* and *CYP11B2* (table 3.5). The IWmit trait's heritability was accounted for by variants in *ACE1* and *AGT* (table 3.5); however, only variants in *AGT* appeared to account for all the heritability of the PWmit trait (table 3.5).

The variants in *ACE1*, *AGT* and *CYP11B2* contributed to the heritability of the pIVSpap trait (table 3.6). Interestingly, variants in *AGT* and *CYP11B2* had a stronger effect by converting all the polygenic variance to an additive variance (table 3.6). The variants in *ACE1*, *CMA* and *CYP11B2* each contributed to the heritability of the aIVSpap trait (table 3.6). Interestingly, variants in *ACE1* and *CYP11B2* had a stronger effect by converting all the polygenic variance to additive variance (table 3.6). The heritability of the aWpap trait was accounted for, in part, by variants in *ACE1*, *CMA* and *AGTR1* (table 3.6). Interestingly, variants in *ACE1* had a stronger effect compared to *CMA* and *AGTR1* by converting all the polygenic variance to additive variance (table 3.6). Additionally, the genetic component of the LWpap trait was comprised of variants in *ACE1*, *AGTR1*, *CYP11B2* and *CMA* (table 3.6). Only variants in *ACE1* and *CYP11B2* accounted for the heritability of the IWpap trait (table 3.6). The markers in *CYP11B2*, rs4539 and rs3097, and *ACE1* and *AGT* accounted for a large proportion of the heritability of the PWpap trait (table 3.6). Interestingly, variants in *ACE1* appeared to have a stronger effect than those

in *CYP11B2* and *AGT* by converting all the polygenic variance to additive variance, respectively (table 3.6).

The variants in *ACE1*, *CYP11B2* and *AGT* accounted for all of the heritability of the IVSapx trait (table 3.7). The variants in the latter three genes converted all the polygenic variance to additive variance (table 3.7). Additionally, all the heritability of the aWapx trait was contributed by variants in *ACE1*, *AGT* and *CYP11B2* (table 3.7). The markers in *CYP11B2* (rs2640539) and *AGTR1* (rs3772627) and the *ACE1* accounted for a small proportion of the heritability of the LWapx trait (table 3.7). However, only variants in the *ACE1* and *CYP11B2* accounted for all of the heritability of the pWapx trait (table 3.7).

4.7. Linkage analysis

In the present study, significant evidence for linkage was found between the *ACE1* rs4340, rs4303 and rs4356 polymorphisms and the PWpap trait (table 3.20). Similarly, significant linkage was observed between the *ACE1* rs4340 polymorphism and the AWapx trait (table 3.22). This suggests that the region in which *ACE1* resides either contains, or is closely linked to, a functional allele involved in hypertrophy development in this segment.

4.8. Association analyses

4.8.1. *ACE1* rs4298, rs4303, rs4340 (I/D) and rs4365 polymorphisms

The results of the within-family association tests with single markers suggested that none of the markers investigated in *ACE1* was associated with any of the 17 LVH parameters (tables 3.8 to 3.24). However, there was evidence of statistically significant *linkage in the presence of association* to PWpap and AWapx *ACE1* SNPs (tables 3.20 and 3.22). This result indicates that the linkage data observed are not accounted for by association and suggests that these SNPs in themselves are not the susceptibility loci, but are in LD with the causal variant.

In LVH

The *ACE1* (I/D) polymorphism has frequently been previously studied in LVH and hypertension and the results are discrepant (see section 1.4.4.1). Schunkert et al., (1994) suggested the *ACE1* locus to be a QTL for determining an individual's genetic susceptibility to LVH, however subsequent studies by Lindpaintner et al., (1996), Wu et al., (2000) and Shlyakhto et al., (2001) negated this. This polymorphism was found to be associated with LVH and has been reported to correlate with variation in plasma and tissue ACE levels (Zhu et al., 2001; Kuznetsova etal., 2004; Saeed et al., 2005). There are mixed results for the association studies of the *ACE1* I/D polymorphism and LVH; in some there are associations between the *II* genotype and increased LVMI and in others the *DD* genotype is associated with increased LVMI (Kuznetsova etal., 2004). The variability of the association results of the *ACE1* I/D polymorphism and LVH suggests that this polymorphism is not a functional variant but is in LD with a functional variant (Ganau et al., 1990), as the actual allele conferring susceptibility or protection of the *ACE1* I/D polymorphism varies depending on ethnicity and geographical region and may be a reflection of the LD structure of the particular population (van Berlo et al., 2003).

In hypertrophy and HCM

The findings from Yoneya et al., (1995) suggest that the *ACE1 DD* genotype is associated with cardiac hypertrophy in HCM. The findings of Yoneya et al., (1995) suggested that in solitary cases of HCM the *DD* genotype of *ACE1* partially determines an individual's genetic disposition for HCM. The main difference between the present study and both the Lechin et al., (1995) and Yoneya et al., (1995) studies is the inclusion of a healthy panel of related individuals that did not develop hypertrophy (table 2.1). The inclusion of phenotypically unaffected individuals gives a better estimate of the influence of the *ACE1* genotype on hypertrophy development. Several other investigators considered the *ACE1 DD* genotype an allelic LVH modifier in HCM (Marian et al., 1993; Lechin et al., 1995; Tesson et al., 1997; Ortlepp et al., 2002) despite the use of smaller numbers in their study and not indexing LVM to BSA, as was done in the present study. Smaller HCM cohort studies found overall overrepresentation of the *D*-allele (Yoneya et al., 1995; Lopez-Haldon et al., 1999) but did not include family members as controls. Larger studies have

shown a LVH-modifying effect of *DD-ACE1* while adjusting for covariates such as age, sex, weight, height, BSA, BMI and *ACE1* genotypes (Lechin et al., 1995; Ortlepp et al., 2002). However, contrary to the present study, the Lechin et al., (1995) and Ortlepp et al., (2002) studies, only investigated single SNPs within individual genes that encode RAAS components.

Animal models

Higaki et al., (2000a) demonstrated that *"in vivo"* transfection of human *ACE1* into rat myocardium resulted in elevated cardiac ACE activity, increased left ventricular wall thickness and collagen content independent of systemic and haemodynamic effects. Furthermore, administration of perindopril (an ACE inhibitor) abolished the cardiac hypertrophy observed in the rat hearts (Higaki et al., 2000a). Xiao and colleagues (2004) observed in a genetic mouse model with cardiac restricted *ACE1* (called *ACE* 8/8) atrial enlargement, cardiac arrhythmia and SCD, but no ventricular hypertrophy or fibrosis, indicating that ANGII in the heart is not sufficient to induce LVH or fibrosis.

It is speculated that although no known HCM loci are genetically linked to the *ACE1* locus, *ACE1* may be playing the role of a co-acting modifier gene (Pfeufer et al., 1996). This implies that the *ACE1 DD* genotype serves as an indicative marker to assess risk to an individual for development of LVH. However, the present association data does not support a significant independent role for the *ACE1 DD* genotype in contributing to the development or severity of hypertrophy in the South African HCM population (tables 3.8 to 3.24).

On the other hand, interestingly, in the present study variants in *ACE1* showed linkage to two traits (PWpap and AWapx) (table 3.20 and 3.22) but showed no association with these particular traits. The reason for this perhaps could be that linkage extends over substantial genetic distances, and is therefore suited for long-range mapping, and in the present study might be picking up signals of a more distant locus. Association on the other hand relies on either the presence of LD between the investigated marker and trait locus, or on the marker locus being the trait locus itself and is therefore useful for short

range-mapping. If there is linkage, but no association, the marker itself can be discarded as having an effect. In the present study, there was therefore no evidence that the I/D locus itself influences cardiac hypertrophy after other covariates of hypertrophy had been taken into account.

4.8.2. *AGT* rs5051, rs4762, rs699, rs11122575 and rs1926723 polymorphisms

The localised expression of *AGT* occurs within the myocardium and makes it a good candidate gene to assess its influence on cardiac growth (Dzau et al., 1987; Baker et al., 1992; de Mello and Danser, 2000). Consequently, it has been suggested that variants within *AGT* might be potential independent mediators or determinants of LVM. Numerous investigators have reported on positive and negative findings regarding the relationship between the M235T (rs699) polymorphism and LVM (Kauma et al., 1998; Jeng et al., 1999; Staessen et al., 1999).

The M235T (rs699) polymorphism has been shown to be in LD with -6*G/A* (rs5051), with the latter having been shown to influence *AGT* concentrations (Inoue et al., 1997; Paillard et al., 1999). The -6*G/A* (rs5051) polymorphism, particularly the *A*-allele, influences basal transcription of *AGT* and stimulate transcription due to receptor binding (Zhao et al., 1999). According to Cooper et al., (1998), a key site for the transcriptional effect of -20*A/C* and -6*G/A* variants might be the adipose tissue because *AGT* is highly expressed within this tissue. The -6*G/A* (rs5051) polymorphism were shown to be associated with plasma AGT concentrations in subjects of African ancestry (Rotimi et al., 1997).

In the present study, the results of the within-family association tests with single markers suggested that the *AGT* rs5051, rs4762, rs699, rs11122575 and rs1926723 polymorphisms showed no association with any of the 17 LVH parameters (tables 3.8 to 3.24). These results are in agreement with a study by Yamada et al., (1997) that found no relationship between *AG*T M235T (rs699) and T174M (rs4762) variants and HCM or DCM. In contrast, other studies did reveal a statistically significant association between *AG*T M235T and LVM or LVH (Karjalainen et al., 1999; Tang et al., 2002; Kurland et

al., 2002; Ortlepp et al., 2002). Reasons for differences in results between the present study and latter mentioned studies could be the use of different ethnic groups and not compensating for covariates that determine LVH.

4.8.3. *CYP11B2* rs1799998, rs4539 and rs3097 polymorphisms

The results of the within-family association tests with single markers suggested that the *CYP11B2* rs3097 polymorphism in exon 9 (3'UTR) was associated with PWmit, IWpap and PWpap (tables 3.14, 319 and 3.20). Further statistical analysis of rs3097 revealed that subjects homozygous for the *G*-allele were at a lower risk of developing increased PWmit, IWpap and PWpap compared to homozygotes for the *A*-allele, indicating a protective role for the *G*-allele in LVH susceptibility. The *CYP11B2* rs3097 polymorphism has been previously studied in LVH and has shown association with cardiac wall thickness (Mayosi et al., 2003) as well as with hypertension in a study by Kumar et al., (2003). Mayosi and colleagues (2003) observed that a haplotype containing the minor *A*-allele of rs3097 polymorphism was associated with increased septal wall thickness (SWT) in a panel of Caucasian hypertensive probands. The *G*-allele of rs3097 polymorphism was strongly associated with hypertension particularly in females, with the *GG* genotype frequency being three-fold higher in hypertensives compared to normotensives (Kumar et al., 2003). The *CYP11B2* rs3097 polymorphism is located in the 3'UTR and might play a role in mRNA stability (Pesole et al., 2002).

In the present study, the other previously investigated polymorphisms in the promoter region (rs1799998) and exon 3 (rs4539) of *CYP11B2* showed no association with any of the 17 LVH parameters investigated (tables 3.8 to 3.24), while only the rs1799998 polymorphism did show association with hypertension in the Kumar et al., (2003) study. In a different study, the rs4539 (2713 *A/G* or Arg173Lys) polymorphism was associated with stage-2 hypertension in Han-Chinese women (Gu et al., 2004), while Zhu et al., (2003) found no association with hypertension in black Afro-Caribbean origin subjects from South-West London. The rs4539 polymorphism is suggested to have a negligible physiological significance on *CYP11B2* transcription as it does not alter enzyme activity

"in vitro" and is in LD with the functional -344*C/T* polymorphism, as discussed below (Davies et al., 2003).

Several studies have found an association between *CYP11B2* rs1799998 (-344 *C/T*) polymorphism and LV structure and LVM (Kupari et al., 1998; Delles et al., 2001), although Schunkert et al., (1998) could not confirm the association in a larger study cohort. The current findings are in agreement with the Schunkert et al., (1998) study, as no such an association was found between LVH and rs1799998 (-344 *C/T*) polymorphism allele status. The *CYP11B2* rs1799998 polymorphism is an attractive choice for association studies as it resides at a putative binding site for steroidogenic transcription factor (SF-1) (White and Slutker et al., 1995). Additionally, it is associated with a four-fold increase in SF-1 binding to -344 *C*-allele carriers and increased aldosterone synthesis associated with plasma aldosterone concentrations and urinary aldosterone excretion rates (Pojoga et al., 1998; Davies et al., 1999; Paillard et al., 1999).

A reason for the association between the *CYP11B2* rs1799998 (-344 *C/T*) polymorphism and hypertension in other studies could be that *CYP11B2* is known to influence aldosterone production, and thereby blood pressure. This influence may not be directly correlated with an influence on hypertrophy, as it has been shown that there is an imperfect correlation between BP and LVM (Wachtell et al., 2000).

In vitro, the -344 *C*-allele binds SF-1 four fold stronger, than the *T*-allele (White and Slutker et al., 1995). Consequently, the *C*-allele results in there being less SF-1 available to bind to other promoter sites and thus might lead to reduced transcription, while the -344 *T*-allele increases transcription (Clyne et al., 1997). This polymorphism could be in LD with an, as yet, unidentified functional variant near or adjacent to *CYP11B2*. Discrepancies in other studies that investigated hypertension support such a notion (Brand et al., 1998; Davies et al., 1999; Matsubara et al., 2001; Russo et al., 2002).

Animal model

Tsybouleva et al., (2004) demonstrated that aldosterone is involved in the cardiac phenotype observed in a genetically engineered mouse model with HCM. Blockade of mineralocorticoid receptors in a transgenic animal model of human HCM (cTnT-Q92) had beneficial effects by reducing myocytic disarray and interstitial fibrosis indicating aldosterone is an important molecular link between cardiac phenotype and the HCM phenotype (Tsybouleva et al., 2004).

The present *CYP11B2* rs3097 data supports a significant independent role for *CYP11B2* in contributing to the development or severity of hypertrophy in the SA HCM population.

4.8.4. *CMA* rs1800875 and rs1885108 polymorphisms

The results of the within-family association tests with single markers suggested that none of the polymorphisms within *CMA* was associated with any of the 17 LVH parameters investigated.

The present study's results are in agreement with studies of Pfeufer et al., (1996) and Ortlepp et al., (2002), where no independent association was found between *CMA* rs1800875 and LVH in HCM subjects. Similarly, no evidence was found to support a possible association between the *CMA* rs1800875 polymorphism and regression of LVH when 157 unrelated Chinese hypertensive patients were treated with the same antihypertensive drug benazepril (He et al., 2005). The usual arguments are discussed in the He et al., (2005) paper to explain why their observed results are different from the studies of Pfeufer et al., (1996) and Gumprecht et al., (2002). Reasons such as the different population groups used, genetic heterogeneity, different diagnosis criteria and the level of hypertension and LVH were posited. Finally, the dosage of the ACE inhibitor (ACEI) and other medicines used could also have contributed to spurious results observed between studies where treatment of LVH was investigated. These findings indicate that LVH formation/development is a complex process with the possibility of various genes interacting to produce a clinical phenotype, for example *CMA* and other genes encoding RAAS products.

4.8.5. *AGTR1* rs2640539, rs3772627and rs5182 polymorphisms

The *AGTR1* rs2640539 and rs3772627 polymorphisms have not been previously studied in LVH or in hypertension, as far as is known. In the present study, the results of the within-family association tests with single markers suggested that the *AGTR1* rs2640539 polymorphism in intron 1 is associated with AWmit (table 3.11), and the *AGTR1* rs3772627 polymorphism in intron 2 is associated with aIVSpap (table 3.16). Additionally, the association tests with single markers suggested that the *AGTR1* rs5182 polymorphism in exon 3 is associated with IWpap and AWapx (tables 3.19 and 3.23). Further statistical analysis of rs2640539 polymorphism revealed that subjects homozygous for the *G*-allele were at a lower risk of developing increased AWmit compared to homozygotes for the *A*-allele, indicating a protective role for the *G*-allele in LVH susceptibility. Moreover, statistical analysis of rs3772627 polymorphism revealed that subjects homozygous for the *A*-allele were at a higher risk of developing increased aIVSpap compared to homozygotes for the *G*-allele, making the *A*-allele the risk allele. Statistical analysis of rs5182 polymorphism revealed that subjects homozygous for the *T*-allele were at a lower risk of developing increased IWpap and AWapx compared to homozygotes for the *C*-allele, indicating a protective role for the *T*-allele of the rs5182 polymorphism.

The *T*-allele of the *AGTR1* rs5182 (573 C/T) polymorphism was shown to be a protective factor for urinary albumin excretion in EHT patients (Chaves et al., 2001). Additionally, Chaves et al., (2001) demonstrated evidence of linkage disequilibrium between rs5186 (1166*A/C*) and 573*C/T* polymorphisms. However, Redon and colleagues (2005) found no association between rs5182 and rs5186 polymorphisms and BP reduction following telmisartan treatment. The present results are in contrast with those of Kupari et al., (1994), Lindpaintner et al., (1996) and Hamon et al., (1997) that showed no significant influence of *AGTR1* genotypes on LVH.

It should be noted that genotyping the rs5182 polymorphism using restriction enzyme *Mnl*I generated incongruous results. For technical reasons the fragment sizes differed on the gel from the expected digested product sizes generated using the software package

DNAMAN. A possible reason for the banding pattern is that the *Mnl*I enzyme has the ability to remain associated with the cleaved DNA, thus resulting in DNA band shifting during electrophoresis (http://www.fermentas.com/catalog/re/mnli.htm). Sequencing the *AGTR1* fragment 3 containing the SNP revealed that the correct region of the gene containing the SNP of interest was amplified. These discrepancies were also confirmed by another laboratory (Dr P. Fernandez, Department of Urology, US), which also found that the 325bp and 422bp fragments did not migrate as expected by size. Additionally, no disparities between the *AGTR1* nucleotide sequence of fragment 3 and the NCBI database sequence were observed. After confirming that the different sized fragment on the gels was not due to technical errors, and that mobilities were constant, the genotyping of rs5182 polymorphism was continued.

The functional significance of the association of the *AGTR1* rs5182 polymorphism with cardiac hypertrophy remains unknown. This polymorphism is a synonymous substitution and does not alter the sense codon 191 (CTC →CTT) that codes for leucine but it can be speculated that it is in LD with an unknown polymorphism in *AGTR1* or adjacent genes. The *AGTR1* rs2640539 and rs3772627 polymorphisms are intronic and so far the functional significance of these polymorphic alleles is unclear. However, introns may contain regulatory elements that affect gene transcription and translation (Ying and Lin et al., 2006). Moreover, microRNAs derived from introns (Ying and Lin et al., 2006) have the ability to bind mRNA, interfering with transcription, degrading mRNA and thereby reducing transcript levels (Sevignani et al., 2006), indicating that intronic polymorphisms can also influence gene splicing by affecting a splice donor or acceptor sites. Of course, these polymorphisms could also be in LD with other functional variants that affect mRNA and protein expression.

Animal models

In a rat model, it was demonstrated that overexpression of AGTR1 under physiological conditions resulted in no change of the cardiac structure, whereas pressure and volume overload produced hypertrophic growth in the model (Hoffmann et al., 2001). In a study by Harada et al., (1999), AGTR1 subtype a (AGTR1a) specific receptor knockout mice

displayed less LV remodelling and improved survival rates at four weeks after myocardial infarction (MI) compared to wild type (WT) MI mice. However, Yoshiyama et al., (2005) demonstrated that ACE inhibitors prevent LV remodelling in a non-*AGTR1* mediated mechanism after MI in *AGTR1* knockout mice. In experimental animal model studies, ACE inhibitors and AGTR1 antagonists induce regression and prevent cardiac hypertrophy development in hypertensive animal models (Pfeffer et al., 1982 and 1983; Dunn et al., 1984; Nakashima et al., 1984). Harada et al., (1998) demonstrated that *AGTR1* is not essential for the development of pressure overload-induced cardiac hypertrophy in transgenic animal models. Similarly, Katada and colleagues (2005) observed elevated cardiac CYP11B2 levels in AGTR1a receptor knockout mice after MI inducing cardiac remodelling via ANGII- independent mechanisms.

Tsuchida and colleagues (1998) generated *AGTR1* knockout mice by gene target disruption creating a null mutation gene. Similarly to homozygous (AGT -/-) knockout mice, (*AGTR1* subtype a and b specific receptors, are products of different genes) double null-zygote mice developed a ventricular septum defect in combination with abnormal phenotypes, namely, severe hypotension (low BP), growth retardation, renal arterial wall thickening and hypolastic papilla (Tsuchida et al 1998). Paradis et al., (2000) generated transgenic mice expressing human *AGTR1* under the control of the mouse (α-MHC) promoter and observed ventricular hypertrophy and fibrosis in cardiac overexpressed AGTR1 (by more than 200 fold) in absence of external stimuli.

4.8.6. *ACE2* rs1978124, rs2285666, rs879922 and rs4646179 polymorphisms

The results of the association tests with single markers suggested that the rs879922 polymorphism in intron 11 of *ACE2* was associated with both pIVSmit and LWmit (table 3.25). Further statistical analysis of rs879922 (28330*C/G*) polymorphism revealed that subjects homozygous for the *G*-allele were at an increased risk of developing increased pIVSmit compared to homozygotes for the *C*-allele, indicating a dominant role for the *G*-allele in LVH susceptibility. Similarly, further statistical analysis of rs879922 polymorphism revealed that subjects homozygous for the *G*-allele were at an increased risk of developing increased LWmit compared to homozygotes for the *C*-allele,

indicating a recessive role for the *G*-allele in LVH susceptibility. However, the previously investigated polymorphisms rs1978124, rs2285666 and rs4646179 of *ACE2* showed no association with any of the 17 LVH parameters investigated in the current study.

The present study's results are similar to findings by Lieb et al., (2006) where an independent association was found between the rs879922 polymorphism (the *G*-allele) and LVMI, SWT and LVH. Lieb and coworker's (2006) results were found in male subjects with HCM, while our positive association results were only found in women. The association observed in women should be verified by haplotype analysis. In contrast, Benjafield et al., (2004) found no evidence of association between the rs879922 polymorphism and EHT in Australian populations. Whether the *ACE2* rs879922 intronic polymorphism has any functional significance is yet to be determined, and thus the explanation for the described association can only be speculative. One explanation for the association could be that the intronic polymorphism is in LD with a functional polymorphism, either within *ACE2* or an adjacent gene that is associated with LVH development. The preliminary data should be seen as experimental evidence suggestive of the role of *ACE2* as a modifier of cardiac structure and function.

Animal models

In response to pressure overload, mice that lacked *ACE2* developed cardiac hypertrophy and dilatation compared to WT mice (Yamamoto et al., 2000). Furthermore, treatment with candesartan (an AGTR1 subtype receptor blocker) resulted in the reduction of the hypertrophic response and suppression of MAP kinase in *ACE2* knockout mice (Yamamoto et al., 2000). Furthermore, Crackower et al., (2002) showed that *ACE2* is involved in the regulation of cardiac structure and function in *ACE2* knockout mice generated by target gene disruption. However, in *ACE2* null mutant mice the BP levels were high compared to WT mice, while the cardiac structure and function was normal (Gurley et al., 2006). They suggest that *ACE2* is an important regulator of BP but not of cardiac structure and function.

4.9. Limitations of the present study

The default association test used by QTDT assumes that the trait values, after adjusting for all covariates, will have normal distributions inside genotype groups. It is not possible to test this assumption, which must be valid for each combination of trait and polymorphism tested. That is why it was decided to use QTDT's permutation tests, which are valid for any quantitative variable, and also protect against multiple testing, to test the significance of those combinations yielding significant p-values with default QTDT association tests. An alternative would have been to transform the trait values to an overall normal distribution. This would make it equally difficult to confirm normal distributions of adjusted trait values inside genotype groups; there is no recommended single approach (Ziegler A and König IR., 2006).

The particular genes and SNPs chosen in this study might have contributed to the negative findings obtained. A selection of SNPs spread through the candidate genes was targeted; these included those previously reported to be associated with HCM phenotypes in other populations, as well as SNPs chosen in this study. It is not known whether these SNPs represent tag SNPs in the population studied, and thus it remains unclear what extent of variation within each candidate gene was captured by these SNPs.

In the absence of knowledge of tag SNPs, it may be useful to attempt to capture the variation in each candidate gene by analysis of haplotypes constructed for the investigated SNPs. The investigator was not aware of a program which could construct most probable haplotypes in general pedigrees and either analyse these haplotypes for association, or export them in a suitable format for analysis in a separate program. However, an upgraded version of the program UNPHASED is allegedly proposed to be able to do this, but due to time constraints this was not investigated.

Furthermore, it is speculated that a combination of alleles at different loci may act in unison to produce a much stronger effect on the clinical phenotype of a condition (Souery et al., 2001). These effects, called epistatic interactions, may be a potential reason for the failure to replicate association studies. It is quite likely that components of the RAAS

system interact epistatically (Ortlepp et al., 2002; Perkins et al., 2005). The RAAS cascade is a hormonal system with several components, each interacting to produce independent or synergistic effects and thus it is prudent to investigate not one component on its own, but several together. Time constraints did not allow the investigation of epistatic effects or of additional components of the RAAS, as the focus was on those that have been frequently investigated for a role in LVH in the literature.

Additionally, as others suggested, failure to replicate positive association studies could be due to statistical issues such as reduced power, correcting for multiple testing, procedural issues involving population stratification and differences in diagnostic procedures and sample ascertainment. Association studies in the general population are prone to spurious results because of genetic heterogeneity of a population. Thus specific studies should be repeated in different populations to ensure that any association of a gene with a disease is pathogenically significant (Komiya et al., 2000).

Each of the genotyping techniques used in this study has its advantages and disadvantages. SNaPshot analysis of a large number of SNPs in many subjects is costly; and although multiplexing will reduce costs considerably, optimising conditions can be technically challenging. Additionally, there may be a lack of correlation between SNaPshot and sequencing data, as shown for the two SNPs, rs4342 and rs3434, in fragment 2 of *ACE1*. There were discrepancies when the data generated by SNaPshot and sequencing were compared in 10% of the samples (see section 3.6). Whereas ASREA is presently cheaper than SNaPshot analysis, it is manual and time-consuming. A potential limitation to this technique is the generation of partial digests, if the restriction enzyme does not completely cleave the diagnostic site, for example because of the presence of inhibitors, inactive enzyme or other technical conditions. This will obviously confound interpretation of genotype and lead to errors. In the present study, this was circumvented wherever possible, by designing amplicons with additional invariant restriction enzyme sites (section 3.5.3 pg 114, *AGTR1* fragment 2 rs3772627 polymorphism).

4.10. New resources for association studies

HapMap project

The human genome differs by approximately 0.1% between individuals (one variant per 1000 nucleotide bases on average), with an estimated 10 million SNPs contributing to 90% of the variation (Li et al., 1991; Wang et al., 1998; Cargill et al., 1999; Halushka et al., 1999). Thus, with current technology, genotyping all the SNPs within the genome is not feasible, due to cost and labour intensity. As an alternative, the use of HapMap data will reduce genotyping workload by targeting SNPs that show association and tag SNPs that capture linkage information. Genotyping one SNP in a haplotype block will provide enough information about the remainder of the common SNPs in that region. It is thus feasible to only investigate or type a few of the tag SNPs in a gene to represent other common haplotypes in the region.

At the commencement of the present study, the HapMap project data was in its infancy, and the current wealth of SNP data for these candidate genes was not available. Additionally, it was uncertain whether HapMap tag SNPs documented for other populations will be relevant for the South African HCM cohort. However, the results of a study by Willer et al., (2006) demonstrated that selecting tag SNPs for individuals from Finland using the CEPH Utah samples from the HapMap database provided useful information, as a strong correlation was observed between the two samples. This study indicated that the HapMap database may be a useful tool for selecting tag SNPs for association studies, beyond the populations from which they were sampled (Willer et al., 2006), although it could be argued that the Finish population and the CEPH Utah population would be expected to be genetically fairly similar. Moreover, a study by Service et al., (2007) confirmed that HapMap tag SNPs performed well in several population isolates that differed significantly from HapMap samples in levels of LD or SNP allele frequency. The study by Service and colleagues (2007) examined 12 populations (CAU-Caucasian reference sample from the Human Variation Panel of the Coriell Institute, FIP-Finland nationwide, FIC-Northern Finland Birth cohort, ERF-a village in South Western Netherlands, NFL-Newfoundland, FIK-Finnish subisolate of Kuusamo, ASH-Ashkenazi, SAR-Province of Nuoro in Sardinia, SAF-Afrikaner, CR-

Central Valley of Costa Rica, AZO-Azores, ANT-Antioquia), populations not included in the HapMap. However, most individuals in the present study were of Mixed Ancestry (Coloured individuals from the Western Cape), a population with roots in the Khoi-San, European, African and Malaysian populations. Thus, it remains uncertain which HAPMAP tag SNPs to target, as tag SNPs and tagged haplotypes do differ to some extent between HAPMAP populations.

By chance, some of the SNPs chosen in the present study were HapMap tag SNPs (*ACE1* rs4343 in CEU population, *CMA* rs1885108 in CEU and Yoruba population, *AGT* rs699 in CEU, *CYP11B2* rs3097 in CEU, *AGTR1* rs5182 in CEU, *ACE2* rs4646179 in CEU). Although tag SNPs was not used as a selection priority in the present study, it would add value to future studies. As for the present study, certain tag SNPs did show association with particular traits, but *CYP11B2* rs3097 captured no other SNPs in the CEU, *AGTR1* rs5182 captured no other SNPs in the CEU population and *ACE2* rs879922 had no available data from HapMap database, thus providing no additional information on how much of the relevant gene regions had been covered.

4.11. Future research

The present study yielded interesting preliminary results that need to be followed up with additional gene variants in RAAS-associated genes. For example, more polymorphisms in a gene that contain HapMap tag SNPs should be screened in order to capture all the information within a whole gene. Additionally, LD tests and functional assays on the SNPs investigated need to be employed to verify associations observed. Prospective studies should measure the levels of *AGTR1*, *CYP11B2* and *ACE2* in HCM patients, which could add better value to the study. From the statistical results, it is difficult to draw conclusions as to whether *ACE1*, *CMA* and *AGT* are modifiers of the HCM phenotype because of the lack of statistically significant associations found. Genes that did not show association should not be excluded but be re-examined in other population groups. Although, previous association studies looked at the same genes investigated in this study, perhaps other novel genes may play a much larger role in the pathogenesis of this condition. Future research should include haplotype analysis, which might also make

an existing association more credible and clearer and should also be extended to candidate genes which showed no association in the current study.

The present research forms part of a larger, ongoing research endeavour to elucidate the molecular mechanisms of LVH development in South African HCM families. Identification of HCM-modifying genes will not only give insight into the phenotypic variability among South African HCM patients, but also the molecular causes of diseases, such as hypertension, of which LVH is a feature. In future, these results might contribute to the development of new drug targets for reducing cardiac hypertrophy in the general population.

4.12. Conclusions

The present study failed to show significant linkage between any candidate markers within *AGT*, *AGTR1*, *CYP11B2* and *CMA* and any of the 16 traits and the CWT-score studied. However, significant associations were found between some of these traits and candidate markers within *AGTR1*, *CYP11B2* and markers within *ACE2*, while findings for association for *ACE1*, *AGT* and *CMA* were all statistically non-significant. The main findings of the present study are that some *CYP11B2*, *AGTR1* and *ACE2* polymorphisms are associated with LVH in patients with HCM, independent of covariates such as blood pressure. This study provides simultaneous evidence of linkage in the absence and presence of association, confirming that *ACE1*, but not the ACE I/D polymorphism per se, is a QTL for LVH development. The preliminary data should also be seen as experimental evidence suggestive of the role for *AGTR1*, *CYP11B2* and *ACE2* as modifiers of cardiac structure.

The present study followed a strict design and included a larger sample size compared to many other studies, thus it may more accurately indicate the mechanisms involved at a molecular level in the development of LVH. In future, these results might have beneficial pharmacogenetics applications to aid new drug development.

APPENDIX I

BUFFERS, MARKERS AND SOLUTIONS

1.   BUFFERS

1.1.   CELL LYSIS

Sucrose (Merck, RSA)	0.32M
Triton-X-100 (Sigma, RSA)	1%
$MgCl_2$ (Merck, RSA)	5mM
Tris-HCl (Merck, RSA)	10mM
H_2O	1L

1.2.   DNA EXTRACTION BUFFER

NaCl (Meck, RSA)	0.1M
Tris-HCl	0.01M
EDTA (pH 8) (Merck, RSA)	0.025M
SDS (Sigma, RSA)	0.5%
Proteinase K (Sigma, RSA)	0.1mg/ml

1.3.   TBE-BUFFER (10X stock)

Tris-HCl	0.89M
Boric Acid (Whitehead, Scientific, RSA)	0.89M
Na_2EDTA (pH 8)	20mM

1.4.   SB-BUFFER (20X stock)

di-sodium tetraborate decahydrate (Merck, RSA)	38.137g/mol
H_2O	1L

1.5.   WASH BUFFER

Tris-EDTA Buffer	10mM
Absolute ethanol	48ml

1.6. ELUTION BUFFER

Tris-HCl (pH 8)   10mM

Autoclaved ddH$_2$O   50μl

2. MOLECULAR WEIGHT MARKER

2.1. LAMBDA PST1

Bacteriophage Lambda DNA (250μg) (Promega, USA)   100μl

Buffer M (Boerhinger Mannheim, GmbH)   15μl

PstI (Boerhinger Mannheim, GmbH)   11μl

H$_2$O   32μl

Incubate at 37 ^{o}C for 2 hours followed by heat inactivation at 65°C for 5 minutes. Load 4μl onto 2% Agarose gel.

2.2. 100 bp DNA ladder (Promega, USA)   4μl

3. SOLUTIONS

3.1. SOLUTIONS FOR POLYACRYLAMIDE GELS

3.1.1 40% Acrylamide/ 0.8% BIS (ready to use) stock solution for polyacrylamide gels supplied by Merck (37:5:1)

3.1.2. 10% AMMONIUMPERSULPHATE (APS)

APS   2g

H$_2$O   20ml

3.2. SOLUTIONS FOR SILVER STAINING

3.2.1. 0.1% AgNO$_3$ (Solution B)

AgNO$_3$ 1g

H$_2$O   1L

3.2.2. DEVELOPING SOLUTION (Solution C)

NaOH (Merck, RSA)	15g
NaBH$_4$ (Whitehead, Scientific, RSA)	0.1g
Formaldehyde (Merck, RSA)	4ml
H$_2$O	1L

4. GELS

4.1. AGAROSE GEL SOLUTIONS

Concentration	1.5%	2%	3%
Agarose (g)	1.5	2	3
1X TBE stock (ml)	100	100	100
or			
1X SB stock (ml)	100	100	100

Melt agarose in microwave oven until completely dissolved, let cool for 3' and add 5µl Ethidium Bromide.

4.2. 12% POLYACRYLAMIDE GEL

40% Acrylamide-Bis (ready to use) solution	3ml
10X TBE	1ml
Distilled H$_2$O	6ml
APS	80ml
TEMED	30ml

5. LOADING DYES

5.1. BROMOPHENOL BLUE

Bromophenol blue (Merck, RSA)	0.2% (w/v)
Glycerol (Merck, RSA)	50%
Tris (pH 8)	10mM

6. **Glossary of terms used:**

Endocrine system = A system of glands that release their secretions (peptide/hormones) directly into the circulatory system and produces its' effects on target organs located at a distance.

Paracrine system = After secretion, the peptide acts on its' receptors located on adjacent cells.

Autocrine system = After secretion, the peptide acts on its' own receptors.

7. **SNaPshot analysis**

7.1. **50CM CAPILLARY CUSTOMADE RUN MODULE CONDITIONS**

	Parameter name	Value	Range
1	Run temp	60	int 18 - 65 °C
2	Cap fill volume	46	int 1-200 steps
3	Current tolerance	100	int 1-100μAmps
4	Run current	100	int 1-100μAmps
5	Voltage tollerance	0.6	float 0.25-2 kVolts
6	Pre run voltage	15	float 1-15 kVolts
7	Pre run time	60	int 1-1000sec
8	Injection voltage	2	float 1-15 kVolts
9	Injection time	15	int 1-600sec
10	Run voltage	15	float 0-15 kVolts
11	Number of steps	10	int 1-100nk
12	Voltage step interval	20	int 1-60sec
13	Data delay time	200	int 1-3600sec
14	Run time	1900	int 300-14000sec

8.   Summary of echocardiographic traits measured at three levels and composite score.

Abbreviations used: overall, at three levels and composite scores.

<u>Overall measures:</u>

LVM-left ventricle mass

mIVS-maximum interventricular septum thickness

mLVWT-maximum left ventricle wall thickness

mPWT-maximum posterior wall thickness

<u>AT mitral valve level:</u>

mLVWTmit-maximum left ventricle wall thickness at level of mitral valve

mIVSmit-maximum interventricular septum thickness at level of mitral valve

pIVSmit-posterior interventricular septum thickness at level of mitral valve

aIVSmit-anterior interventricular septum thickness at level of mitral valve

AWmit-anterior wall thickness at level of mitral valve

LWmit-lateral wall thickness at level of mitral valve

IWmit-inferior wall thickness at level of mitral valve

PWmit-posterior wall thickness at level of mitral valve

<u>At level of papillary muscle:</u>

mLVWTpap-maximum left ventricle wall thickness at level of papillary muscles

mIVSp-maximum interventricular septum thickness at level of papillary muscles

pIVSpap-posterior interventricular septum thickness at level of papillary muscles

aIVSpap-anterior interventricular septum thickness at level of papillary muscles

AWpap-anterior wall thickness at level of papillary muscles

LWpap-lateral wall thickness at level of papillary muscles

IWpap-inferior wall thickness at level of papillary muscles

PWpap-posterior wall thickness at level of papillary muscles

<u>At level of apex:</u>

mLVWTapx-maximum left ventricle wall thickness at level of apex

IVSapx-interventricular septum thickness at level of apex

AWapx-anterior wall thickness at level of apex

LWapx-lateral wall thickness at level of apex

PWapx-posterior wall thickness at level of apex

Composite score:

CWTscore-cumulative wall thickness score

References:

Abecasis GR, Cookson WO and Cardon LR. Pedigree tests of transmission disequilibrium. *Eur J Hum Genet* 8: 545-551, 2000.

Abecasis GR, Cookson WO and Cardon LR. The power to detect linkage disequilibrium with quantitative traits in selected samples. *Am J Hum Genet* 68: 1463-1474, 2001.

Adams TD, Yanowitz FG, Fisher AG, Ridges JD, Nelson AG, Hagan AD, Williams RR and Hunt SC. Heritability of cardiac size: an echocardiographic and electrocardiographic study of monozygotic and dizygotic twins. *Circulation* 71: 39-44, 1985.

Agachan B, Isbir T, Yilmaz H and Akoglu E. Angiotensin converting enzyme I/D, angiotensinogen T174M-M235T and angiotensin II type 1 receptor A1166C gene polymorphisms in Turkish hypertensive patients. *Exp Mol Med* 35: 545-549, 2003.

Akey J, Jin L and Xiong M. Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur J Hum Genet* 9: 291-300, 2001.

Alhenc-Gelas F, Richard J, Courbon D, Warnet JM and Corvol P. Distribution of plasma angiotensin I-converting enzyme levels in healthy men: relationship to environmental and hormonal parameters. *J Lab Clin Med* 117: 33-39, 1991.

Alvarez R, Reguero JR, Batalla A, Iglesias-Cubero G, Cortina A, Alvarez V and Coto E. Angiotensin-converting enzyme and angiotensin II receptor 1 polymorphisms: association with early coronary disease. *Cardiovasc Res* 40: 375-379, 1998.

Anan R, Shono H, Kisanuki A, Arima S, Nakao S and Tanaka H. Patients with familial hypertrophic cardiomyopathy caused by a Phe110Ile missense mutation in the cardiac troponin T gene have variable cardiac morphologies and a favorable prognosis. *Circulation* 98: 391-397, 1998.

Arad M, Benson DW, Perez-Atayde AR, McKenna WJ, Sparks EA, Kanter RJ, McGarry K, Seidman JG and Seidman CE. Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy. *J Clin Invest* 109: 357-362, 2002.

Arad M, Maron BJ, Gorham JM, Johnson WH, Jr., Saul JP, Perez-Atayde AR, Spirito P, Wright GB, Kanter RJ, Seidman CE and Seidman JG. Glycogen storage diseases presenting as hypertrophic cardiomyopathy. *N Engl J Med* 352: 362-372, 2005.

Arai M, Yoguchi A, Iso T, Takahashi T, Imai S, Murata K and Suzuki T. Endothelin-1 and its binding sites are upregulated in pressure overload cardiac hypertrophy. *Am J Physiol* 268: H2084-H2091, 1995.

Arnal JF, Battle T, Rasetti C, Challah M, Costerousse O, Vicaut E, Michel JB and Alhenc-Gelas F. ACE in three tunicae of rat aorta: expression in smooth muscle and effect of renovascular hypertension. *Am J Physiol* 267: H1777-H1784, 1994.

Asano K, Dutcher DL, Port JD, Minobe WA, Tremmel KD, Roden RL, Bohlmeyer TJ, Bush EW, Jenkin MJ, Abraham WT, Raynolds MV, Zisman LS, Perryman MB and Bristow MR. Selective downregulation of the angiotensin II AT1-receptor subtype in failing human ventricular myocardium. *Circulation* 95: 1193-1200, 1997.

Bader M and Ganten D. Regulation of renin: new evidence from cultured cells and genetically modified mice. *J Mol Med* 78: 130-139, 2000.

Bader M and Ganden D. Role of Local Renin Angiotensin System in Cardiac Damage. In: Renin Angiotensin System and the Heart. De Mello W, ed. pp. 47-62, 2004. John Wiley and Sons Ltd. Hoboken, New Jersey.

Baker KM, Booz GW and Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. *Annu Rev Physiol* 54: 227-241, 1992.

Barlassina C, Schork NJ, Manunta P, Citterio L, Sciarrone M, Lanella G, Bianchi G and Cusi D. Synergistic effect of alpha-adducin and ACE genes causes blood pressure changes with body sodium and volume expansion. *Kidney Int* 57: 1083-1090, 2000.

Barrett JC, Fry B, Maller J and Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263-265, 2005.

Baudin B. Polymorphism in angiotensin II receptor genes and hypertension. *Exp Physiol* 90: 277-282, 2005.

Baxevanis AD. The Molecular Biology Database Collection: 2003 update. *Nucleic Acids Res* 31: 1-12, 2003.

Baxevanis AD. Using genomic databases for sequence-based biological discovery. *Mol Med* 9: 185-192, 2003.

Beige J, Zilch O, Hohenbleicher H, Ringel J, Kunz R, Distler A and Sharma AM. Genetic variants of the renin-angiotensin system and ambulatory blood pressure in essential hypertension. *J Hypertens* 15: 503-508, 1997.

Bella JN, MacCluer JW, Roman MJ, Almasy L, North KE, Best LG, Lee ET, Fabsitz RR, Howard BV and Devereux RB. Heritability of left ventricular dimensions and mass in American Indians: The Strong Heart Study. *J Hypertens* 22: 281-286, 2004.

Benjafield AV, Wang WY and Morris BJ. No association of angiotensin-converting enzyme 2 gene (ACE2) polymorphisms with essential hypertension. *Am J Hypertens* 17: 624-628, 2004.

Bennett CL, Schrader AP and Morris BJ. Cross-sectional analysis of Met235-->Thr variant of angiotensinogen gene in severe, familial hypertension. *Biochem Biophys Res Commun* 197: 833-839, 1993.

Benter IF, Ferrario CM, Morris M and Diz DI. Antihypertensive actions of angiotensin-(1-7) in spontaneously hypertensive rats. *Am J Physiol* 269: H313-H319, 1995.

Berry EM, Coustere-Yakir C and Grover NB. The significance of non-significance. *QJM* 91: 647-653, 1998.

Bielen E, Fagard R and Amery A. The inheritance of left ventricular structure and function assessed by imaging and Doppler echocardiography. *Am Heart J* 121: 1743-1749, 1991.

Blair E, Redwood C, Ashrafian H, Oliveira M, Broxholme J, Kerr B, Salmon A, Ostman-Smith I and Watkins H. Mutations in the gamma(2) subunit of AMP-activated protein kinase cause familial hypertrophic cardiomyopathy: evidence for the central role of energy compromise in disease pathogenesis. *Hum Mol Genet* 10: 1215-1220, 2001.

Bland JM and Altman DG. Multiple significance tests: the Bonferroni method. *BMJ* 310: 170, 1995.

Bloem LJ, Manatunga AK, Tewksbury DA and Pratt JH. The serum angiotensinogen concentration and variants of the angiotensinogen gene in white and black children. *J Clin Invest* 95: 948-953, 1995.

Bloem LJ, Manatunga AK and Pratt JH. Racial difference in the relationship of an angiotensin I-converting enzyme gene polymorphism to serum angiotensin I-converting enzyme activity. *Hypertension* 27: 62-66, 1996.

Bohlender J, Menard J, Wagner J, Luft FC and Ganten D. Human renin-dependent hypertension in rats transgenic for human angiotensinogen. *Hypertension* 27: 535-540, 1996.

Bonnardeaux A, Davies E, Jeunemaitre X, Fery I, Charru A, Clauser E, Tiret L, Cambien F, Corvol P and Soubrier F. Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension* 24: 63-69, 1994.

Bonne G, Carrier L, Bercovici J, Cruaud C, Richard P, Hainque B, Gautel M, Labeit S, James M, Beckmann J, Weissenbach J, Vosberg HP, Fiszman M, Komajda M and Schwartz K. Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. *Nat Genet* 11: 438-440, 1995.

Bonvalet JP, Doignon I, Blot-Chabaud M, Pradelles P and Farman N. Distribution of 11 beta-hydroxysteroid dehydrogenase along the rabbit nephron. *J Clin Invest* 86: 832-837, 1990.

Borland JA, Kelsall C, Yacoub MH and Chester AH. Expression, localisation and function of ACE and chymase in normal and atherosclerotic human coronary arteries. *Vascul Pharmacol* 42: 99-108, 2005.

Brachfeld N and Gorlin R. Subaortic stenosis: a revised concept of the disease. *Medicine (Baltimore)* 38: 415-433, 1959.

Brand E, Chatelain N, Mulatero P, Fery I, Curnow K, Jeunemaitre X, Corvol P, Pascoe L and Soubrier F. Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* 32: 198-204, 1998.

Brand E, Schorr U, Ringel J, Beige J, Distler A and Sharma AM. Aldosterone synthase gene (CYP11B2) C-344T polymorphism in Caucasians from the Berlin Salt-Sensitivity Trial (BeSST). *J Hypertens* 17: 1563-1567, 1999.

Brent LB, Aburano A, Fisher DL, Moran TJ, Myers JD and Taylor WJ. Familial muscular subaortic stenosis: an unrecognized form of "idiopathic heart diseases," with clinical and autopsy observations. *Circulation* 21: 167-180, 1960.

Brink AJ, Cassim S, Brink PA, Gregory MA, Naidoo DP, Seedat YK, Motyer R and Desai DK. Myocardial hypertrophy--Part II. *S Afr Med J* 86 Suppl 2: C104-C110, 1996.

Brugada R, Kelsey W, Lechin M, Zhao G, Yu QT, Zoghbi W, Quinones M, Elstein E, Omran A, Rakowski H, Wigle D, Liew CC, Sole M, Roberts R and Marian AJ. Role of candidate modifier genes on the phenotypic expression of hypertrophy in patients with hypertrophic cardiomyopathy. *J Investig Med* 45: 542-551, 1997.

Burrell LM, Risvanis J, Kubota E, Dean RG, MacDonald PS, Lu S, Tikellis C, Grant SL, Lew RA, Smith AI, Cooper ME and Johnston CI. Myocardial infarction increases ACE2 expression in rat and humans. *Eur Heart J* 26: 369-375, 2005.

Campbell DJ and Habener JF. Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. *J Clin Invest* 78: 31-39, 1986.

Campbell DJ and Habener JF. Cellular localization of angiotensinogen gene expression in brown adipose tissue and mesentery: quantification of messenger ribonucleic acid abundance using hybridization in situ. *Endocrinology* 121: 1616-1626, 1987.

Cardon LR and Palmer LJ. Population stratification and spurious allelic association. *Lancet* 361: 598-604, 2003.

Carey RM and Siragy HM. Newly recognized components of the renin-angiotensin system: potential roles in cardiovascular and renal regulation. *Endocr Rev* 24: 261-271, 2003.

Cargill M, Altshuler D, Ireland J, Sklar P, Ardlie K, Patil N, Shaw N, Lane CR, Lim EP, Kalyanaraman N, Nemesh J, Ziaugra L, Friedland L, Rolfe A, Warrington J, Lipshutz R, Daley GQ and Lander ES. Characterization of single-nucleotide polymorphisms in coding regions of human genes. *Nat Genet* 22: 231-238, 1999.

Carrier L, Bonne G, Bahrend E, Yu B, Richard P, Niel F, Hainque B, Cruaud C, Gary F, Labeit S, Bouhour JB, Dubourg O, Desnos M, Hagege AA, Trent RJ, Komajda M, Fiszman M and Schwartz K. Organization and sequence of human cardiac myosin binding protein C gene (MYBPC3) and identification of mutations predicted to produce truncated proteins in familial hypertrophic cardiomyopathy. *Circ Res* 80: 427-434, 1997.

Castellano M, Muiesan ML, Beschi M, Rizzoni D, Cinelli A, Salvetti M, Pasini G, Porteri E, Bettoni G, Zulli R and Agabiti-Rosei E. Angiotensin II type 1 receptor A/C1166 polymorphism. Relationships with blood pressure and cardiovascular structure. *Hypertension* 28: 1076-1080, 1996.

Catt KJ, Cain MD, Coghlan JP, Zimmet PZ, Cran E and Best JB. Metabolism and blood levels of angiotensin II in normal subjects, renal disease, and essential hypertension. *Circ Res* 27: Suppl, 1970.

Caulfield M, Lavender P, Farrall M, Munroe P, Lawson M, Turner P and Clark AJ. Linkage of the angiotensinogen gene to essential hypertension. *N Engl J Med* 330: 1629-1633, 1994.

Caulfield M, Lavender P, Newell-Price J, Farrall M, Kamdar S, Daniel H, Lawson M, De Freitas P, Fogarty P and Clark AJ. Linkage of the angiotensinogen gene locus to human essential hypertension in African Caribbeans. *J Clin Invest* 96: 687-692, 1995.

Charron P, Dubourg O, Desnos M, Isnard R, Hagege A, Millaire A, Carrier L, Bonne G, Tesson F, Richard P, Bouhour JB, Schwartz K and Komajda M. Diagnostic value of electrocardiography and echocardiography for familial hypertrophic cardiomyopathy in a genotyped adult population. *Circulation* 96: 214-219, 1997.

Charron P, Dubourg O, Desnos M, Bennaceur M, Carrier L, Camproux AC, Isnard R, Hagege A, Langlard JM, Bonne G, Richard P, Hainque B, Bouhour JB, Schwartz K and Komajda M. Clinical features and prognostic implications of familial hypertrophic cardiomyopathy related to the cardiac myosin-binding protein C gene. *Circulation* 97: 2230-2236, 1998.

Chaves FJ, Pascual JM, Rovira E, Armengod ME and Redon J. Angiotensin II AT1 receptor gene polymorphism and microalbuminuria in essential hypertension. *Am J Hypertens* 14: 364-370, 2001.

Cheung BM, Leung R, Shiu S, Tan KC, Lau CP and Kumana CR. M235T polymorphism of the angiotensinogen gene and hypertension in Chinese. *J Hypertens* 16: 1137-1140, 1998.

Chien KL, Hsu HC, Su TC, Chen MF and Lee YT. Heritability and major gene effects on left ventricular mass in the Chinese population: a family study. *BMC Cardiovasc Disord* 6: 37, 2006.

Clyne CD, Zhang Y, Slutsker L, Mathis JM, White PC and Rainey WE. Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements. *Mol Endocrinol* 11: 638-649, 1997.

Cohen SL, Jhetam D, Da Silva J, Milne FJ and van der WA. Sodium and potassium status, plasma renin and aldosterone profiles in normotensive and hypertensive Johannesburg blacks. *S Afr Med J* 62: 941-944, 1982.

Cooper R, McFarlane-Anderson N, Bennett FI, Wilks R, Puras A, Tewksbury D, Ward R and Forrester T. ACE, angiotensinogen and obesity: a potential pathway leading to hypertension. *J Hum Hypertens* 11: 107-111, 1997.

Corfield VA, Moolman JC, Martell R and Brink PA. Polymerase chain reaction-based detection of MN blood group-specific sequences in the human genome. *Transfusion* 33: 119-124, 1993.

Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, Chappell MC, Backx PH, Yagil Y and Penninger JM. Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* 417: 822-828, 2002.

Curnow KM, Pascoe L and White PC. Genetic analysis of the human type-1 angiotensin II receptor. *Mol Endocrinol* 6: 1113-1118, 1992.

Czubryt MP and Olson EN. Balancing contractility and energy production: the role of myocyte enhancer factor 2 (MEF2) in cardiac hypertrophy. *Recent Prog Horm Res* 59: 105-124, 2004.

Daly MJ, Rioux JD, Schaffner SF, Hudson TJ and Lander ES. High-resolution haplotype structure in the human genome. *Nat Genet* 29: 229-232, 2001.

Danilczyk U, Eriksson U, Crackower MA and Penninger JM. A story of two ACEs. *J Mol Med* 81: 227-234, 2003.

Danser AH, Koning MM, Admiraal PJ, Sassen LM, Derkx FH, Verdouw PD and Schalekamp MA. Production of angiotensins I and II at tissue sites in intact pigs. *Am J Physiol* 263: H429-H437, 1992.

Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA and Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation* 92: 1387-1388, 1995.

Dausse E, Komajda M, Fetler L, Dubourg O, Dufour C, Carrier L, Wisnewsky C, Bercovici J, Hengstenberg C, al Mahdawi S, Isnard R, Hagege A, Bouhour J, Desnos M, Beckmann J, Weissenbach J, Schwartz K and Guicheney P. Familial hypertrophic cardiomyopathy. Microsatellite haplotyping and identification of a hot spot for mutations in the beta-myosin heavy chain gene. *J Clin Invest* 92: 2807-2813, 1993.

Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C, Anderson NH, Fraser R and Connell JM. Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. *Hypertension* 33: 703-707, 1999.

Davies E and Kenyon CJ. CYP11B2 polymorphisms and cardiovascular risk factors. *J Hypertens* 21: 1249-1253, 2003.

Davies MJ. The current status of myocardial disarray in hypertrophic cardiomyopathy. *Br Heart J* 51: 361-363, 1984.

De Mello WC and Danser AH. Angiotensin II and the heart : on the intracrine renin-angiotensin system. *Hypertension* 35: 1183-1188, 2000.

Delles C, Erdmann J, Jacobi J, Hilgers KF, Fleck E, Regitz-Zagrosek V and Schmieder RE. Aldosterone synthase (CYP11B2) -344 C/T polymorphism is associated with left ventricular structure in human arterial hypertension. *J Am Coll Cardiol* 37: 878-884, 2001.

Denfield SW and Garson A, Jr. Sudden death in children and young adults. *Pediatr Clin North Am* 37: 215-231, 1990.

Deng HW. Population admixture may appear to mask, change or reverse genetic effects of genes underlying complex traits. *Genetics* 159: 1319-1323, 2001.

Deschepper CF, Mellon SH, Cumin F, Baxter JD and Ganong WF. Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat. *Proc Natl Acad Sci U S A* 83: 7552-7556, 1986.

Dessi-Fulgheri P, Catalini R, Sarzani R, Sturbini S, Siragusa N, Guazzarotti F, Offidani M, Tamburrini P, Zingaretti O and Rappelli A. Angiotensin converting enzyme gene polymorphism and carotid atherosclerosis in a low-risk population. *J Hypertens* 13: 1593-1596, 1995.

Diao G and Lin DY. Improving the power of association tests for quantitative traits in family studies. *Genet Epidemiol* 30: 301-313, 2006.

Diet F, Graf C, Mahnke N, Wassmer G, Predel HG, Palma-Hohmann I, Rost R and Bohm M. ACE and angiotensinogen gene genotypes and left ventricular mass in athletes. *Eur J Clin Invest* 31: 836-842, 2001.

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE and Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ Res* 87: E1-E9, 2000.

Doolan G, Nguyen L, Chung J, Ingles J and Semsarian C. Progression of left ventricular hypertrophy and the angiotensin-converting enzyme gene polymorphism in hypertrophic cardiomyopathy. *Int J Cardiol* 96: 157-163, 2004.

Drayer JI, Gardin JM and Weber MA. Echocardiographic left ventricular hypertrophy in hypertension. *Chest* 84: 217-221, 1983.

Drayer JI, Weber MA, Gardin JM and Lipson JL. Effect of long-term antihypertensive therapy on cardiac anatomy in patients with essential hypertension. *Am J Med* 75: 116-120, 1983.

Dunn FG, Oigman W, Ventura HO, Messerli FH, Kobrin I and Frohlich ED. Enalapril improves systemic and renal hemodynamics and allows regression of left ventricular mass in essential hypertension. *Am J Cardiol* 53: 105-108, 1984.

Dzau VJ, Ellison KE, Brody T, Ingelfinger J and Pratt RE . A comparative study of the distributions of renin and angiotensinogen messenger ribonucleic acids in rat and mouse tissues. *Endocrinology* 120: 2334-2338, 1987.

Dzau VJ. Multiple pathways of angiotensin production in the blood vessel wall: evidence, possibilities and hypotheses. *J Hypertens* 7: 933-936, 1989.

Dzau VJ. Tissue renin-angiotensin system in myocardial hypertrophy and failure. *Arch Intern Med* 153: 937-942, 1993.

Easterbrook PJ, Berlin JA, Gopalan R and Matthews DR. Publication bias in clinical research. *Lancet* 337: 867-872, 1991.

Egger M, Ebrahim S and Smith GD. Where now for meta-analysis? *Int J Epidemiol* 31: 1-5, 2002.

Elston RC. Introduction and overview. Statistical methods in genetic epidemiology. *Stat Methods Med Res* 9: 527-541, 2000.

Epstein ND, Fananapazir L, Lin HJ, Mulvihill J, White R, Lalouel JM, Lifton RP, Nienhuis AW and Leppert M. Evidence of genetic heterogeneity in five kindreds with familial hypertrophic cardiomyopathy. *Circulation* 85: 635-647, 1992 (a).

Epstein ND, Cohn GM, Cyran F and Fananapazir L. Differences in clinical expression of hypertrophic cardiomyopathy associated with two distinct mutations in the beta-myosin heavy chain gene. A 908Leu----Val mutation and a 403Arg----Gln mutation. *Circulation* 86: 345-352, 1992 (b).

Erdmann J, Hegemann N, Weidemann A, Kallisch H, Hummel M, Hetzer R, Fleck E and Regitz-Zagrosek V. Screening the human bradykinin B2 receptor gene in patients with cardiovascular diseases: identification of a functional mutation in the promoter and a new coding variant (T21M). *Am J Med Genet* 80: 521-525, 1998.

Erdmann J, Raible J, Maki-Abadi J, Hummel M, Hammann J, Wollnik B, Frantz E, Fleck E, Hetzer R and Regitz-Zagrosek V. Spectrum of clinical phenotypes and gene variants in cardiac myosin-binding protein C mutation carriers with hypertrophic cardiomyopathy. *J Am Coll Cardiol* 38: 322-330, 2001.

Fananapazir L and Epstein ND. Genotype-phenotype correlations in hypertrophic cardiomyopathy. Insights provided by comparisons of kindreds with distinct and identical beta-myosin heavy chain gene mutations. *Circulation* 89: 22-32, 1994.

Fardella CE, Claverie X, Vignolo P, Montero J and Villarroel L. T235 variant of the angiotensinogen gene and blood pressure in the Chilean population. *J Hypertens* 16: 829-833, 1998.

Farrall M, Keavney B, McKenzie C, Delepine M, Matsuda F and Lathrop GM. Fine-mapping of an ancestral recombination breakpoint in DCP1. *Nat Genet* 23: 270-271, 1999.

Fatini C, Abbate R, Pepe G, Battaglini B, Gensini F, Ruggiano G, Gensini GF and Guazzelli R. Searching for a better assessment of the individual coronary risk profile. The role of angiotensin-converting enzyme, angiotensin II type 1 receptor and angiotensinogen gene polymorphisms. *Eur Heart J* 21: 633-638, 2000.

Fatkin D, McConnell BK, Mudd JO, Semsarian C, Moskowitz IG, Schoen FJ, Giewat M, Seidman CE and Seidman JG. An abnormal Ca(2+) response in mutant sarcomere protein-mediated familial hypertrophic cardiomyopathy. *J Clin Invest* 106: 1351-1359, 2000.

Fernandez-Llama P, Poch E, Oriola J, Botey A, Rivera F and Revert L. Angiotensinogen gene M235T and T174M polymorphisms in essential hypertension: relation with target organ damage. *Am J Hypertens* 11: 439-444, 1998.

Fisher ND, Hurwitz S, Ferri C, Jeunemaitre X, Hollenberg NK and Williams GH. Altered adrenal sensitivity to angiotensin II in low-renin essential hypertension. *Hypertension* 34: 388-394, 1999.

Fleming I. Signaling by the angiotensin-converting enzyme. *Circ Res* 98: 887-896, 2006.

Fogari R, Zoppi A, Mugellini A, Tettamanti F, Lusardi P and Corradi L. Effects of lisinopril vs hydralazine on left ventricular hypertrophy and ambulatory blood pressure monitoring in essential hypertension. *Eur Heart J* 16: 1120-1125, 1995.

Folkow B. Physiological aspects of primary hypertension. *Physiol Rev* 62: 347-504, 1982.

Forissier JF, Charron P, Tezenas du MS, Hagege A, Isnard R, Carrier L, Richard P, Desnos M, Bouhour JB, Schwartz K, Komajda M and Dubourg O. Diagnostic accuracy of a 2D left ventricle hypertrophy score for familial hypertrophic cardiomyopathy. *Eur Heart J* 26: 1882-1886, 2005.

Fornage M, Amos CI, Kardia S, Sing CF, Turner ST and Boerwinkle E. Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males. *Circulation* 97: 1773-1779, 1998.

Freeman EJ, Chisolm GM, Ferrario CM and Tallant EA. Angiotensin-(1-7) inhibits vascular smooth muscle cell growth. *Hypertension* 28: 104-108, 1996.

Frohlich ED and Sasaki O. Dissociation of changes in cardiovascular mass and performance with angiotensin-converting enzyme inhibitors in Wistar-Kyoto and spontaneously hypertensive rats. *J Am Coll Cardiol* 16: 1492-1499, 1990.

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ and Altshuler D. The structure of haplotype blocks in the human genome. *Science* 296: 2225-2229, 2002.

Galperin MY. The Molecular Biology Database Collection: 2007 update. *Nucleic Acids Res* 35: D3-D4, 2007.

Gambaro G, Anglani F and D'Angelo A. Association studies of genetic polymorphisms and complex disease. *Lancet* 355: 308-311, 2000.

Ganau A, Devereux RB, Pickering TG, Roman MJ, Schnall PL, Santucci S, Spitzer MC and Laragh JH. Relation of left ventricular hemodynamic load and contractile performance to left ventricular mass in hypertension. *Circulation* 81: 25-36, 1990.

Ganau A, Devereux RB, Roman MJ, de Simone G, Pickering TG, Saba PS, Vargiu P, Simongini I and Laragh JH. Patterns of left ventricular hypertrophy and geometric remodeling in essential hypertension. *J Am Coll Cardiol* 19: 1550-1558, 1992.

Ganten D, Lang RE, Lehmann E and Unger T. Brain angiotensin: on the way to becoming a well-studied neuropeptide system. *Biochem Pharmacol* 33: 3523-3528, 1984.

Garner C, Lecomte E, Visvikis S, Abergel E, Lathrop M and Soubrier F. Genetic and environmental influences on left ventricular mass. A family study. *Hypertension* 36: 740-746, 2000.

Geier C, Perrot A, Ozcelik C, Binner P, Counsell D, Hoffmann K, Pilz B, Martiniak Y, Gehmlich K, Van der Ven PF, Furst DO, Vornwald A, von Hodenberg E, Nurnberg P, Scheffold T, Dietz R and Osterziel KJ. Mutations in the human muscle LIM protein gene in families with hypertrophic cardiomyopathy. *Circulation* 107: 1390-1395, 2003.

Geisterfer-Lowrance AA, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE and Seidman JG. A molecular basis for familial hypertrophic cardiomyopathy: a beta cardiac myosin heavy chain gene missense mutation. *Cell* 62: 999-1006, 1990.

Ghali JK, Liao Y, Simmons B, Castaner A, Cao G and Cooper RS. The prognostic role of left ventricular hypertrophy in patients with or without coronary artery disease. *Ann Intern Med* 117: 831-836, 1992.

Gharavi AG, Lipkowitz MS, Diamond JA, Jhang JS and Phillips RA. Deletion polymorphism of the angiotensin-converting enzyme gene is independently associated with left ventricular mass and geometric remodeling in systemic hypertension. *Am J Cardiol* 77: 1315-1319, 1996.

Gharavi AG, Lipkowitz ML, Diamond JA, Chamie R and Phillips RA. Ambulatory blood pressure monitoring for detecting the relation between angiotensinogen gene polymorphism and hypertension. *Am J Hypertens* 10: 687-691, 1997.

Glass GV. Primary, secondary and meta-analysis of research. *Educ Res* 5: 3-8, 1976.

Gollob MH, Green MS, Tang AS, Gollob T, Karibe A, Ali Hassan AS, Ahmad F, Lozado R, Shah G, Fananapazir L, Bachinski LL and Roberts R. Identification of a gene responsible for familial Wolff-Parkinson-White syndrome. *N Engl J Med* 344: 1823-1831, 2001.

Goulter AB, Goddard MJ, Allen JC and Clark KL. ACE2 gene expression is up-regulated in the human failing heart. *BMC Med* 2: 19, 2004.

Granger CB, Karimeddini MK, Smith VE, Shapiro HR, Katz AM and Riba AL. Rapid ventricular filling in left ventricular hypertrophy: I. Physiologic hypertrophy. *J Am Coll Cardiol* 5: 862-868, 1985.

Gregory MA, Brouckaert CJ and Whitton ID. Characterisation of normal human myocardium by means of morphometric analysis. *Cardiovasc Res* 17: 177-183, 1983.

Griendling KK, Murphy TJ and Alexander RW. Molecular biology of the renin-angiotensin system. *Circulation* 87: 1816-1828, 1993.

Grossman W, Jones D and McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Invest* 56: 56-64, 1975.

Gu D, Ge D, He J, Li B, Chen J, Liu D, Chen J and Chen R . Haplotypic analyses of the aldosterone synthase gene CYP11B2 associated with stage-2 hypertension in northern Han Chinese. *Clin Genet* 66: 409-416, 2004.

Gumprecht J, Zychma M, Grzeszczak W, LAcka B, Burak W, Mosur M, Kaczmarski J, Otulski I, Stoklosa T and Czank P. Angiotensin I-converting enzyme and chymase gene polymorphisms - relationship to left ventricular mass in type 2 diabetes patients. *Med Sci Monit* 8: CR603-CR606, 2002.

Gurley SB, Allred A, Le TH, Griffiths R, Mao L, Philip N, Haystead TA, Donoghue M, Breitbart RE, Acton SL, Rockman HA and Coffman TM. Altered blood pressure responses and normal cardiac phenotype in ACE2-null mice. *J Clin Invest* 116: 2218-2225, 2006.

Hall JE, Mizelle HL and Woods LL. The renin-angiotensin system and long-term regulation of arterial pressure. *J Hypertens* 4: 387-397, 1986.

Hall JE, Guyton AC and Mizelle HL. Role of the renin-angiotensin system in control of sodium excretion and arterial pressure. *Acta Physiol Scand Suppl* 591: 48-62, 1990.

Hall JE, Guyton AC and Brands MW. Pressure-volume regulation in hypertension. *Kidney Int Suppl* 55: S35-S41, 1996.

Halushka MK, Fan JB, Bentley K, Hsie L, Shen N, Weder A, Cooper R, Lipshutz R and Chakravarti A. Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* 22: 239-247, 1999.

Hamon M, Amant C, Bauters C, Richard F, Helbecque N, McFadden E, Lablanche JM, Bertrand M and Amouyel P. Association of angiotensin converting enzyme and angiotensin II type 1 receptor genotypes with left ventricular function and mass in patients with angiographically normal coronary arteries. *Heart* 77: 502-505, 1997.

Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K and Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin II type 1A receptor knockout mice. *Circulation* 97: 1952-1959, 1998.

Harada K, Sugaya T, Murakami K, Yazaki Y and Komuro I. Angiotensin II type 1A receptor knockout mice display less left ventricular remodeling and improved survival after myocardial infarction. *Circulation* 100: 2093-2099, 1999.

Harrap SB, Dominiczak AF, Fraser R, Lever AF, Morton JJ, Foy CJ and Watt GC. Plasma angiotensin II, predisposition to hypertension, and left ventricular size in healthy young adults. *Circulation* 93: 1148-1154, 1996.

Hata A, Namikawa C, Sasaki M, Sato K, Nakamura T, Tamura K and Lalouel JM. Angiotensinogen as a risk factor for essential hypertension in Japan. *J Clin Invest* 93: 1285-1287, 1994.

Hatakeyama H, Miyamori I, Takeda Y, Yamamoto H and Mabuchi H. The expression of steroidogenic enzyme genes in human vascular cells. *Biochem Mol Biol Int* 40: 639-645, 1996.

Haywood GA, Gullestad L, Katsuya T, Hutchinson HG, Pratt RE, Horiuchi M and Fowler MB. AT1 and AT2 angiotensin receptor gene expression in human heart failure. *Circulation* 95: 1201-1206, 1997.

He H, Li LM, Cao WH, Sun NL, Liu MZ and Hu YH. A study of the relationships between angiotensin- converting enzyme gene, chymase gene polymorphisms, pharmacological treatment with ACE inhibitor and regression of left ventricular hypertrophy in essential hypertension patients treated with benazepril. *Ann Hum Biol* 32: 30-43, 2005.

Hegele RA, Harris SB, Hanley AJ, Sun F, Connelly PW and Zinman B. -6A promoter variant of angiotensinogen and blood pressure variation in Canadian Oji-Cree. *J Hum Genet* 43: 37-41, 1998.

Hengstenberg C, Holmer SR, Mayer B, Lowel H, Engel S, Hense HW, Riegger GA and Schunkert H. Evaluation of the aldosterone synthase (CYP11B2) gene polymorphism in patients with myocardial infarction. *Hypertension* 35: 704-709, 2000.

Hernandez D, de la RA, Barragan A, Barrios Y, Salido E, Torres A, Martin B, Laynez I, Duque A, De Vera A, Lorenzo V and Gonzalez A. The ACE/DD genotype is associated with the extent of exercise-induced left ventricular growth in endurance athletes. *J Am Coll Cardiol* 42: 527-532, 2003.

Higaki J, Aoki M, Morishita R, Kida I, Taniyama Y, Tomita N, Yamamoto K, Moriguchi A, Kaneda Y and Ogihara T. In vivo evidence of the importance of cardiac angiotensin-converting enzyme in the pathogenesis of cardiac hypertrophy. *Arterioscler Thromb Vasc Biol* 20: 428-434, 2000 (a).

Higaki J, Baba S, Katsuya T, Sato N, Ishikawa K, Mannami T, Ogata J and Ogihara T. Deletion allele of angiotensin-converting enzyme gene increases risk of essential hypertension in Japanese men: the Suita Study. *Circulation* 101: 2060-2065, 2000 (b).

Higashimori K, Gante J, Holzemann G and Inagami T. Significance of vascular renin for local generation of angiotensins. *Hypertension* 17: 270-277, 1991.

Hingorani AD, Sharma P, Jia H, Hopper R and Brown MJ. Blood pressure and the M235T polymorphism of the angiotensinogen gene. *Hypertension* 28: 907-911, 1996.

Hirschhorn JN and Daly MJ. Genome-wide association studies for common diseases and complex traits. *Nat Rev Genet* 6: 95-108, 2005.

Ho CY and Seidman CE. A contemporary approach to hypertrophic cardiomyopathy. *Circulation* 113: e858-e862, 2006.

Hoffmann B, Schmidt-Traub H, Perrot A, Osterziel KJ and Gessner R. First mutation in cardiac troponin C, L29Q, in a patient with hypertrophic cardiomyopathy. *Hum Mutat* 17: 524, 2001.

Hoffmann S, Krause T, van Geel PP, Willenbrock R, Pagel I, Pinto YM, Buikema H, van Gilst WH, Lindschau C, Paul M, Inagami T, Ganten D and Urata H. Overexpression of the human angiotensin II type 1 receptor in the rat heart augments load induced cardiac hypertrophy. *J Mol Med* 79: 601-608, 2001.

Hsueh WA and Baxter JD. Human prorenin. *Hypertension* 17: 469-477, 1991.

Iles M. In: Camp NJ and Cox A. Quantitative trait loci: Methods and Protocols. Methods in Molecular Biology Vol 195 Chapter 5. pp. 135, 2002. Humana Press Inc. Totowa, New Jersey.

Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear R, Powers M, Cheng T, Ludwig EH, Sharma AM, Hata A, Jeunemaitre X and Lalouel JM. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J Clin Invest* 99: 1786-1797, 1997.

International HapMap Consortium. The International HapMap Project. Nature 426: 789-796, (2003).

International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature 409, 860-921 (2001).

Isaji M, Mune T, Takada N, Yamamoto Y, Suwa T, Morita H, Takeda J and White PC. Correlation between left ventricular mass and urinary sodium excretion in specific genotypes of CYP11B2. *J Hypertens* 23: 1149-1157, 2005.

Ishanov A, Okamoto H, Yoneya K, Watanabe M, Nakagawa I, Machida M, Onozuka H, Mikami T, Kawaguchi H, Hata A, Kondo K and Kitabatake A. Angiotensinogen gene polymorphism in Japanese patients with hypertrophic cardiomyopathy. *Am Heart J* 133: 184-189, 1997.

Ishanov A, Okamoto H, Watanabe M, Yoneya K, Nakagawa I, Kumamoto H, Chiba S, Hata A, Kawaguchi H and Kitabatake A. Angiotensin II type 1 receptor gene polymorphisms in patients with cardiac hypertrophy. *Jpn Heart J* 39: 87-96, 1998.

Ishigami T, Umemura S, Tamura K, Hibi K, Nyui N, Kihara M, Yabana M, Watanabe Y, Sumida Y, Nagahara T, Ochiai H and Ishii M. Essential hypertension and 5' upstream core promoter region of human angiotensinogen gene. *Hypertension* 30: 1325-1330, 1997.

Ito H, Hiroe M, Hirata Y, Fujisaki H, Adachi S, Akimoto H, Ohta Y and Marumo F. Endothelin ETA receptor antagonist blocks cardiac hypertrophy provoked by hemodynamic overload. *Circulation* 89: 2198-2203, 1994.

Iwai N, Ohmichi N, Nakamura Y and Kinoshita M. DD genotype of the angiotensin-converting enzyme gene is a risk factor for left ventricular hypertrophy. *Circulation* 90: 2622-2628, 1994.

Jeng JR. Left ventricular mass, carotid wall thickness, and angiotensinogen gene polymorphism in patients with hypertension. *Am J Hypertens* 12: 443-450, 1999.

Jeunemaitre X, Lifton RP, Hunt SC, Williams RR and Lalouel JM. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. *Nat Genet* 1: 72-75, 1992.

Jeunemaitre X, Inoue I, Williams C, Charru A, Tichet J, Powers M, Sharma AM, Gimenez-Roqueplo AP, Hata A, Corvol P and Lalouel JM. Haplotypes of angiotensinogen in essential hypertension. *Am J Hum Genet* 60: 1448-1460, 1997.

Jiang Z, Zhao W, Yu F and Xu G. Association of angiotensin II type 1 receptor gene polymorphism with essential hypertension. *Chin Med J (Engl)* 114: 1249-1251, 2001.

Jin W, Liu Y, Sheng HH, Jin L, Shen YY, Hua Q, Lu L, Yu JD and Huang W. Single nucleotide polymorphisms in promoter of angiotensin II type 1 receptor gene associated with essential hypertension and coronary heart disease in Chinese population. *Acta Pharmacol Sin* 24: 1083-1088, 2003.

Jorde LB. Linkage disequilibrium as a gene-mapping tool. *Am J Hum Genet* 56: 11-14, 1995.

Juo SH, Di Tullio MR, Lin HF, Rundek T, Boden-Albala B, Homma S and Sacco RL. Heritability of left ventricular mass and other morphologic variables in Caribbean Hispanic subjects: the Northern Manhattan Family Study. *J Am Coll Cardiol* 46: 735-737, 2005.

Kannel WB. Hypertension, blood lipids, and cigarette smoking as co-risk factors for coronary heart disease. *Ann N Y Acad Sci* 304: 128-139, 1978.

Karjalainen J, Kujala UM, Stolt A, Mantysaari M, Viitasalo M, Kainulainen K and Kontula K. Angiotensinogen gene M235T polymorphism predicts left ventricular hypertrophy in endurance athletes. *J Am Coll Cardiol* 34: 494-499, 1999.

Katada J, Meguro T, Saito H, Ohashi A, Anzai T, Ogawa S and Yoshikawa T. Persistent cardiac aldosterone synthesis in angiotensin II type 1A receptor-knockout mice after myocardial infarction. *Circulation* 111: 2157-2164, 2005.

Kato N, Sugiyama T, Morita H, Kurihara H, Yamori Y and Yazaki Y. Angiotensinogen gene and essential hypertension in the Japanese: extensive association study and meta-analysis on six reported studies. *J Hypertens* 17: 757-763, 1999.

Kato N, Sugiyama T, Morita H, Kurihara H, Furukawa T, Isshiki T, Sato T, Yamori Y and Yazaki Y. Comprehensive analysis of the renin-angiotensin gene polymorphisms with relation to hypertension in the Japanese. *J Hypertens* 18: 1025-1032, 2000.

Kauma H, Ikaheimo M, Savolainen MJ, Kiema TR, Rantala AO, Lilja M, Reunanen A and Kesaniemi YA. Variants of renin-angiotensin system genes and echocardiographic left ventricular mass. *Eur Heart J* 19: 1109-1117, 1998.

Keavney B, McKenzie CA, Connell JM, Julier C, Ratcliffe PJ, Sobel E, Lathrop M and Farrall M. Measured haplotype analysis of the angiotensin-I converting enzyme gene. *Hum Mol Genet* 7: 1745-1751, 1998.

Kidd KK, Morar B, Castiglione CM, Zhao H, Pakstis AJ, Speed WC, Bonne-Tamir B, Lu RB, Goldman D, Lee C, Nam YS, Grandy DK, Jenkins T and Kidd JR. A global survey of haplotype frequencies and linkage disequilibrium at the DRD2 locus. *Hum Genet* 103: 211-227, 1998.

Kiema TR, Kauma H, Rantala AO, Lilja M, Reunanen A, Kesaniemi YA and Savolainen MJ. Variation at the angiotensin-converting enzyme gene and

angiotensinogen gene loci in relation to blood pressure. *Hypertension* 28: 1070-1075, 1996.

Kim HS, Krege JH, Kluckman KD, Hagaman JR, Hodgin JB, Best CF, Jennette JC, Coffman TM, Maeda N and Smithies O. Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A* 92: 2735-2739, 1995.

Kimura A, Harada H, Park JE, Nishi H, Satoh M, Takahashi M, Hiroi S, Sasaoka T, Ohbuchi N, Nakamura T, Koyanagi T, Hwang TH, Choo JA, Chung KS, Hasegawa A, Nagai R, Okazaki O, Nakamura H, Matsuzaki M, Sakamoto T, Toshima H, Koga Y, Imaizumi T and Sasazuki T. Mutations in the cardiac troponin I gene associated with hypertrophic cardiomyopathy. *Nat Genet* 16: 379-382, 1997.

Koch-Weser J. Myocardial actions of angiotensin. *Circ Res* 14: 337-344, 1964.

Komiya I, Yamada T, Takara M, Asawa T, Shimabukuro M, Nishimori T and Takasu N. Lys(173)Arg and -344T/C variants of CYP11B2 in Japanese patients with low-renin hypertension. *Hypertension* 35: 699-703, 2000.

Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, Takaku F and Yazaki Y. Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem* 265: 3595-3598, 1990.

Koren MJ, Devereux RB, Casale PN, Savage DD and Laragh JH. Relation of left ventricular mass and geometry to morbidity and mortality in uncomplicated essential hypertension. *Ann Intern Med* 114: 345-352, 1991.

Korner PI and Jennings GL. Assessment of prevalence of left ventricular hypertrophy in hypertension. *J Hypertens* 16: 715-723, 1998.

Kotchen TA, Kotchen JM, Grim CE, George V, Kaldunski ML, Cowley AW, Hamet P and Chelius TH. Genetic determinants of hypertension: identification of candidate phenotypes. *Hypertension* 36: 7-13, 2000.

Krege JH, John SW, Langenbach LL, Hodgin JB, Hagaman JR, Bachman ES, Jennette JC, O'Brien DA and Smithies O. Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* 375: 146-148, 1995.

Kumar NN, Benjafield AV, Lin RC, Wang WY, Stowasser M and Morris BJ. Haplotype analysis of aldosterone synthase gene (CYP11B2) polymorphisms shows association with essential hypertension. *J Hypertens* 21: 1331-1337, 2003.

Kupari M, Perola M, Koskinen P, Virolainen J and Karhunen PJ. Left ventricular size, mass, and function in relation to angiotensin-converting enzyme gene polymorphism in humans. *Am J Physiol* 267: H1107-H1111, 1994.

Kupari M, Hautanen A, Lankinen L, Koskinen P, Virolainen J, Nikkila H and White PC. Associations between human aldosterone synthase (CYP11B2) gene polymorphisms and left ventricular size, mass, and function. *Circulation* 97: 569-575, 1998.

Kurland L, Melhus H, Karlsson J, Kahan T, Malmqvist K, Ohman P, Nystrom F, Hagg A and Lind L. Polymorphisms in the angiotensinogen and angiotensin II type 1 receptor gene are related to change in left ventricular mass during antihypertensive treatment: results from the Swedish Irbesartan Left Ventricular Hypertrophy Investigation versus Atenolol (SILVHIA) trial. *J Hypertens* 20: 657-663, 2002.

Kuznetsova T, Staessen JA, Wang JG, Gasowski J, Nikitin Y, Ryabikov A and Fagard R. Antihypertensive treatment modulates the association between the D/I ACE gene polymorphism and left ventricular hypertrophy: a meta-analysis. *J Hum Hypertens* 14: 447-454, 2000.

Kuznetsova T, Staessen JA, Thijs L, Kunath C, Olszanecka A, Ryabikov A, Tikhonoff V, Stolarz K, Bianchi G, Casiglia E, Fagard R, Brand-Herrmann SM, Kawecka-Jaszcz K, Malyutina S, Nikitin Y and Brand E. Left ventricular mass in relation to genetic variation in angiotensin II receptors, renin system genes, and sodium excretion. *Circulation* 110: 2644-2650, 2004.

Laragh JH. Renovascular hypertension: a paradigm for all hypertension. *J Hypertens Suppl* 4: S79-S88, 1986.

Lechin M, Quinones MA, Omran A, Hill R, Yu QT, Rakowski H, Wigle D, Liew CC, Sole M, Roberts R and . Angiotensin-I converting enzyme genotypes and left ventricular hypertrophy in patients with hypertrophic cardiomyopathy. *Circulation* 92: 1808-1812, 1995.

Lemos VS, Cortes SF, Silva DM, Campagnole-Santos MJ and Santos RA. Angiotensin-(1-7) is involved in the endothelium-dependent modulation of phenylephrine-induced contraction in the aorta of mRen-2 transgenic rats. *Br J Pharmacol* 135: 1743-1748, 2002.

Levy D, Anderson KM, Savage DD, Kannel WB, Christiansen JC and Castelli WP. Echocardiographically detected left ventricular hypertrophy: prevalence and risk factors. The Framingham Heart Study. *Ann Intern Med* 108: 7-13, 1988.

Levy D, Garrison RJ, Savage DD, Kannel WB and Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* 322: 1561-1566, 1990.

Li WH and Sadler LA. Low nucleotide diversity in man. *Genetics* 129: 513-523, 1991.

Lieb W, Graf J, Gotz A, Konig IR, Mayer B, Fischer M, Stritzke J, Hengstenberg C, Holmer SR, Doring A, Lowel H, Schunkert H and Erdmann J. Association of angiotensin-converting enzyme 2 (ACE2) gene polymorphisms with parameters of left

ventricular hypertrophy in men. Results of the MONICA Augsburg echocardiographic substudy. *J Mol Med* 84: 88-96, 2006.

Liebson PR and Serry RD. Optimal antihypertensive therapy for prevention and treatment of left ventricular hypertrophy. *Curr Hypertens Rep* 2: 260-270, 2000.

Lim DS, Lutucuta S, Bachireddy P, Youker K, Evans A, Entman M, Roberts R and Marian AJ. Angiotensin II blockade reverses myocardial fibrosis in a transgenic mouse model of human hypertrophic cardiomyopathy. *Circulation* 103: 789-791, 2001.

Lin SL, Chang D, Wu DY and Ying SY. A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. *Biochem Biophys Res Commun* 310: 754-760, 2003.

Lindpaintner K, Lee M, Larson MG, Rao VS, Pfeffer MA, Ordovas JM, Schaefer EJ, Wilson AF, Wilson PW, Vasan RS, Myers RH and Levy D. Absence of association or genetic linkage between the angiotensin-converting-enzyme gene and left ventricular mass. *N Engl J Med* 334: 1023-1028, 1996.

Linz W, Scholkens BA and Ganten D. Converting enzyme inhibition specifically prevents the development and induces regression of cardiac hypertrophy in rats. *Clin Exp Hypertens A* 11: 1325-1350, 1989.

Lombes M, Alfaidy N, Eugene E, Lessana A, Farman N and Bonvalet JP. Prerequisite for cardiac aldosterone action. Mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase in the human heart. *Circulation* 92: 175-182, 1995.

Lopez-Haldon J, Garcia-Lozano JR, Martinez MA, Nunez-Roldan A and Burgos CJ. The effect of polymorphisms of the angiotensin-converting enzyme and angiotensinogen genes on the phenotypic expression of Spanish patients with hypertrophic cardiomyopathy. *Med Clin (Barc)* 113: 161-163, 1999.

Makridakis NM and Reichardt JK. Multiplex automated primer extension analysis: simultaneous genotyping of several polymorphisms. *Biotechniques* 31: 1374-1380, 2001.

Mann DL and Spinale FG. Activation of matrix metalloproteinases in the failing human heart: breaking the tie that binds. *Circulation* 98: 1699-1702, 1998.

Marcus ML, Mueller TM and Eastham CL. Effects of short- and long-term left ventricular hypertrophy on coronary circulation. *Am J Physiol* 241: H358-H362, 1981.

Marian AJ, Yu QT, Workman R, Greve G and Roberts R. Angiotensin-converting enzyme polymorphism in hypertrophic cardiomyopathy and sudden cardiac death. *Lancet* 342: 1085-1086, 1993.

Marian AJ and Roberts R. Recent advances in the molecular genetics of hypertrophic cardiomyopathy. *Circulation* 92: 1336-1347, 1995.

Marian AJ and Roberts R. The molecular genetic basis for hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 33: 655-670, 2001.

Marian AJ. Modifier genes for hypertrophic cardiomyopathy. *Curr Opin Cardiol* 17: 242-252, 2002.

Maron BJ, Gottdiener JS, Bonow RO and Epstein SE. Hypertrophic cardiomyopathy with unusual locations of left ventricular hypertrophy undetectable by M-mode echocardiography. Identification by wide-angle two-dimensional echocardiography. *Circulation* 63: 409-418, 1981.

Maron BJ, Nichols PF, III, Pickle LW, Wesley YE and Mulvihill JJ. Patterns of inheritance in hypertrophic cardiomyopathy: assessment by M-mode and two-dimensional echocardiography. *Am J Cardiol* 53: 1087-1094, 1984.

Maron BJ, Olivotto I, Spirito P, Casey SA, Bellone P, Gohman TE, Graham KJ, Burton DA and Cecchi F. Epidemiology of hypertrophic cardiomyopathy-related death: revisited in a large non-referral-based patient population. *Circulation* 102: 858-864, 2000.

Maron BJ. Hypertrophic cardiomyopathy: a systematic review. *JAMA* 287: 1308-1320, 2002.

Matsubara M, Omori F, Fujita S, Metoki H, Kikuya M, Fujiwara T, Araki T and Imai Y. Haplotypes of aldosterone synthase (CYP11B2) gene in the general population of Japan: the Ohasama study. *Clin Exp Hypertens* 23: 603-610, 2001.

Matsubara M, Kikuya M, Ohkubo T, Metoki H, Omori F, Fujiwara T, Suzuki M, Michimata M, Hozawa A, Katsuya T, Higaki J, Tsuji I, Araki T, Ogihara T, Satoh H, Hisamichi S, Nagai K, Kitaoka H and Imai Y. Aldosterone synthase gene (CYP11B2) C-334T polymorphism, ambulatory blood pressure and nocturnal decline in blood pressure in the general Japanese population: the Ohasama Study. *J Hypertens* 19: 2179-2184, 2001.

Mayosi BM, Keavney B, Kardos A, Davies CH, Ratcliffe PJ, Farrall M and Watkins H. Electrocardiographic measures of left ventricular hypertrophy show greater heritability than echocardiographic left ventricular mass. *Eur Heart J* 23: 1963-1971, 2002.

Mayosi BM, Keavney B, Watkins H and Farrall M. Measured haplotype analysis of the aldosterone synthase gene and heart size. *Eur J Hum Genet* 11: 395-401, 2003.

Mazzolai L, Nussberger J, Aubert JF, Brunner DB, Gabbiani G, Brunner HR and Pedrazzini T. Blood pressure-independent cardiac hypertrophy induced by locally activated renin-angiotensin system. *Hypertension* 31: 1324-1330, 1998.

McIntyre LM, Martin ER, Simonsen KL and Kaplan NL. Circumventing multiple testing: a multilocus Monte Carlo approach to testing for association. *Genet Epidemiol* 19: 18-29, 2000.

McKenzie CA, Abecasis GR, Keavney B, Forrester T, Ratcliffe PJ, Julier C, Connell JM, Bennett F, Farlane-Anderson N, Lathrop GM and Cardon LR. Trans-ethnic fine mapping of a quantitative trait locus for circulating angiotensin I-converting enzyme (ACE). *Hum Mol Genet* 10: 1077-1084, 2001.

McKenzie CA, Julier C, Forrester T, McFarlane-Anderson N, Keavney B, Lathrop GM, Ratcliffe PJ and Farrall M. Segregation and linkage analysis of serum angiotensin I-converting enzyme levels: evidence for two quantitative-trait loci. *Am J Hum Genet* 57: 1426-1435, 1995.

Mogensen J, Klausen IC, Pedersen AK, Egeblad H, Bross P, Kruse TA, Gregersen N, Hansen PS, Baandrup U and Borglum AD. Alpha-cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy. *J Clin Invest* 103: R39-R43, 1999.

Moolman-Smook JC, Mayosi B, Brink P and Corfield VA. Identification of a new missense mutation in MyBP-C associated with hypertrophic cardiomyopathy. *J Med Genet* 35: 253-254, 1998.

Moolman-Smook JC, De Lange WJ, Bruwer EC, Brink PA and Corfield VA. The origins of hypertrophic cardiomyopathy-causing mutations in two South African subpopulations: a unique profile of both independent and founder events. *Am J Hum Genet* 65: 1308-1320, 1999.

Moolman-Smook JC, De Lange J, Brink A and Corfield A. Hypertrophic cardiomyopathy repealing tenets in South Africa. *Cardiovasc J S Afr* 11: 202-209, 2000.

Moolman-Smook JC, Mayosi BM, Brink PA and Corfield VA. Molecular genetics of cardiomyopathy: changing times, shifting paradigms. *Cardiovasc J S Afr* 14: 145-155, 2003.

Moolman JC, Brink PA and Corfield VA. Identification of a new missense mutation at Arg403, a CpG mutation hotspot, in exon 13 of the beta-myosin heavy chain gene in hypertrophic cardiomyopathy. *Hum Mol Genet* 2: 1731-1732, 1993.

Moolman JC, Brink PA and Corfield VA. Identification of a novel Ala797Thr mutation in exon 21 of the beta-myosin heavy chain gene in hypertrophic cardiomyopathy. *Hum Mutat* 6: 197-198, 1995.

Moolman JC, Corfield VA, Posen B, Ngumbela K, Seidman C, Brink PA and Watkins H. Sudden death due to troponin T mutations. *J Am Coll Cardiol* 29: 549-555, 1997.

Morishita R, Gibbons GH, Ellison KE, Lee W, Zhang L, Yu H, Kaneda Y, Ogihara T and Dzau VJ. Evidence for direct local effect of angiotensin in vascular hypertrophy. In vivo gene transfer of angiotensin converting enzyme. *J Clin Invest* 94: 978-984, 1994.

Muller DN, Bohlender J, Hilgers KF, Dragun D, Costerousse O, Menard J and Luft FC. Vascular angiotensin-converting enzyme expression regulates local angiotensin II. *Hypertension* 29: 98-104, 1997.

Muller DN, Fischli W, Clozel JP, Hilgers KF, Bohlender J, Menard J, Busjahn A, Ganten D and Luft FC. Local angiotensin II generation in the rat heart: role of renin uptake. *Circ Res* 82: 13-20, 1998.

Naftilan AJ, Williams R, Burt D, Paul M, Pratt RE, Hobart P, Chirgwin J and Dzau VJ. A lack of genetic linkage of renin gene restriction fragment length polymorphisms with human hypertension. *Hypertension* 14: 614-618, 1989 (a).

Naftilan AJ, Pratt RE, Eldridge CS, Lin HL and Dzau VJ. Angiotensin II induces c-fos expression in smooth muscle via transcriptional control. *Hypertension* 13: 706-711, 1989 (b).

Nakahara K, Matsushita S, Matsuoka H, Inamatsu T, Nishinaga M, Yonawa M, Aono T, Arai T, Ezaki Y and Orimo H. Insertion/deletion polymorphism in the angiotensin-converting enzyme gene affects heart weight. *Circulation* 101: 148-151, 2000.

Nakai K, Itoh C, Miura Y, Hotta K, Musha T, Itoh T, Miyakawa T, Iwasaki R and Hiramori K. Deletion polymorphism of the angiotensin I-converting enzyme gene is associated with serum ACE concentration and increased risk for CAD in the Japanese. *Circulation* 90: 2199-2202, 1994.

Nakashima Y, Fouad FM and Tarazi RC. Regression of left ventricular hypertrophy from systemic hypertension by enalapril. *Am J Cardiol* 53: 1044-1049, 1984.

Neitzel H. A routine method for the establishment of permanent growing lymphoblastoid cell lines. *Hum Genet* 73: 320-326, 1986.

Niimura H, Bachinski LL, Sangwatanaroj S, Watkins H, Chudley AE, McKenna W, Kristinsson A, Roberts R, Sole M, Maron BJ, Seidman JG and Seidman CE. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. *N Engl J Med* 338: 1248-1257, 1998.

Niimura H, Patton KK, McKenna WJ, Soults J, Maron BJ, Seidman JG and Seidman CE. Sarcomere protein gene mutations in hypertrophic cardiomyopathy of the elderly. *Circulation* 105: 446-451, 2002.

Niu T, Qin ZS, Xu X and Liu JS. Bayesian haplotype inference for multiple linked single-nucleotide polymorphisms. *Am J Hum Genet* 70: 157-169, 2002.

Norton GR, Tsotetsi J, Trifunovic B, Hartford C, Candy GP and Woodiwiss AJ. Myocardial stiffness is attributed to alterations in cross-linked collagen rather than total collagen or phenotypes in spontaneously hypertensive rats. *Circulation* 96: 1991-1998, 1997.

O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH and Levy D. Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation* 97: 1766-1772, 1998.

Ollivier JP and Bouchet VA. Prospects for cardioreparation. *Am J Cardiol* 70: 27C-36C, 1992.

Olson EN and Molkentin JD. Prevention of cardiac hypertrophy by calcineurin inhibition: hope or hype? *Circ Res* 84: 623-632, 1999.

Olson TM, Doan TP, Kishimoto NY, Whitby FG, Ackerman MJ and Fananapazir L. Inherited and de novo mutations in the cardiac actin gene cause hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 32: 1687-1694, 2000.

Ono K, Mannami T, Baba S, Yasui N, Ogihara T and Iwai N. Lack of association between angiotensin II type 1 receptor gene polymorphism and hypertension in Japanese. *Hypertens Res* 26: 131-134, 2003.

Ortlepp JR, Breithardt O, Ohme F, Hanrath P and Hoffmann R. Lack of association among five genetic polymorphisms of the renin-angiotensin system and cardiac hypertrophy in patients with aortic stenosis. *Am Heart J* 141: 671-676, 2001.

Ortlepp JR, Vosberg HP, Reith S, Ohme F, Mahon NG, Schroder D, Klues HG, Hanrath P and McKenna WJ. Genetic polymorphisms in the renin-angiotensin-aldosterone system associated with expression of left ventricular hypertrophy in

hypertrophic cardiomyopathy: a study of five polymorphic genes in a family with a disease causing mutation in the myosin binding protein C gene. *Heart* 87: 270-275, 2002.

Osterop AP, Kofflard MJ, Sandkuijl LA, ten Cate FJ, Krams R, Schalekamp MA and Danser AH. AT1 receptor A/C1166 polymorphism contributes to cardiac hypertrophy in subjects with hypertrophic cardiomyopathy. *Hypertension* 32: 825-830, 1998.

Ott J. Genetic loci and genetic polymorphisms. In: Ott J. Analysis of human genetic linkage 3rd ed. pp 24-36, 1999. The Johns Hopskins University Press. Baltimore, Maryland.

Paillard F, Chansel D, Brand E, Benetos A, Thomas F, Czekalski S, Ardaillou R and Soubrier F. Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. *Hypertension* 34: 423-429, 1999.

Paradis P, Dali-Youcef N, Paradis FW, Thibault G and Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc Natl Acad Sci U S A* 97: 931-936, 2000.

Patel R, Lim DS, Reddy D, Nagueh SF, Lutucuta S, Sole MJ, Zoghbi WA, Quinones MA, Roberts R and Marian AJ. Variants of trophic factors and expression of cardiac hypertrophy in patients with hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 32: 2369-2377, 2000.

Patel R, Nagueh SF, Tsybouleva N, Abdellatif M, Lutucuta S, Kopelen HA, Quinones MA, Zoghbi WA, Entman ML, Roberts R and Marian AJ. Simvastatin induces regression of cardiac hypertrophy and fibrosis and improves cardiac function in a transgenic rabbit model of human hypertrophic cardiomyopathy. *Circulation* 104: 317-324, 2001.

Perkins MJ, Van Driest SL, Ellsworth EG, Will ML, Gersh BJ, Ommen SR and Ackerman MJ. Gene-specific modifying effects of pro-LVH polymorphisms involving

the renin-angiotensin-aldosterone system among 389 unrelated patients with hypertrophic cardiomyopathy. *Eur Heart J* 26: 2457-2462, 2005.

Perneger TV. What's wrong with Bonferroni adjustments. *BMJ* 316: 1236-1238, 1998.

Perticone F, Ceravolo R, Cosco C, Trapasso M, Zingone A, Malatesta P, Perrotti N, Tramontano D and Mattioli PL. Deletion polymorphism of angiotensin-converting enzyme gene and left ventricular hypertrophy in southern Italian patients. *J Am Coll Cardiol* 29: 365-369, 1997.

Pesole G, Liuni S, Grillo G, Licciulli F, Mignone F, Gissi C and Saccone C. UTRdb and UTRsite: specialized databases of sequences and functional elements of 5' and 3' untranslated regions of eukaryotic mRNAs. Update 2002. *Nucleic Acids Res* 30: 335-340, 2002.

Petrie MC, Padmanabhan N, McDonald JE, Hillier C, Connell JM and McMurray JJ. Angiotensin converting enzyme (ACE) and non-ACE dependent angiotensin II generation in resistance arteries from patients with heart failure and coronary heart disease. *J Am Coll Cardiol* 37: 1056-1061, 2001.

Pfeffer JM, Pfeffer MA, Mirsky I and Braunwald E. Regression of left ventricular hypertrophy and prevention of left ventricular dysfunction by captopril in the spontaneously hypertensive rat. *Proc Natl Acad Sci U S A* 79: 3310-3314, 1982.

Pfeffer JM, Pfeffer MA, Mirsky I and Braunwald E. Prevention of the development of heart failure and the regression of cardiac hypertrophy by captopril in the spontaneously hypertensive rat. *Eur Heart J* 4 Suppl A: 143-148, 1983.

Pfeufer A, Osterziel KJ, Urata H, Borck G, Schuster H, Wienker T, Dietz R and Luft FC. Angiotensin-converting enzyme and heart chymase gene polymorphisms in hypertrophic cardiomyopathy. *Am J Cardiol* 78: 362-364, 1996.

Pinheiro J, Bates D, DebRoy S and Sarkar D. Linear and Nonlinear Mixed Effects Models (nlme). R package version 3.1-80, 2007.

Poetter K, Jiang H, Hassanzadeh S, Master SR, Chang A, Dalakas MC, Rayment I, Sellers JR, Fananapazir L and Epstein ND. Mutations in either the essential or regulatory light chains of myosin are associated with a rare myopathy in human heart and skeletal muscle. *Nat Genet* 13: 63-69, 1996.

Pojoga L, Gautier S, Blanc H, Guyene TT, Poirier O, Cambien F and Benetos A. Genetic determination of plasma aldosterone levels in essential hypertension. *Am J Hypertens* 11: 856-860, 1998.

Posen BM, Moolman JC, Corfield VA and Brink PA. Clinical and prognostic evaluation of familial hypertrophic cardiomyopathy in two South African families with different cardiac beta myosin heavy chain gene mutations. *Br Heart J* 74: 40-46, 1995.

Rapp JP, Wang SM and Dene H. A genetic polymorphism in the renin gene of Dahl rats cosegregates with blood pressure. *Science* 243: 542-544, 1989.

Redon J, Luque-Otero M, Martell N and Chaves FJ. Renin-angiotensin system gene polymorphisms: relationship with blood pressure and microalbuminuria in telmisartan-treated hypertensive patients. *Pharmacogenomics J* 5: 14-20, 2005.

Reich DE, Gabriel SB and Altshuler D. Quality and completeness of SNP databases. *Nat Genet* 33: 457-458, 2003.

Reichek N, Helak J, Plappert T, Sutton MS and Weber KT. Anatomic validation of left ventricular mass estimates from clinical two-dimensional echocardiography: initial results. *Circulation* 67: 348-352, 1983.

Reid IA, Morris BJ and Ganong WF. The renin-angiotensin system. *Annu Rev Physiol* 40: 377-410, 1978.

Ren Y, Garvin JL and Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension* 39: 799-802, 2002.

Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P and Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 86: 1343-1346, 1990.

Risch N and Merikangas K. The future of genetic studies of complex human diseases. *Science* 273: 1516-1517, 1996.

Rotimi C, Morrison L, Cooper R, Oyejide C, Effiong E, Ladipo M, Osotemihen B and Ward R. Angiotensinogen gene in human hypertension. Lack of an association of the 235T allele among African Americans. *Hypertension* 24: 591-594, 1994.

Rotimi C, Cooper R, Ogunbiyi O, Morrison L, Ladipo M, Tewksbury D and Ward R. Hypertension, serum angiotensinogen, and molecular variants of the angiotensinogen gene among Nigerians. *Circulation* 95: 2348-2350, 1997.

Russo P, Siani A, Venezia A, Iacone R, Russo O, Barba G, D'Elia L, Cappuccio FP and Strazzullo P. Interaction between the C(-344)T polymorphism of CYP11B2 and age in the regulation of blood pressure and plasma aldosterone levels: cross-sectional and longitudinal findings of the Olivetti Prospective Heart Study. *J Hypertens* 20: 1785-1792, 2002.

Sadoshima J and Izumo S. Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ Res* 73: 413-423, 1993.

Saeed M, Saleheen D, Siddiqui S, Khan A, Butt ZA and Frossard PM. Association of angiotensin converting enzyme gene polymorphisms with left ventricular hypertrophy. *Hypertens Res* 28: 345-349, 2005.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA and Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354, 1985.

Sato N, Katsuya T, Rakugi H, Takami S, Nakata Y, Miki T, Higaki J and Ogihara T. Association of variants in critical core promoter element of angiotensinogen gene with increased risk of essential hypertension in Japanese. *Hypertension* 30: 321-325, 1997.

Satoh M, Takahashi M, Sakamoto T, Hiroe M, Marumo F and Kimura A. Structural analysis of the titin gene in hypertrophic cardiomyopathy: identification of a novel disease gene. *Biochem Biophys Res Commun* 262: 411-417, 1999.

Say YH, Ling KH, Duraisamy G, Isaac S and Rosli R. Angiotensinogen M235T gene variants and its association with essential hypertension and plasma renin activity in Malaysian subjects: a case control study. *BMC Cardiovasc Disord* 5: 7, 2005.

Schelling P, Fischer H and Ganten D. Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J Hypertens* 9: 3-15, 1991.

Schiavone MT, Santos RA, Brosnihan KB, Khosla MC and Ferrario CM. Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. *Proc Natl Acad Sci U S A* 85: 4095-4098, 1988.

Schiller NB, Shah PM, Crawford M, DeMaria A, Devereux R, Feigenbaum H, Gutgesell H, Reichek N, Sahn D, Schnittger I, et al. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. American Society of Echocardiography Committee on Standards, Subcommittee on Quantitation of Two-Dimensional Echocardiograms. *J Am Soc Echocardiogr* 2: 358-367, 1989.

Schmieder RE, Erdmann J, Delles C, Jacobi J, Fleck E, Hilgers K and Regitz-Zagrosek V. Effect of the angiotensin II type 2-receptor gene (+1675 G/A) on left ventricular structure in humans. *J Am Coll Cardiol* 37: 175-182, 2001.

Schmitt JP, Semsarian C, Arad M, Gannon J, Ahmad F, Duffy C, Lee RT, Seidman CE and Seidman JG. Consequences of pressure overload on sarcomere protein mutation-induced hypertrophic cardiomyopathy. *Circulation* 108: 1133-1138, 2003 (a).

Schmitt JP, Kamisago M, Asahi M, Li GH, Ahmad F, Mende U, Kranias EG, MacLennan DH, Seidman JG and Seidman CE. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. *Science* 299: 1410-1413, 2003 (b).

Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS and Lorell BH. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J Clin Invest* 86: 1913-1920, 1990.

Schunkert H, Hense HW, Holmer SR, Stender M, Perz S, Keil U, Lorell BH and Riegger GA. Association between a deletion polymorphism of the angiotensin-converting-enzyme gene and left ventricular hypertrophy. *N Engl J Med* 330: 1634-1638, 1994.

Schunkert H, Hense HW, Muscholl M, Luchner A and Riegger GA. Association of angiotensin converting enzyme activity and arterial blood pressure in a population-based sample. *J Hypertens* 14: 571-575, 1996.

Schunkert H. Polymorphism of the angiotensin-converting enzyme gene and cardiovascular disease. *J Mol Med* 75: 867-875, 1997.

Schunkert H, Hense HW, Doring A, Riegger GA and Siffert W. Association between a polymorphism in the G protein beta3 subunit gene and lower renin and elevated diastolic blood pressure levels. *Hypertension* 32: 510-513, 1998.

Schunkert H, Hengstenberg C, Holmer SR, Broeckel U, Luchner A, Muscholl MW, Kurzinger S, Doring A, Hense HW and Riegger GA. Lack of association between a

polymorphism of the aldosterone synthase gene and left ventricular structure. *Circulation* 99: 2255-2260, 1999 (a).

Schunkert H, Brockel U, Hengstenberg C, Luchner A, Muscholl MW, Kurzidim K, Kuch B, Doring A, Riegger GA and Hense HW. Familial predisposition of left ventricular hypertrophy. *J Am Coll Cardiol* 33: 1685-1691, 1999 (b).

Sebkhi A, Zhao L, Lu L, Haley CS, Nunez DJ and Wilkins MR. Genetic determination of cardiac mass in normotensive rats: results from an F344xWKY cross. *Hypertension* 33: 949-953, 1999.

Semsarian C, Healey MJ, Fatkin D, Giewat M, Duffy C, Seidman CE and Seidman JG. A polymorphic modifier gene alters the hypertrophic response in a murine model of familial hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 33: 2055-2060, 2001.

Sen S, Tarazi RC, Khairallah PA and Bumpus FM. Cardiac hypertrophy in spontaneously hypertensive rats. *Circ Res* 35: 775-781, 1974.

Sen S and Tarazi RC. Regression of myocardial hypertrophy and influence of adrenergic system. *Am J Physiol* 244: H97-101, 1983.

Service S, Sabatti C and Freimer N. Tag SNPs chosen from HapMap perform well in several population isolates. *Genet Epidemiol* 2007.

Sethi AA, Nordestgaard BG, Agerholm-Larsen B, Frandsen E, Jensen G and Tybjaerg-Hansen A. Angiotensinogen polymorphisms and elevated blood pressure in the general population: the Copenhagen City Heart Study. *Hypertension* 37: 875-881, 2001.

Sevignani C, Calin GA, Siracusa LD and Croce CM. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome* 17: 189-202, 2006.

Sharov VG, Sabbah HN, Shimoyama H, Goussev AV, Lesch M and Goldstein S. Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am J Pathol* 148: 141-149, 1996.

Shirani J, Pick R, Roberts WC and Maron BJ. Morphology and significance of the left ventricular collagen network in young patients with hypertrophic cardiomyopathy and sudden cardiac death. *J Am Coll Cardiol* 35: 36-44, 2000.

Shlyakhto EV, Shwartz EI, Nefedova YB, Zukova AV, Vinnic TA and Conrady AO. Lack of association of the renin-angiotensin system genes polymorphisms and left ventricular hypertrophy in hypertension. *Blood Press* 10: 135-141, 2001.

Silverman EK and Palmer LJ. Case-control association studies for the genetics of complex respiratory diseases. *Am J Respir Cell Mol Biol* 22: 645-648, 2000.

Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM, Swynghedauw B and Delcayre C. Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. *J Biol Chem* 273: 4883-4891, 1998.

Silvestre JS, Heymes C, Oubenaissa A, Robert V, Aupetit-Faisant B, Carayon A, Swynghedauw B and Delcayre C. Activation of cardiac aldosterone production in rat myocardial infarction: effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation* 99: 2694-2701, 1999.

Smithies O. Theodore Cooper Memorial Lecture. A mouse view of hypertension. *Hypertension* 30: 1318-1324, 1997.

Sobel E, Sengul H and Weeks DE. Multipoint estimation of identity-by-descent probabilities at arbitrary positions among marker loci on general pedigrees. *Hum Hered* 52: 121-131, 2001.

Souery D, Rivelli SK and Mendlewicz J. Molecular genetic and family studies in affective disorders: state of the art. *J Affect Disord* 62: 45-55, 2001.

Spielman RS, McGinnis RE and Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52: 506-516, 1993.

Spirito P and Maron BJ. Relation between extent of left ventricular hypertrophy and occurrence of sudden cardiac death in hypertrophic cardiomyopathy. *J Am Coll Cardiol* 15: 1521-1526, 1990.

Spirito P, Seidman CE, McKenna WJ and Maron BJ. The management of hypertrophic cardiomyopathy. *N Engl J Med* 336: 775-785, 1997.

Spirito P, Bellone P, Harris KM, Bernabo P, Bruzzi P and Maron BJ. Magnitude of left ventricular hypertrophy and risk of sudden death in hypertrophic cardiomyopathy. *N Engl J Med* 342: 1778-1785, 2000.

Staessen JA, Ginocchio G, Wang JG, Saavedra AP, Soubrier F, Vlietinck R and Fagard R. Genetic variability in the renin-angiotensin system: prevalence of alleles and genotypes. *J Cardiovasc Risk* 4: 401-422, 1997.

Staessen JA, Kuznetsova T, Wang JG, Emelianov D, Vlietinck R and Fagard R. M235T angiotensinogen gene polymorphism and cardiovascular renal risk. *J Hypertens* 17: 9-17, 1999.

Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhager WH, Herrmann SM, Fagard R, Tizzoni L and Bianchi G. Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. *J Hypertens* 19: 1349-1358, 2001.

Stec DE, Davisson RL and Sigmund CD. Transgenesis and gene targeting in the mouse; tools for studying genetic determinants of hypertension. *Trends Cardiovasc Med* 8: 256-264, 1998.

Stowasser M. Primary aldosteronism: rare bird or common cause of secondary hypertension? *Curr Hypertens Rep* 3: 230-239, 2001.

Takahashi N, Murakami H, Kodama K, Kasagi F, Yamada M, Nishishita T and Inagami T. Association of a polymorphism at the 5'-region of the angiotensin II type 1 receptor with hypertension. *Ann Hum Genet* 64: 197-205, 2000.

Takai E, Akita H, Kanazawa K, Shiga N, Terashima M, Matsuda Y, Iwai C, Miyamoto Y, Kawai H, Takarada A and Yokoyama M. Association between aldosterone synthase (CYP11B2) gene polymorphism and left ventricular volume in patients with dilated cardiomyopathy. *Heart* 88: 649-650, 2002.

Takai S, Jin D, Muramatsu M and Miyazaki M. Chymase as a novel target for the prevention of vascular diseases. *Trends Pharmacol Sci* 25: 518-522, 2004.

Takami S, Katsuya T, Rakugi H, Sato N, Nakata Y, Kamitani A, Miki T, Higaki J and Ogihara T. Angiotensin II type 1 receptor gene polymorphism is associated with increase of left ventricular mass but not with hypertension. *Am J Hypertens* 11: 316-321, 1998.

Takemoto M, Node K, Nakagami H, Liao Y, Grimm M, Takemoto Y, Kitakaze M and Liao JK. Statins as antioxidant therapy for preventing cardiac myocyte hypertrophy. *J Clin Invest* 108: 1429-1437, 2001.

Tan LB, Jalil JE, Pick R, Janicki JS and Weber KT. Cardiac myocyte necrosis induced by angiotensin II. *Circ Res* 69: 1185-1195, 1991.

Tanaka M, Fujiwara H, Onodera T, Wu DJ, Hamashima Y and Kawai C. Quantitative analysis of myocardial fibrosis in normals, hypertensive hearts, and hypertrophic cardiomyopathy. *Br Heart J* 55: 575-581, 1986.

Tang W, Devereux RB, Rao DC, Oberman A, Hopkins PN, Kitzman DW and Arnett DK. Associations between angiotensinogen gene variants and left ventricular mass and function in the HyperGEN study. *Am Heart J* 143: 854-860, 2002.

Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW and Nadal-Ginard B. Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca2+ mobilization and protein kinase C activation. *J Biol Chem* 264: 526-530, 1989.

Tesson F, Dufour C, Moolman JC, Carrier L, al Mahdawi S, Chojnowska L, Dubourg O, Soubrier E, Brink P, Komajda M, Guicheney P, Schwartz K and Feingold J. The influence of the angiotensin I converting enzyme genotype in familial hypertrophic cardiomyopathy varies with the disease gene mutation. *J Mol Cell Cardiol* 29: 831-838, 1997.

Thierfelder L, Watkins H, MacRae C, Lamas R, McKenna W, Vosberg HP, Seidman JG and Seidman CE. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* 77: 701-712, 1994.

Thomas DC. Statistical Methods in Genetic Epidemiology, 2004. New York: Oxford University Press.

Thomas DC and Witte JS. Point: population stratification: a problem for case-control studies of candidate-gene associations? *Cancer Epidemiol Biomarkers Prev* 11: 505-512, 2002.

Tiago AD, Badenhorst D, Skudicky D, Woodiwiss AJ, Candy GP, Brooksbank R, Sliwa K, Sareli P and Norton GR. An aldosterone synthase gene variant is associated

with improvement in left ventricular ejection fraction in dilated cardiomyopathy. *Cardiovasc Res* 54: 584-589, 2002.

Tiago AD, Badenhorst D, Nkeh B, Candy GP, Brooksbank R, Sareli P, Libhaber E, Samani NJ, Woodiwiss AJ and Norton GR. Impact of renin-angiotensin-aldosterone system gene variants on the severity of hypertension in patients with newly diagnosed hypertension. *Am J Hypertens* 16: 1006-1010, 2003.

Tian B, Meng QC, Chen YF, Krege JH, Smithies O and Oparil S. Blood pressures and cardiovascular homeostasis in mice having reduced or absent angiotensin-converting enzyme gene function. *Hypertension* 30: 128-133, 1997.

Tian XL, Pinto YM, Costerousse O, Franz WM, Lippoldt A, Hoffmann S, Unger T and Paul M. Over-expression of angiotensin converting enzyme-1 augments cardiac hypertrophy in transgenic rats. *Hum Mol Genet* 13: 1441-1450, 2004.

Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G and Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J Biol Chem* 275: 33238-33243, 2000.

Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F and Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet* 51: 197-205, 1992.

Tiret L, Bonnardeaux A, Poirier O, Ricard S, Marques-Vidal P, Evans A, Arveiler D, Luc G, Kee F, Ducimetiere P, Soubrier F and Cambien F. Synergistic effects of angiotensin-converting enzyme and angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *Lancet* 344: 910-913, 1994.

Tiret L, Blanc H, Ruidavets JB, Arveiler D, Luc G, Jeunemaitre X, Tichet J, Mallet C, Poirier O, Plouin PF and Cambien F. Gene polymorphisms of the renin-angiotensin

system in relation to hypertension and parental history of myocardial infarction and stroke: the PEGASE study. Projet d'Etude des Genes de l'Hypertension Arterielle Severe a moderee Essentielle. *J Hypertens* 16: 37-44, 1998.

Tsai CT, Fallin D, Chiang FT, Hwang JJ, Lai LP, Hsu KL, Tseng CD, Liau CS and Tseng YZ. Angiotensinogen gene haplotype and hypertension: interaction with ACE gene I allele. *Hypertension* 41: 9-15, 2003.

Tsotetsi OJ, Woodiwiss AJ, Netjhardt M, Qubu D, Brooksbank R and Norton GR. Attenuation of cardiac failure, dilatation, damage, and detrimental interstitial remodeling without regression of hypertrophy in hypertensive rats. *Hypertension* 38: 846-851, 2001.

Tsuchida S, Matsusaka T, Chen X, Okubo S, Niimura F, Nishimura H, Fogo A, Utsunomiya H, Inagami T and Ichikawa I. Murine double nullizygotes of the angiotensin type 1A and 1B receptor genes duplicate severe abnormal phenotypes of angiotensinogen nullizygotes. *J Clin Invest* 101: 755-760, 1998.

Tsukada K, Ishimitsu T, Teranishi M, Saitoh M, Yoshii M, Inada H, Ohta S, Akashi M, Minami J, Ono H, Ohrui M and Matsuoka H. Positive association of CYP11B2 gene polymorphism with genetic predisposition to essential hypertension. *J Hum Hypertens* 16: 789-793, 2002.

Tsybouleva N, Zhang L, Chen S, Patel R, Lutucuta S, Nemoto S, DeFreitas G, Entman M, Carabello BA, Roberts R and Marian AJ. Aldosterone, through novel signaling proteins, is a fundamental molecular bridge between the genetic defect and the cardiac phenotype of hypertrophic cardiomyopathy. *Circulation* 109: 1284-1291, 2004.

Ueno H, Takata M, Yasumoto K, Tomita S and Inoue H. Angiotensin-converting enzyme gene polymorphism and geometric patterns of hypertensive left ventricular hypertrophy. *Jpn Heart J* 40: 589-598, 1999.

Urata H, Kinoshita A, Misono KS, Bumpus FM and Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem* 265: 22348-22357, 1990.

Urata H, Kinoshita A, Perez DM, Misono KS, Bumpus FM, Graham RM and Husain A. Cloning of the gene and cDNA for human heart chymase. *J Biol Chem* 266: 17173-17179, 1991.

Urata H, Nishimura H and Ganten D. Chymase-dependent angiotensin II forming systems in humans. *Am J Hypertens* 9: 277-284, 1996.

van Berlo JH and Pinto YM. Polymorphisms in the RAS and cardiac function. *Int J Biochem Cell Biol* 35: 932-943, 2003.

van Geel PP, Pinto YM, Buikema H and van Gilst WH. Is the A1166C polymorphism of the angiotensin II type 1 receptor involved in cardiovascular disease? *Eur Heart J* 19 Suppl G: G13-G17, 1998.

van Geel PP, Pinto YM, Voors AA, Buikema H, Oosterga M, Crijns HJ and van Gilst WH. Angiotensin II type 1 receptor A1166C gene polymorphism is associated with an increased response to angiotensin II in human arteries. *Hypertension* 35: 717-721, 2000.

Vane JR. *In: Angiotensin. Handbook of experimental pharmacology*. I.H. Page and F.M.Bumpus, editors., 1974. Springer-Verlag, New York.

Varnava AM, Elliott PM, Mahon N, Davies MJ and McKenna WJ. Relation between myocyte disarray and outcome in hypertrophic cardiomyopathy. *Am J Cardiol* 88: 275-279, 2001.

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, et al. The sequence of the human genome. *Science* 291: 1304-1351, 2001.

Vikstrom KL and Leinwand LA. Contractile protein mutations and heart disease. *Curr Opin Cell Biol* 8: 97-105, 1996.

Villard E, Tiret L, Visvikis S, Rakotovao R, Cambien F and Soubrier F. Identification of new polymorphisms of the angiotensin I-converting enzyme (ACE) gene, and study of their relationship to plasma ACE levels by two-QTL segregation-linkage analysis. *Am J Hum Genet* 58: 1268-1278, 1996.

Voight BF and Pritchard JK. Confounding from cryptic relatedness in case-control association studies. *PLoS Genet* 1: e32, 2005.

Wacholder S, Rothman N and Caporaso N. Counterpoint: bias from population stratification is not a major threat to the validity of conclusions from epidemiological studies of common polymorphisms and cancer. *Cancer Epidemiol Biomarkers Prev* 11: 513-520, 2002.

Wachtell K, Bella JN, Liebson PR, Gerdts E, Dahlof B, Aalto T, Roman MJ, Papademetriou V, Ibsen H, Rokkedal J and Devereux RB. Impact of different partition values on prevalences of left ventricular hypertrophy and concentric geometry in a large hypertensive population : the LIFE study. *Hypertension* 35: 6-12, 2000.

Walker WG, Whelton PK, Saito H, Russell RP and Hermann J . Relation between blood pressure and renin, renin substrate, angiotensin II, aldosterone and urinary sodium and potassium in 574 ambulatory subjects. *Hypertension* 1: 287-291, 1979.

Wang D, Yang H, Quinones MJ, Bulnes-Enriquez I, Jimenez X, De La RR, Modilevsky T, Yu K, Li Y, Taylor KD, Hsueh WA, Hodis HN and Rotter JI. A

genome-wide scan for carotid artery intima-media thickness: the Mexican-American Coronary Artery Disease family study. *Stroke* 36: 540-545, 2005.

Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein L, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburn D, Rioux J, Nusbaum C, Rozen S, Hudson TJ, Lipshutz R, Chee M and Lander ES. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science* 280: 1077-1082, 1998.

Wang JG and Staessen JA. Genetic polymorphisms in the renin-angiotensin system: relevance for susceptibility to cardiovascular disease. *Eur J Pharmacol* 410: 289-302, 2000.

Wang WY, Zee RY and Morris BJ. Association of angiotensin II type 1 receptor gene polymorphism with essential hypertension. *Clin Genet* 51: 31-34, 1997.

Wang WY, Glenn CL, Zhang W, Benjafield AV, Nyholt DR and Morris BJ. Exclusion of angiotensinogen gene in molecular basis of human hypertension: sibpair linkage and association analyses in Australian anglo-caucasians. *Am J Med Genet* 87: 53-60, 1999.

Watkins H, Rosenzweig A, Hwang DS, Levi T, McKenna W, Seidman CE and Seidman JG. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. *N Engl J Med* 326: 1108-1114, 1992.

Watkins H, McKenna WJ, Thierfelder L, Suk HJ, Anan R, O'Donoghue A, Spirito P, Matsumori A, Moravec CS, Seidman JG and . Mutations in the genes for cardiac troponin T and alpha-tropomyosin in hypertrophic cardiomyopathy. *N Engl J Med* 332: 1058-1064, 1995.

Watt GC, Harrap SB, Foy CJ, Holton DW, Edwards HV, Davidson HR, Connor JM, Lever AF and Fraser R. Abnormalities of glucocorticoid metabolism and the renin-angiotensin system: a four-corners approach to the identification of genetic determinants of blood pressure. *J Hypertens* 10: 473-482, 1992.

Weber KT. The what, why and how of hypertensive heart disease. *J Hum Hypertens* 8: 665-675, 1994.

White PC and Slutsker L. Haplotype analysis of CYP11B2. *Endocr Res* 21: 437-442, 1995.

Wigginton JE and Abecasis GR. PEDSTATS: descriptive statistics, graphics and quality assessment for gene mapping data. *Bioinformatics* 21: 3445-3447, 2005.

Wigle ED, Sasson Z, Henderson MA, Ruddy TD, Fulop J, Rakowski H and Williams WG. Hypertrophic cardiomyopathy. The importance of the site and the extent of hypertrophy. A review. *Prog Cardiovasc Dis* 28: 1-83, 1985.

Wigle ED, Rakowski H, Kimball BP and Williams WG. Hypertrophic cardiomyopathy. Clinical spectrum and treatment. *Circulation* 92: 1680-1692, 1995.

Wigle ED. Cardiomyopathy: The diagnosis of hypertrophic cardiomyopathy. *Heart* 86: 709-714, 2001.

Willer CJ, Scott LJ, Bonnycastle LL, Jackson AU, Chines P, Pruim R, Bark CW, Tsai YY, Pugh EW, Doheny KF, Kinnunen L, Mohlke KL, Valle TT, Bergman RN, Tuomilehto J, Collins FS and Boehnke M. Tag SNP selection for Finnish individuals based on the CEPH Utah HapMap database. *Genet Epidemiol* 30: 180-190, 2006.

Wollert KC, Studer R, Doerfer K, Schieffer E, Holubarsch C, Just H and Drexler H. Differential effects of kinins on cardiomyocyte hypertrophy and interstitial collagen

matrix in the surviving myocardium after myocardial infarction in the rat. *Circulation* 95: 1910-1917, 1997.

Wu S, Hong J, Li H, Feng S, Qiao D, Gao M, Shi J and Nie H. No correlation of polymorphism of angiotensin-converting enzyme genes with left ventricular hypertrophy in essential hypertension. *Hypertens Res* 23: 261-264, 2000.

Xiao F, Puddefoot JR and Vinson GP. Aldosterone mediates angiotensin II-stimulated rat vascular smooth muscle cell proliferation. *J Endocrinol* 165: 533-536, 2000.

Xiao HD, Fuchs S, Campbell DJ, Lewis W, Dudley SC, Jr., Kasi VS, Hoit BD, Keshelava G, Zhao H, Capecchi MR and Bernstein KE. Mice with cardiac-restricted angiotensin-converting enzyme (ACE) have atrial enlargement, cardiac arrhythmia, and sudden death. *Am J Pathol* 165: 1019-1032, 2004.

Yamada Y, Ichihara S, Fujimura T and Yokota M. Lack of association of polymorphisms of the angiotensin converting enzyme and angiotensinogen genes with nonfamilial hypertrophic or dilated cardiomyopathy. *Am J Hypertens* 10: 921-928, 1997.

Yamamoto K, Ohishi M, Katsuya T, Ito N, Ikushima M, Kaibe M, Tatara Y, Shiota A, Sugano S, Takeda S, Rakugi H and Ogihara T. Deletion of angiotensin-converting enzyme 2 accelerates pressure overload-induced cardiac dysfunction by increasing local angiotensin II. *Hypertension* 47: 718-726, 2006.

Yamazaki T, Komuro I, Kudoh S, Zou Y, Shiojima I, Hiroi Y, Mizuno T, Maemura K, Kurihara H, Aikawa R, Takano H and Yazaki Y. Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem* 271: 3221-3228, 1996.

Yamazaki T, Komuro I and Yazaki Y. Role of the renin-angiotensin system in cardiac hypertrophy. *Am J Cardiol* 83: 53H-57H, 1999.

Ying SY and Lin SL. Current perspectives in intronic micro RNAs (miRNAs). *J Biomed Sci* 13: 5-15, 2006.

Yoneya K, Okamoto H, Machida M, Onozuka H, Noguchi M, Mikami T, Kawaguchi H, Murakami M, Uede T and Kitabatake A. Angiotensin-converting enzyme gene polymorphism in Japanese patients with hypertrophic cardiomyopathy. *Am Heart J* 130: 1089-1093, 1995.

Yoshiyama M, Nakamura Y, Omura T, Izumi Y, Matsumoto R, Oda S, Takeuchi K, Kim S, Iwao H and Yoshikawa J. Angiotensin converting enzyme inhibitor prevents left ventricular remodelling after myocardial infarction in angiotensin II type 1 receptor knockout mice. *Heart* 91: 1080-1085, 2005.

Yu H, Harrap SB and Di Nicolantonio R. Cosegregation of spontaneously hypertensive rat renin gene with elevated blood pressure in an F2 generation. *J Hypertens* 16: 1141-1147, 1998.

Zhao YY, Zhou J, Narayanan CS, Cui Y and Kumar A. Role of C/A polymorphism at -20 on the expression of human angiotensinogen gene. *Hypertension* 33: 108-115, 1999.

Zhou J, Allen AM, Yamada H. Localisation and properties of angiotensin-converting enzyme and angiotensin receptors in the heart. In: *The Cardiac Renin-Angiotensin System*, Lindpainter K and Ganten D (eds), pp. 63-88, 1994. Futura Publishing Co., Inc., Armonk, New York.

Zhu H, Sagnella GA, Dong Y, Miller MA, Onipinla A, Markandu ND and MacGregor GA. Contrasting associations between aldosterone synthase gene polymorphisms and essential hypertension in blacks and in whites. *J Hypertens* 21: 87-95, 2003.

Zhu X, Bouzekri N, Southam L, Cooper RS, Adeyemo A, McKenzie CA, Luke A, Chen G, Elston RC and Ward R. Linkage and association analysis of angiotensin I-

233

converting enzyme (ACE)-gene polymorphisms with ACE concentration and blood pressure. *Am J Hum Genet* 68: 1139-1148, 2001.

Zhu X, Chang YP, Yan D, Weder A, Cooper R, Luke A, Kan D and Chakravarti A. Associations between hypertension and genes in the renin-angiotensin system. *Hypertension* 41: 1027-1034, 2003.

Zhu X, Fejerman L, Luke A, Adeyemo A and Cooper RS. Haplotypes produced from rare variants in the promoter and coding regions of angiotensinogen contribute to variation in angiotensinogen levels. *Hum Mol Genet* 14: 639-643, 2005.

Ziegler A and König IR. A Statistical Approach to Genetic Epidemiology, 2006. Wiley VCH. Weinheim, GmbH.

Electronic references/ uniform resource locator (URL)

American Society of Echocardiography

(http://www.asecho.org/guidelines.php)

Fermentas

http://www.fermentas.com/catalog/re/mnli.htm

FHC database

http:// www.angis.org.au/database/heart/heartbreak.html

Genecards

http://bioinfo.weizmann.ac.il/cards/index.shtml

HapMap data coordination Center (DCC)

http://www.hapmap.org

Medline

http:// www.ncbi.nlm.nih.gov/Medline

National Centre for Biotechnology Information database

http://www.ncbi.nlm.nih.gov.

Nucleic Acid Research (NAR) website

http://www.nar.oupjournals.org.

Pubmed

http:// www.ncbi.nlm.nih.gov/Pubmed

R Development Core Team (2007) website.

http://www.R-project.org.

Single nucleotide polymorphism database

http://www.ncbi.nlm.nih.gov/ query.fcgi? db = SNP

University of California Santa Cruz (UCSC) database

http://www.genome.ucsc.edu

Addendum I

A representative example of a portion of a pedigree file for selected families showing covariates, three composite scores, five left ventricle wall thickness measurements and the genotype code for *AGTR1* rs2640539.

family	indiv id	father id	mother id	sex	age	mutation	BSA (m2)	Syst BP	diast BP	HR bpm	Maron-Spirito score	CWT score	Wigle score	post IVSmit	ant IVSmit	ant wallmit	lat wallmit	inf wallmit	*AGTR1* rs2640539
F101	1	3	4	1	67	T797	2.0	130.0	80.0	65.0	X	261.4	7.0	11.5	16.4	14.0	9.1	8.0	1 2
F101	2	7	8	2	55	T797	X	120.0	80.0	44.0	37.7	134.3	X	X	X	X	X	X	0 0
F101	3	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	4	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	5	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	6	5	4	2	53	1	1.9	160.0	90.0	60.0	38.2	191.9	X	9.4	9.0	10.8	8.0	8.3	1 1
F101	7	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	8	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	9	1	2	2	X	T797	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	10	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	11	10	9	1	22	1	2.0	120.0	80.0	55.0	43.4	213.1	X	11.2	10.3	10.8	9.3	8.6	1 1
F101	12	10	9	2	22	T797	1.8	100.0	60.0	80.0	118.8	398.4	10.0	25.0	32.2	21.4	12.9	7.8	1 1
F101	13	1	2	1	X	T797	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	14	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	15	13	14	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	16	1	2	1	42	T797	2.5	X	X	90.0	X	X	X	15.5	15.5	X	X	X	1 1
F101	17	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	18	16	17	2	19	1	2.1	100.0	70.0	85.0	31.4	185.1	X	7.1	6.6	8.6	7.6	7.6	1 1
F101	19	16	17	2	X	T797	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F101	20	1	2	1	37	T797	2.5	130.0	90.0	90.0	95.5	386.7	10.0	21.4	32.3	20.2	19.4	11.6	2 2
F101	21	7	8	2	62	1	1.6	180.0	120.0	70.0	48.5	234.0	X	14.3	11.8	13.5	9.0	8.4	1 2
F101	22	7	8	2	59	T797	1.5	100.0	70.0	60.0	36.1	210.7	X	8.6	8.1	11.1	9.6	8.5	1 1
F101	23	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	24	23	22	2	36	T797	1.8	100.0	70.0	60.0	35.6	199.6	X	7.6	9.5	9.0	8.0	7.6	1 1
F101	25	23	22	1	31	1	2.2	130.0	85.0	70.0	37.2	204.5	X	8.1	9.5	8.4	8.0	7.4	1 1
F101	26	23	22	2	26	1	1.6	90.0	60.0	65.0	25.1	169.9	X	6.0	6.7	5.6	6.7	5.9	1 2
F101	27	7	8	2	45	T797	X	150.0	90.0	80.0	X	X	X	X	X	X	X	X	1 1
F101	28	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F101	29	28	27	2	37	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F101	30	28	27	2	34	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F101	31	7	8	2	46	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F103	1	0	0	2	61	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F103	2	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F103	3	2	1	1	42	W92	1.5	160.0	80.0	85.0	74.8	312.5	6.0	17.4	22.0	22.4	17.4	13.0	1 1
F103	4	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F103	5	3	4	2	22	1	1.6	115.0	70.0	77.0	29.5	198.2	X	8.1	7.7	10.3	5.8	5.4	1 1
F103	6	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F103	7	3	6	2	19	W92	1.6	110.0	80.0	74.0	40.2	227.8	X	10.8	10.3	11.4	7.5	5.7	1 1
F103	8	3	4	2	23	1	1.7	120.0	70.0	67.0	35.9	213.0	X	9.6	9.1	8.8	8.2	8.0	1 1
F104	1	0	0	2	70	T797	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F104	2	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F104	3	0	0	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F104	4	3	1	1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F104	5	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F104	6	2	1	1	56	T797	1.7	180.0	100.0	57.0	111.1	434.1	10.0	24.5	32.7	24.2	15.2	10.8	1 2
F104	7	2	1	1	55	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F104	8	2	1	1	X	T797	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F104	9	2	1	2	47	1	1.8	150.0	90.0	80.0	49.3	266.4	X	11.4	13.8	12.7	9.4	8.6	0 0
F104	10	2	1	2	43	1	1.9	120.0	80.0	60.0	37.4	232.6	X	8.4	8.9	9.5	9.8	7.5	1 2
F104	11	2	1	2	41	T797	1.3	110.0	70.0	70.0	42.6	244.5	X	9.8	11.7	11.5	8.6	7.4	1 2
F104	12	0	0	1	X	1	X	X	X	X	X	X	X	X	X	X	X	X	1 1
F104	13	12	11	2	18	T797	X	X	X	X	X	X	X	X	X	X	X	X	1 2
F104	14	0	0	2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	0 0
F104	15	8	14	2	29	1	2.0	120.0	80.0	75.0	38.4	236.4	X	10.5	9.1	8.8	7.8	8.4	1 2
F104	16	8	14	2	24	T797	1.9	120.0	80.0	65.0	36.4	238.8	X	9.8	10.1	9.0	8.6	7.0	1 2