Renin-angiotensin-aldosterone system genes and the complex hypertrophic phenotype of hypertrophic cardiomyopathy

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Dissertation presented for the degree of Doctor of Philosophy in Medical Sciences (Human Genetics) at the Faculty of Medicine and Health Sciences, Stellenbosch University

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December 2012

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ABSTRACT

Left ventricular hypertrophy (LVH) is a strong independent predictor of cardiovascular morbidity and mortality, while its regression is associated with an improved clinical prognosis. It is, therefore, vital to elucidate and fully comprehend the mechanisms that contribute to LVH development and to identify markers that indicate a strong predisposition to the development of severe cardiac hypertrophy, before its occurrence.

Hypertrophic cardiomyopathy (HCM) serves as a model to investigate LVH development. This primary cardiac disease is characterised by LVH in the absence of increased external loading conditions and is caused by defective sarcomeric proteins, as a result of mutations within the genes encoding these proteins. However, the hypertrophic phenotype of HCM is largely complex, as we see strong variability in the extent and distribution of LVH in HCM, even in individuals with the same disease-causing mutation from the same family; this points toward the involvement of additional genetic and environmental modifiers.

Components of the renin-angiotensin-aldosterone system (RAAS) influence LVH indirectly, through their key role in blood pressure regulation, but also directly, due to the direct cellular hypertrophic effects of some RAAS components. Previous genetic association studies aimed at investigating the contribution of RAAS variants to LVH were largely centred on a subset of polymorphisms within the genes encoding the angiotensin converting enzyme (ACE) and angiotensin II type 1 receptor genes, while the renin section and RAAS components downstream from ACE remained largely neglected. In addition, most previous studies have reported relatively small individual effects for a small subset of RAAS variants on LVH.

In the present study we, therefore, employ a family-based genetic association analysis approach to investigate the contribution of the entire RAAS to this complex hypertrophic phenotype by exploring both the individual as well as the compound effects of 84 variants within 22 RAAS genes, in a cohort of 388 individuals from 27 HCM families, in which either of three HCM-founder mutations segregate.

During the course of this explorative study, we identified a number of RAAS variants that had significant effects on hypertrophy in HCM, whether alone or within the context of a multi-variant haplotype. Through single variant association analyses, we identified variants within the genes encoding angiotensinogen, renin-binding protein, the mannose-6-phosphate receptor, ACE, ACE2, angiotensin receptors 1 and 2, the mineralocorticoid receptor, as well as the

epithelial sodium channel and the Na^+/K^+-ATPase β-subunits, that contribute to hypertrophy in HCM. Using haplotype-based association analyses, we were able to identify haplotypes within the genes encoding for renin, the mannose-6-phosphate receptor, angiotensin receptor 1, the mineralocorticoid receptor, epithelial sodium channel and Na^+/K^+-ATPase α- and β subunits, as well as the CYP11B1/B2 locus, that contribute significantly to LVH. In addition, we found that some RAAS variants and haplotypes had statistically significantly different effects in the three HCM founder mutation groups.

Finally, we used stepwise selection to identify a set of nine risk-alleles that together predicted a 127.80 g increase in left ventricular mass, as well as a 13.97 mm increase in maximum interventricular septal thickness and a 14.67 mm increase in maximum left ventricular wall thickness in the present cohort. In contrast, we show that a set of previously identified "pro-LVH" polymorphisms rather poorly predicted LVH in the present South African cohort.

This is the first RAAS investigation, to our knowledge, to provide clear quantitative effects for a subset of RAAS variants indicative of a risk for LVH development that are representative of the entire pathway. Our findings suggest that the eventual hypertrophic phenotype of HCM is modulated by the compound effect of a number of RAAS modifier loci, where each polymorphism makes a modest contribution towards the eventual phenotype. Research such as that presented here provides a basis on which future studies can build improved risk profiles for LVH development within the context of HCM, and ultimately in all patients with a risk of cardiac hypertrophy.

OPSOMMING

Linker ventrikulêre hipertrofie (LVH) is 'n sterk onafhanklike voorspeller van kardiovaskulêre morbiditeit en mortaliteit, terwyl LVH regressie verband hou met ‘n verbeterde kliniese voorspelling. Dit is dus noodsaaklik om die meganismes wat bydra to LVH ontwikkeling ten volle te verstaan en merkers wat 'n sterk geneigdheid tot die ontwikkeling van ernstige kardiale hipertrofie te identifiseer, voordat dit voorkom.

Hipertrofiese kardiomiopatie (HKM) dien as 'n model om LVH ontwikkeling te ondersoek. Hierdie primêre hartsiekte word gekenmerk deur LVH en word meestal veroorsaak deur foutiewe sarkomeer proteïene as gevolg van mutasies binne die gene wat kodeer vir hierdie proteïene. Die hipertrofiese fenotipe van HKM is egter grootliks kompleks; ons sien, by voorbeeld, sterk veranderlikheid in die omvang en die verspreiding van LVH in HKM, selfs in individue met dieselfde siekte-veroorsakende mutasie binne dieselfde gesin, wat dui op die betrokkenheid van addisionele genetiese en omgewing modifiseerders.

Komponente van die renien-angiotensien-aldosteroon sisteem (RAAS) beïnvloed LVH indirek, deur middel van hul belangrike rol in bloeddruk regulasie, maar ook direk, as gevolg van die direkte sellulêre hipertrofiese gevolge van sommige RAAS komponente. Vorige genetiese assosiasie studies wat daarop gemik was om die bydrae van RAAS variante LVH te ondersoek, was hoofsaaklik gesentreer op 'n groepie polimorfismes binne die gene wat kodeer vir die “angiotensin converting enzyme” (ACE) en angiotensien II tipe 1-reseptor gene, terwyl die renien gedeelte en RAAS komponente stroomaf van ACE meestal nie ondersoek was nie. Daarbenewens het die meeste vorige studies relatief klein individuele gevolge gerapporteer vir 'n klein groepie RAAS variante op LVH.

In die huidige studie het ons dus 'n familie-gebaseerde genetiese assosiasie-analise benadering gebruik om die bydrae van die hele RAAS tot hierdie komplekse hipertrofiese fenotipe te ondersoek deur 'n studie van die individuele-, sowel as die saamgestelde effekte van 84 variante binne 22 RAAS gene, in 'n groep van 388 individue vanaf 27 HKM families, waarin een van drie HCM-stigter mutasies seggregeer.

Gedurende die loop van hierdie studie het ons 'n aantal RAAS variante wat ‘n beduidende uitwerking op HKM hipertrofie geïdentifiseer, hetsy alleen of binne die konteks van' n multi-variant haplotipe. Deur middel van enkele variant assosiasie toetsing het ons variante geïdentifiseer binne die gene wat kodeer vir angiotensinogen, renien-bindende proteïen, die

mannose-6-fosfaat reseptor, ACE, ACE2, angiotensien reseptore 1 en 2, die mineralokortikoïd reseptor, sowel as die epiteel natrium kanaal en Na^+/ K^+-ATPase β-subeenhede, wat bydra tot HKM hipertrofie. Deur die gebruik van haplotipe-gebaseerde assosiasie ontleding was ons in staat om haplotipes te identifiseer binne die gene wat kodeer vir renien, die mannose-6-fosfaat reseptor angiotensien reseptor 1, die mineralokortikoïd reseptor, epiteel natrium kanaal en die Na^+/ K^+-ATPase α-en β subeenhede, sowel as die CYP11B1/B2 lokus, wat aansienlik bydra tot LVH. Verder het ons bevind dat sommige RAAS variante en haplotipes statisties beduidende verskillende effekte gehad het in die drie HKM stigter mutasie groepe.

Laastens, het ons stapsgewyse seleksie gebruik om 'n stel van nege risiko-allele wat saam' n toename van 127.80 g in linker ventrikulêre massa, sowel as 'n 13.97 mm toename in maksimum ventrikulêre septale dikte, en' n 14.67 mm verhoging in maksimum linker ventrikulêre wanddikte voorspel, te identifiseer in die huidige kohort. In teenstelling hiermee wys ons dat 'n stel van voorheen geïdentifiseerde "pro-LVH" polimorfismes swakker gevaar het as LVH-voorspellers in die huidige Suid-Afrikaanse kohort.

Hierdie is die eerste RAAS ondersoek, tot ons kennis, wat ‘n duidelike kwantitatiewe gevolge vir 'n stel RAAS variante wat ‘n verhoogde risiko tot LVH ontwikkeling aandui, wat verteenwoordigend is van die hele RAAS. Ons bevindinge dui daarop dat die uiteindelike hipertrofiese fenotipe van HKM gemoduleer word deur die saamgestelde effek van 'n aantal RAAS wysiger loki, waar elke polimorfisme ' n beskeie bydrae maak tot die uiteindelike fenotipe. Navorsing soos dié wat hier aangebied word dien as 'n basis waarop toekomstige studies kan bou vir ‘n verbeterde risiko-profiel vir LVH ontwikkeling binne die konteks van die HKM, en uiteindelik in alle pasiënte met' n verhoogde risiko vir kardiale hipertrofie.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people who assisted me during the course of this degree:

Prof Hanlie Moolman-Smook, for her knowledge, time, effort and every bit of mentorship and advice during my years in the MAGIC lab. You helped me grow.

Prof Lize van der Merwe for her time, expertise, endless patience and guidance with the statistical analyses. Under you guidence I gained a true appreciation for genetic association analysis.

Dr Craig Kinnear for his technical expertise, humour, words of encouragement and always being a shout away in times of need.

Prof Paul van Helden, for his support, numerous reference letters and advice.

The members of the MAGIC lab, past and present, for your support, advice, crazy lab humour and for providing the best working environment a student can ask for.

My family and friends, for their words of encouragement, understanding and putting up with my absence and mood swings.

Finally, Adriaan, for love, waiting, understanding and encouraging.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
˚C	degrees Celsius
11β-HSD2	11 β-hydroxysteroid-dehydrogenase type 2
2D	two-dimensional
3'	three prime
5'	five prime
A	adenine
ABI	Applied Biosystems Incorporated
ACE	angiotensin-converting enzyme
ACE2	angiotensin-converting enzyme 2
ACTC1	α-cardiac actin
AGT	angiotensinogen
AGTR1	Angiotensin II type I receptor gene
AGTR2	Angiotensin II type II receptor gene
aIVS	anterior interventricular septum thickness
ALLAY	Aliskiren in Left Ventricular Hypertrophy
AME	apparent mineralocorticoid excess
Ang	angiotensin
ASREA	allele specific restriction enzyme analysis
AT_1 receptor	Angiotensin II type I receptor
AT_2 receptor	Angiotensin II type II receptor
ATP1A1	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide
ATP1A2	Na^+/K^+ transporting, alpha 2 polypeptide
ATP1B1	Na^+/K^+ transporting, beta 1 polypeptide

ATP1B3	Na^+/K^+ transporting, beta 3 polypeptide
ATP6AP2	ATPase, H^+ transporting, lysosomal accessory protein 2
ATPase	adenosine triphosphatase
AV	aortic valve
AW	anterior wall thickness
BP	blood pressure
BSA	body surface area
C	cytosine
Ca^{2+}	calcium
CEU	HapMap population: parent-offspring trios with northern and western European ancestry
CMA	cardiac chymase
CWT	cumulative wall thickness
CYP11B2	aldosterone synthase
DNA	Deoxyribo Nucleic Acid
EDTA	ethylene-diamine-tetra-acetic acid
ENaC	epithelial Na^+ channels
EPHESUS	eplerenone post acute myocardial infarction efficacy and survival study
EPOGH	European Project On Genes in Hypertension
G	guanine
GenSalt	Genetic Epidemiology Network of Salt Sensitivity
GLAECO	Glasgow Heart Scan
GLAEOLD	Glasgow Heart Scan Old
H^+	Hydrogen
HCM	hypertrophic cardiomyopathy
HOPE	Heart Outcomes Prevention Evaluation
HRP	handle region peptide

HSP27	heat-shock protein 27
HyperGEN	Hypertension Genetic Epidemiology Network
HWE	Hardy-Weinberg equilibrium
Hz	Hertz
I2C	intron 2 conversion
I/D	insertion/deletion
IBD	identity-by-decent
IVS	interventricular septum thickness
IW	inferior wall thickness
K^+	potassium
kb	kilo bases
LA	left atrium
LD	linkage disequilibrium
LDU	linkage disequilibrium unit
LIFE	Losartan Intervention for Endpoint reduction
LOD	logarith of odds
LV	left ventricle
LVH	left ventricular hypertrophy
LVM	left ventricular mass
LVOT	left ventricular outflow tract
LVWT	left ventricular wall thickness
LW	lateral wall thickness
M6P	mannose-6-Phosphate
M6PR/IGFII	mannose-6-phosphate/insulin-like growth factor II receptor
MAF	minor allele frequency
MAPK	mitogen-activated protein kinase
MC	mutation carrier

MGB	minor groove binder
min	minute
mIVST	maximal interventricular septum thickness
mLVWT	maximal left ventricular wall thickness
MONICA	MONitoring trends and determinants in CArdiovascular disease
mPWT	maximal posterior wall thickness
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MV	mitral valve
MYBPC3	cardiac myosin-binding protein C
MYH6	α-myosin heavy chain
MYH7	β-myosin heavy chain
Na^+	sodium
Na^+/Ca^{2+} exchanger	sodium calcium exchanger
Na^+/K^+-ATPase	sodium-potassium pump
NaCl	sodium chloride
NAGE	N-acetyl-D-glucosamine 2-epimerase
NC	non-carrier
NCBI	National Center for Bioinformatics
Nedd4-2	neural precursor cell expressed, developmentally downregulated-4-2
NFQ	nonfluorescent quencher
NR3C2	nuclear receptor subfamily 3, group C, member 2
OR	odds-ratio
PAI-1	plasminogen activator inhibitor-1
PC1	first principal component

PCR	polymerase chain reaction
pIVS	posterior interventricular septum thickness
PPARG	peroxisome proliferator-activated receptor gamma
PRA	plasma renin activity
PRR	(pro)renin receptor
PW	posterior wall thickness
QTDT	quantitative transmission disequilibrium test
QTL	quantitative trait locus
RAAS	renin-angiotensin-aldosterone system
RALES	randomized aldactone evaluation study
REN	renin gene
RENBP	renin binding protein gene
RnBP	renin-binding protein
ROS	reactive oxygen species
RSA	Republic of South Africa
RVOT	right ventricular outflow tract
SB	di-sodium tetraborate-decahydrate
SCD	sudden cardiac death
SCNN1A	sodium channel, nonvoltage-gated 1 alpha gene
SCNN1B	sodium channel, nonvoltage-gated 1 beta gene
SCNN1G	sodium channel, nonvoltage-gated 1 gamma gene
SDS	Sequence Detection Systems
sec	second
SF-1	steroidogenic transcription factor-1
SNP	single nucleotide polymorphism
STAT	Signal transducers and activators of transcription
STR	short tandem repeat
SILVHIA	Swedish Irbesartan Left Ventricular Hypertrophy

	Investigation versus Atenolol
T	thymine
TGF-β	transforming growth factor beta
T_m	melting temperature
TNF	tumour necrosis factor
TNNCI	cardiac troponin C
TNNI3	cardiac troponin I
TNNT2	cardiac troponin T
UK	United Kingdom
USA	United States of America
UV	ultra-violet
V	Volts
v	version
WT	wild type
YRI	HapMap population: parent-offspring trios from the Yoruba people in Ibadan, Nigeria

Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

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CHAPTER 1: Introduction[1]

1.1 Left ventricular hypertrophy (LVH)

Left ventricular hypertrophy (LVH) is acknowledged as a major risk factor for cardiovascular morbidity and mortality (Frey and Olson, 2003; Lorell and Carabello, 2000). More specifically, increased LVH has been shown to predict the development of congestive heart failure (Mathew et al., 2001), coronary heart disease (Devereux and Roman, 1993), stroke (Verdecchia et al., 2001), cardiac arrhythmias (McLenachan et al., 1987) and sudden cardiac death (SCD) (Haider et al., 1998). Regression of LVH, on the other hand, is associated with a higher life expectancy (Sharp and Mayet, 2002) and improved clinical prognosis (Muiesan et al., 1995; Verdecchia et al., 1998). It is, therefore, vital to understand the underlying determinants of LVH to eventually facilitate more effective therapeutic intervention; in the meantime, the identification of molecular markers associated with LVH would enable improved risk stratification for cardiac morbidity in susceptible individuals.

Previous studies have shown that LVH is the most common cardiac complication of hypertension (Levy et al., 1990a). The effect of hypertension-control has been evident from studies such as the Heart Outcomes Prevention Evaluation (HOPE) and Losartan Intervention for Endpoint reduction (LIFE) clinical trials that investigated the effect of renin-angiotensin-aldosterone system (RAAS) inhibitors on cardiac hypertrophy in hypertensive cohorts. In the HOPE trial, cardiovascular morbidity and mortality was significantly reduced by regression of LVH with the angiotensin-converting enzyme (ACE) inhibitor ramipril (Mathew et al., 2001). Similarly, the LIFE study reported that the angiotensin receptor-blocker losartan was able to reduce left ventricular mass (LVM), an indicator of LVH, which, in turn, reduced the risk for SCD, myocardial infarction and stroke, independent of systolic blood pressure or other treatment administered (Dahlof et al., 2002a; Devereux et al., 2004). However, antihypertensive treatment has not reduced morbidity and mortality from cardiovascular disease associated with LVH as would be expected by the degree of blood pressure reduction (Koren et al., 1991); furthermore, LVH has also been observed in normotensive subjects (Levy et al., 1990a; Schunkert et al., 1999a). Consequently, LVH is not only attributable to pressure overload, but also to other, non-hemodynamic effects, some of which pertain to direct effects of RAAS components (Barry et al., 2008; Lijnen and Petrov, 1999).

[1] This chapter was accepted in part for publication as a chapter in Angiotensin: New Research (see Appendix I)

Various RAAS components have been shown to individually and collectively influence hypertrophy development (Kim and Iwao, 2000; Yamazaki et al., 1999). For instance, the main effector molecule of the RAAS, Angiotensin (Ang) II, is known to exert hypertrophic effects on neonatal (Baker and Aceto, 1990; Sadoshima and Izumo, 1993) and adult (Ritchie et al., 1998; Schunkert et al., 1995; Wada et al., 1996) cardiomyocytes and has been implicated in numerous pro-hypertrophic cardiac networks (Schluter and Wenzel, 2008). Schunkert et al. found that Ang I to Ang II conversion is increased in rat hearts with adaptive LVH, indicating an involvement of RAAS components in cardiac hypertrophy (Schunkert et al., 1990).

A study by Griffin et al. that showed that Ang II causes vascular hypertrophy in rats, partly by a non-hemodynamic mechanism (Griffin et al., 1991). In addition, Dostal and Baker demonstrated that Ang II-induced cardiac hypertrophy was prevented when an Ang II type 1 receptor (AT_1R)-antagonist was administered, an effect that was not achieved with a reduction in blood pressure, leading the authors to conclude that this effect was blood pressure-independent (Dostal and Baker, 1992). This was later confirmed in double transgenic rats harbouring human renin and human angiotensinogen genes in which end-organ damage can be ascribed to human RAAS components (Luft et al., 1999; Mervaala et al., 2000). These rats were treated with a simultaneous dose of three RAAS-independent drugs, which normalised blood pressure, but only partially prevented cardiac hypertrophy. This blood pressure-independent cardiac hypertrophy was attributed to increased plasma Ang II as plasma Ang II is increased up to 5-fold in these animals, compared with Sprague-Dawley rats, while a human renin inhibitor significantly reduced plasma Ang II concentrations and prevented cardiac hypertrophy (Mervaala et al., 2000).

However, Ang II is involved in complex pathways that influence LVH in a manner that is not yet completely understood, and the full contribution of the different RAAS components to hypertrophy development remains to be elucidated. Such analyses are quite tricky in complex conditions where hypertrophy is but one of the features of the disease, such as hypertension, but slightly easier in more simple conditions.

One such condition is hypertrophic cardiomyopathy (HCM), an inherited condition that is caused primarily by defective sarcomeric proteins, and which is characterised by highly variable extent and distribution of LVH. In this disorder, RAAS gene variants, possibly amongst others, appear to modulate the extent of hypertrophy development (Carstens et al., 2011; Ortlepp et al., 2002; Perkins et al., 2005; Van der Merwe et al., 2008). This disease has proven to be a valuable

model to investigate the molecular mechanisms involved in hypertrophy development, as its strong familial nature makes it amenable to the use of powerful molecular genetic techniques, while its autosomal dominant inheritance pattern ensures at least somewhat larger cohorts of study subjects than some of the other, rarer, genetic disorders in which cardiac hypertrophy is a feature (Watkins et al., 1995b).

1.2. Hypertrophic cardiomyopathy (HCM)

HCM is a primary cardiac disorder characterized clinically by LVH occurring in the absence of increased external loading conditions (Marian, 2002), as well as by diastolic dysfunction, arrhythmias and sudden death (Seidman and Seidman, 2001; Wigle et al., 1995). The prevalence of HCM has been shown to be approximately 1 in 500 in young adults through population-based clinical studies (Maron et al., 1995), although a much higher prevalence is expected in older individuals, based on the fact that HCM penetrance is age-dependent (Niimura et al., 2002).

In HCM, cardiac mass is increased due to left ventricular wall thickening that is frequently asymmetric and most often involves thickening of the interventricular septum (Seidman and Seidman, 2001) (Figure 1.1). Clinical diagnosis of HCM is established most easily with two-dimensional (2D) echocardiography by imaging the hypertrophied, but non-dilated, left ventricular chamber (Maron et al., 2003). However, clinical presentation in patients with HCM varies greatly, some patients present with minimal or no symptoms and have a benign, asymptomatic course, while others develop more serious complications, such as cardiac arrhythmias and heart failure, with one of the most severe endpoints being sudden cardiac death (Seidman and Seidman, 2001; Tsoutsman et al., 2006). This clinical variability is further observed in the extent and distribution of hypertrophy, which ranges from extensive and diffuse to mild and segmental, with no particular pattern considered typical (Klues et al., 1995).

HCM is classically described as a disease of the sarcomere (Thierfelder et al., 1994). Primary HCM is inherited as an autosomal dominant trait, and to date more than a thousand different causal mutations have been identified within 13 functional and structural proteins in the sarcomere and myofilament-related genes, which contribute in part to the heterogeneity of the disease (Ho, 2010a; Seidman and Seidman, 2011). The majority of these mutations are missense mutations that reside in genes encoding regulatory sarcomeric proteins, such as β-myosin heavy chain (β-MHC), actin, cardiac troponin T and I, and tropomyosin, as well as structural proteins, viz. myosin binding protein C (MYBPC) and titin (Alcalai et al., 2008) (Figure 1.2).

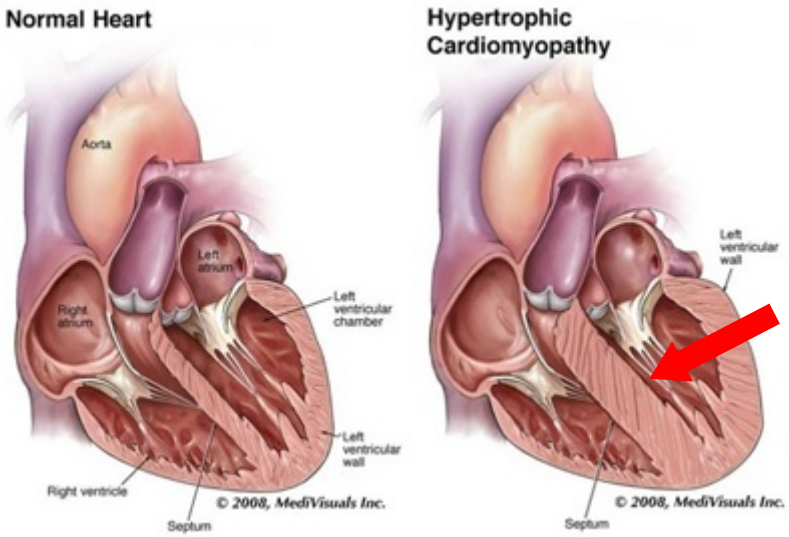

Figure 1.1 Illustration of hypertrophic cardiomyopathy. *Note the severe thickening of the interventricular septum and left ventricular wall as indicated by the red arrow. (Modified from http://cardiology.wustl.edu/details.aspx?NavID=638)*

It has been suggested that the prognostic significance of a given causal mutation is related to its influence on the magnitude of hypertrophy (Spirito et al., 2000; Spirito and Maron, 1990): some mutations are associated with severe hypertrophy, an early onset of disease and higher susceptibility to SCD, while others are associated with a relatively benign outcome (Charron et al., 1998; Erdmann et al., 2001). Furthermore, the dose of these mutant proteins in an individual has been shown to have a strong impact on the clinical course of HCM: individuals with homozygous or compound heterozygous mutations in sarcomere protein genes exhibit more severe clinical phenotypes (Lekanne Deprez et al., 2006; Mohiddin et al., 2003). Even so, the clinical presentation varies even between individuals from the same family with identical causal mutations (Keller et al., 2009), as well as between different families, with intrafamilial and interfamilial variability reaching similar levels (Epstein et al., 1992; Fananapazir and Epstein, 1994; Posen et al., 1995). Thus, sarcomeric mutations account for but a fraction of the diversity of hypertrophic phenotypes seen in HCM (Marian, 2002), suggesting that the clinical heterogeneity of HCM can be viewed as a product of the causal sarcomeric mutation, as well as additional genetic and environmental factors.

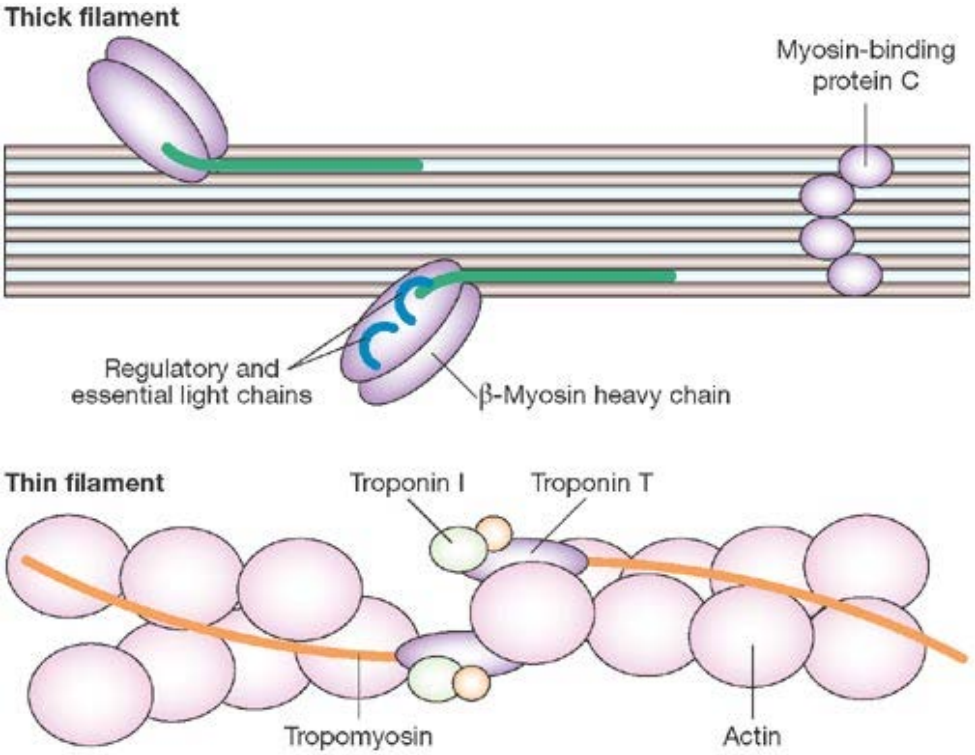

Figure 1.2 Schematic diagram of the cardiac sarcomere, indicating the main causal mutations for HCM*. (Taken from Keren et al., 2008)*

The case for genetic modifiers of HCM is predicated on the fact that a discrepancy exists between sarcomere-related mutations and the resulting cardiac phenotype. For instance, Fananapazir and Epstein (Fananapazir and Epstein, 1994) provided evidence for modifier genes in HCM when they described a Caucasian, as well as a Korean kindred with an identical disease causing mutation (R403Q) in the *MYH7* gene. The R403Q mutation was associated with 100% disease penetrance and a high incidence of SCD in the Caucasian kindred, while no SCD was observed in the Korean kindred; because of the significantly different clinical presentation of HCM between the two families, the authors concluded that the genetic background of the individuals along with environmental factors are responsible for the phenotypic diversity. This was later corroborated by other studies (Epstein et al., 1992; Marian et al., 1995; Marian, 2001; Solomon et al., 1993).

Transgenic animal models have also proven valuable in confirming a role for genetic modifiers on the cardiac phenotype in HCM, aided by the ability to control environmental influences and the genetic background of inbred strains of animals (Geisterfer-Lowrance et al., 1996). Semsarian and co-workers (Semsarian et al., 2001) studied a mouse model of HCM, α-MHC$^{403/+}$; the α-MHC$^{403/+}$ missense mutation in mice is equivalent to the human β-MHC gene (*MYH7*) R403Q mutation. By breeding the α-MHC$^{403/+}$ mice into different genetic backgrounds of

two distinct inbred mouse strains, a range of phenotypic differences in terms of hypertrophy, histopathology and exercise capacity could be identified. Given that the mice strains were housed under the same environmental conditions, the study provided confirmation of the role of genetic modifiers in HCM.

Interestingly, founding mutations have been reported in populations from Europe, the United States of America and South Africa (Moolman-Smook et al., 1999; Seidman and Seidman, 2011). Such populations, in which apparently unrelated families share causal mutations, are particularly valuable for genetic studies, as they offer a more homogeneous population in which to assess the role of additional genes in a clinical phenotype, which, as modifiers, are neither necessary nor sufficient to cause the condition. Thus, although HCM is regarded as a monogenic disease due to the prerequisite for a causative mutation to trigger the development of the phenotype, it can also be regarded as a complex trait due to the variability introduced by the involvement of additional genetic loci and environmental factors, each probably contributing to the phenotype to varying extents.

Various genetic mapping approaches have been employed to identify quantitative trait loci (QTLs) that alter the hypertrophic phenotype of HCM, the most common being candidate gene association analysis. Components of the RAAS are particularly plausible candidate modifiers of LVH in HCM, not only due to their effect on blood pressure, and thus an indirect effect on LVM, but also due to their direct hypertrophic effect on cardiomyocytes (Griendling et al., 1993; Ortlepp et al., 2002; Perkins et al., 2005).

1.3 Renin-angiotensin-aldosterone system (RAAS)

The RAAS exerts its main effect through Ang II, which has the ability to act as a systemic hormone (circulating RAAS) and as a local factor (tissue RAAS) (Paul et al., 2006). A schematic overview of the RAAS is given in Figure 1.3. Briefly, the biologically inert decapeptide Ang I is cleaved from angiotensinogen by the aspartyl-protease, renin, and subsequently hydrolyzed to the active octapeptide Ang II by ACE1 within the circulation, or by ACE1-independent mechanisms, involving, for instance, chymase. A second ACE, ACE2, has also been discovered, which converts Ang I to Ang-(1-7), which has been shown to counteract the vasoconstrictive effects of ACE.

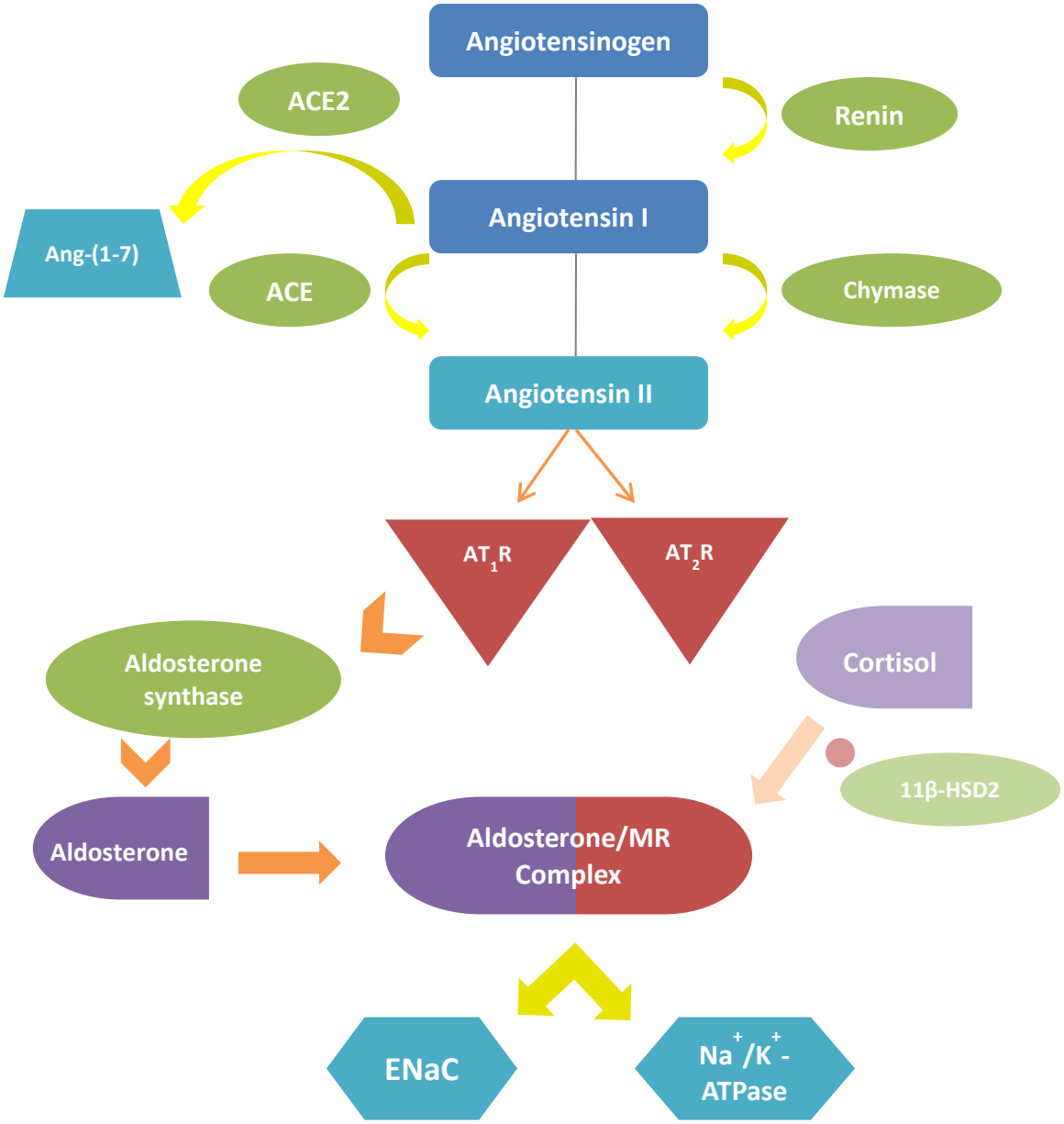

Figure 1.3 Schematic representation of the renin-angiotensin-aldosterone system (RAAS)

Ang II exerts its main biological effects by binding to highly specific Ang II receptors. To date, two main receptors have been characterized in humans: the Ang II type I (AT_1R) and Ang II type II (AT_2R), each with their own signalling cascade and physiological function (Chai and Danser, 2006; De Gasparo et al., 2000). Binding of Ang II to the AT_1R, triggers the synthesis of aldosterone via aldosterone synthase (*CYP11B2*). Aldosterone is a mineralocorticoid that exerts its function by, in turn, binding to the mineralocorticoid receptor (MR), which increases the transcription of MR-responsive genes (Lemarie et al., 2008). The MR binds both aldosterone and glucocorticoids, such as cortisol, with equal affinity. However, the enzyme 11 β-hydroxysteroid-dehydrogenase type 2 (11β-HSD2) increases the MR specificity for aldosterone by inactivating

the glucocorticoids (Tannin et al., 1991). The MR/aldosterone complex then exerts its Na^+-regulating effects in three phases (Eaton et al., 2001; Kamynina and Staub, 2002). The first is a latent period that lasts for about an hour, during which aldosterone-induced transcription and translation takes place. The second is an "early response" phase, lasting up to three hours, during which Na^+ transport is increased, mainly by increasing the open probability and number of active epithelial Na^+ channels (ENaC). A further increase in Na^+ transport is observed during the "late response", that lasts for about 24 hours, and during which expression of ENaC, as well as Na^+/K^+-ATPase subunits are increased (Rossier et al., 2002; Stockand, 2002).

Early investigations of the role of the RAAS pathway in hypertrophy development, in the context of HCM, were largely centred on the genes encoding ACE and the AT_1R, while downstream RAAS genes remained largely neglected. Recent association studies have, however, identified variants in additional RAAS genes that individually and collectively influence the penetrance and extent of LVH in HCM (Carstens et al., 2011; Van der Merwe et al., 2008). Other investigations have provided evidence that local Ang II generation in the myocardium, alternatively named tissue RAAS, is closely linked to the development of cardiac hypertrophy (Bader and Ganten, 2008). Recent studies have identified additional RAAS proteins that impact on hypertrophy development in a blood pressure-dependent, as well as Ang II-independent manner. This calls for an expansion of the "classical" RAAS to include these newly identified RAAS components, as well as a re-evaluation of our current knowledge of the role of RAAS components in the hypertrophic phenotype of HCM. Furthermore, pharmacological inhibition of RAAS in HCM as anti-hypertrophic therapy has recently gained renewed interest with the development of a direct renin inhibitor (Sever et al., 2009; Solomon et al., 2009). Taken together, these studies justify a renewed look at the individual and compound effects of RAAS components on hypertrophy within the context of HCM.

The involvement of specific RAAS components in hypertrophy development will now be discussed, and their hypertrophy-modifying role in HCM further highlighted, with special emphasis on knowledge gained from association studies.

1.4 Angiotensinogen (*AGT*)

Angiotensinogen, which is the first component of the RAAS, is encoded by the *AGT* gene. This gene consists of five exons and four introns and spans 12 kb on chromosome 1q42-43. Angiotensinogen remains a popular candidate modifier for essential hypertension and -asociated end-organ damage, as there exists a significant correlation between plasma *AGT* concentration and blood pressure in humans (Watt et al., 1992). Additionally, mice

overexpressing the *AGT* gene exhibit elevated blood pressure in a dose-dependent manner (Kim et al., 1995; Kimura et al., 1992), while *AGT* gene-knockout mice show reduced blood pressure levels (Tanimoto et al., 1994).

Xu et al. (Xu et al., 2009) reported that transgenic mice overexpressing the rat angiotensinogen gene developed severe chronic hypertension coupled with cardiac hypertrophy and impaired cardiac function. Single nucleotide polymorphisms (SNPs) and related haplotypes in this gene have additionally been associated with essential hypertension and elevated blood pressure in some populations (Brand-Herrmann et al., 2004; Jain et al., 2005; Jeunemaitre et al., 1999; Kumar et al., 2005), but not all (Dickson and Sigmund, 2006). However, two SNPs in *AGT*, T174M and M235T, were related to blood pressure-independent LVM-reductions in hypertensive patients with echocardiographically-diagnosed LVH who were treated with the AT_1R-antagonist irbesartan, but not in such patients treated with the beta-1 adrenergic receptor-blocker atenolol (data from the Swedish Irbesartan Left Ventricular Hypertrophy Investigation versus Atenolol (SILVHIA) trial) (Kurland et al., 2002).

The involvement of *AGT* polymorphisms in LVH development remains controversial, as some studies report significant associations between *AGT* variants and LVH, while other studies fail to replicate these results (Iwai et al., 1995; Jeng, 1999; Karjalainen et al., 1999; Kauma et al., 1998). One explanation for this discrepancy relates to the great variation of *AGT* polymorphism frequencies according to ethnic origin (Staessen et al., 1997a), which makes association studies on these polymorphisms sensitive to false-positive results due to population stratification. In such studies, false-negative results often occur in populations where one allele is largely predominant, due to the limited statistical power of the resultant associations studies (Jeunemaitre et al., 1999). For instance, in a meta-analysis of 69 studies with a combined sample size of 27 906, the overall prevalence of the M235-T allele was 52.1%. The prevalence of the M235-T allele was, however, significantly dependent on race, being 78.0% in Asians, 77% in blacks and only 42.2 % in Caucasians (Staessen et al., 1999).

Kuznetsova et al. (Kuznetsova et al., 2005) studied the European Project On Genes in Hypertension (EPOGH) cohort, which consisted of 221 nuclear families from three Caucasian populations, respectively from Poland, Russia and Italy, to investigate to what extent LVM was associated with the M235T and -6 G/A polymorphisms in the *AGT* gene. They reported that the significant association that they observed between these polymorphisms and LVM were dependent on age, gender, ecogenetic context, and appeared to be modulated by the trophic

effects of salt intake on LVM. These observations point towards the importance of adjusting for relevant confounders in *AGT* association studies to avoid spurious significance of results.

These factors were taken into consideration when Tang et al. investigated the effect of the above polymorphisms in a cohort of 605 predominantly Caucasian patients obtained from the Hypertension Genetic Epidemiology Network (HyperGEN) (Tang et al., 2002). The authors reported that LVM, as well as LVM_{index}, and the M235-T allele were negatively associated in hypertensive patients, but positively associated in normotensive patients, in a model adjusted for the potential confounding effect of weight, height, age, sex, systolic blood pressure, diastolic blood pressure, presence of diabetes, and antihypertensive medication use. The link between hypertrophy and *AGT*, therefore, extends beyond the known impact of angiotensinogen on blood pressure. This concept is also borne out biologically, as angiotensinogen has been shown to be expressed in myocardial tissue, where it is able to induce cardiac hypertrophy, independent of systemic blood pressure (Bader, 2002; Mazzolai et al., 1998; Reudelhuber et al., 2007). Moreover, mice expressing *AGT* exclusively in the liver and brain, showed reduced cardiac hypertrophy when compared to mice expressing *AGT* in the liver, brain and heart with a similar blood pressure (Kang et al., 2002).

Three *AGT* SNPs have been investigated for their role in hypertrophy development in HCM, in particular. These include a threonine to methionine substitution in exon 2 at position 174 of mature angiotensinogen (T174M), a 704 T>C substitution, which results in a methionine to threonine substitution at position 235 (M235T) in the same exon, as well as a promoter variant 6 bp upstream from the transcription initiation site (-6 G/A). Most of the HCM studies focussed on the M235T variant.

Just as in hypertension studies, the involvement of the M235T variant in HCM is controversial, as some studies report a correlation between this polymorphism and HCM (Cai et al., 2004; Ishanov et al., 1997; Kawaguchi, 2003; Manohar Rao et al., 2010), whereas other studies do not (Lopez-Haldon et al., 1999; Perkins et al., 2005; Yamada et al., 1997). Ishanov et al. (Ishanov et al., 1997) revealed that the M235-T allele frequency was higher in Japanese patients with sporadic HCM, than in their unaffected siblings and offspring. These findings were replicated in a study on 96 Japanese HCM patients (43 with familial HCM and 53 with sporadic HCM) and 105 of their unaffected siblings and children (Kawaguchi, 2003). Another study (Manohar Rao et al., 2010) reported similar results from an investigation of 150 South Indian HCM (90 sporadic HCM and 60 familial HCM) patients and 165 age- and sex-matched healthy controls, without known hypertension or LVH. Significant differences were detected in genotypic distribution, as

well as the allelic frequencies of the M235T polymorphism between patients with sporadic HCM and controls, although these findings were not replicated in patients with familial HCM (Manohar Rao et al., 2010).

In contrast, Yamada et al. found no significant association between this variant and non-familial HCM in a Japanese cohort (Yamada et al., 1997). No significant association was found between M235T and cardiac hypertrophy indices in a cohort of 389 unrelated patients with HCM (Perkins et al., 2005). Similarly, Coto et al. reported no significant association between the M235T variant and cardiac hypertrophy in a study on 245 echocardiographically-diagnosed HCM-patients and 300 healthy controls (Coto et al., 2010). Furthermore, none of the most commonly studied *AGT* SNPs (M235T, T174M, and -6 G/A) had a significant influence on a composite LVH score or LVM in a cohort of 108 genetically independent HCM patients (Brugada et al., 1997).

In addition to relatively small sample sizes, none on these studies accounted for the confounding effects of the primary HCM causal mutation or any other known hypertrophy covariates in their analyses.

Furthermore, the question regarding the functionality of the M235T SNP remains. This polymorphism was associated with a stepwise-increase in angiotensinogen levels in Caucasian subjects, as well as a corresponding moderate increase in risk of hypertension in both Caucasian and Asian subjects in a meta-analysis of 127 publications (Sethi et al., 2003). However, the M235-T genotype did not predict plasma angiotensinogen levels, or blood pressure, risk of ischemic heart disease, or myocardial infarction in either Asian or black subjects (Sethi et al., 2003).

The M235T variant is in tight linkage disequilibrium (LD) with the -6G/A variant in the proximal promoter of the *AGT* gene (Inoue et al., 1997; Tang et al., 2002). This substitution affects specific interactions between at least one trans-acting nuclear factor and the promoter of *AGT*, thereby influencing the basal rate of transcription of the gene, which was initially thought to explain why T235-homozygotes have plasma angiotensinogen levels that are 10–20% higher than M235-homozygotes (Danser and Schunkert, 2000).

However, later analyses of transgenic mice expressing either the -6G/235M or the -6A/235T haplotype in the 13.5-kb human *AGT* gene showed that both transgenes exhibited the same transcriptional activity and produced similar plasma levels of human angiotensinogen

(Cvetkovic et al., 2002). These results suggest that variation at the -6-position may only be a marker, and may not, in itself, be functional. However, mice carrying the -6G/235M haplotype showed a slight but significant increase in blood pressure and relative heart weight, as well as compensatory downregulation of endogenous renin expression, which led the authors to speculate that these haplotypes might affect the cardiovascular system and the regulation of blood pressure differently (Cvetkovic et al., 2002).

Jain et al. found that -6G/A can act as a marker for three other promoter SNPs, as well as for three additional intragenic SNPs, where the -6G and -6A variants each tag a different haplotype of these polymorphisms. To inspect the physiological effect of these haplotypes, they generated double transgenic mice containing either the -6A or -6G haplotype of the human *AGT* gene, and also the human renin gene (*REN*). Transgenic mice containing -6A haplotype had increased plasma *AGT* levels and increased blood pressure, compared with those with the -6G haplotype (Jain et al., 2010).

Grobe et al. developed triple-transgenic mice carrying a null mutation in the endogenous murine angiotensinogen gene, while expressing either the -6G/235M or -6A/235T haplotype of the human *AGT* gene, and either an overexpressed and poorly regulated, or a tightly regulated human *REN* gene. Mice expressing the -6G/235M haplotype on the well-controlled renin background exhibited increased blood pressure and cardiac hypertrophy. In contrast, mice with the -6A/235T haplotype in a poorly regulated renin background exhibited increased cardiac and renal growth and increased blood pressure sensitivity to a high-salt diet, leading the authors to conclude that the differential effects of these haplotypes on cardiovascular end-points are context dependent and sensitive to genetic background and environmental influences (Grobe et al., 2010).

There is, however, a lack of studies that explore the *physiological* effects of *AGT* variants on hypertrophy development specifically in HCM.

1.5 Renin and renin-associated genes

Renin is a rate-limiting component of the RAAS, as it controls the initial conversion of angiotensinogen to Ang I. While there is a paucity of research on the role of renin and its associated proteins in hypertrophy development in HCM, it remains an exciting and promising field of research, which is currently offering promising prospects for hypertrophy research that might be transferable to hypertrophy in HCM. In addition, the recent development of a direct renin inhibitor, aliskiren, renewed interest in renin as a potential therapeutic target in cardiac

hypertrophy management (Sever et al., 2009; Verdecchia et al., 2008). As this direct renin inhibitor controls the rate-limiting step of the RAAS and decreases plasma renin activity (PRA), it is thought to offer superior benefits to ACE- and AT_1R blockers in treating cardiovascular disorders. These latter blockers interfere with the negative feedback loop exerted by Ang II on renin formation that elicits a rise in plasma renin concentration (Balakumar and Jagadeesh, 2010a).

Aliskiren ameliorated cardiac hypertrophy in rats expressing both human renin and angiotensinogen (Pilz et al., 2005) and was proven to be at least as effective as ACE inhibition and Ang receptor blockade in LVH reduction in spontaneously hypertensive rats (Van Esch et al., 2010). Aliskiren was also shown to ameliorate cardiac remodelling and hypertrophy after myocardial infarction with doses that did not affect blood pressure in mice (Westermann et al., 2008a). The recent Aliskiren in Left Ventricular Hypertrophy (ALLAY) study reported that aliskiren was as effective as the Ang-receptor blocker losartan in LVM regression, making aliskiren a potential treatment option in patients with LVH (Solomon et al., 2009).

Renin is generated from preprorenin in a number of steps: prorenin is generated from preprorenin in the juxtaglomerular cells of the kidney by the removal of 23 amino acids, and is later converted into mature renin. Recent research has identified three additional proteins that associate with renin and prorenin *in vivo*. This includes a protein that is able to inhibit renin upon binding to it, namely the renin-binding protein (RnBP), as well as two receptors for renin. The mannose-6-phosphate/insulin-like growth factor II receptor (M6PR/IGFII) has been suggested as a clearance receptor in cardiomyocytes, as it only binds glycosylated forms of prorenin and facilitates its subsequent internalisation and degradation (Saris et al., 2001b). The (pro)renin receptor (PRR), on the other hand, is a promising candidate for tissue uptake of renin, as it binds both renin and prorenin and activates prorenin non-proteolytically (Nguyen and Muller, 2010).

The presence of renin in the heart is a matter of great controversy, as evidence for local renin synthesis has not been conclusive. It is now widely accepted that cardiac renin is taken up from the circulation, either due to diffusion into the interstitium (Danser and Saris, 2002; De Lannoy et al., 1997), or through specific functional binding sites and renin receptors (Catanzaro, 2005; Nguyen et al., 2004). In addition, the heart can generate renin locally from circulating prorenin by proteolytic cleavage and non-proteolytic activation through the PRRs in myocardial tissues (Nguyen et al., 2002; Nguyen and Danser, 2008; Reudelhuber et al., 1994). Interestingly, the plasma concentration of prorenin is ten times greater than that of renin (Danser et al., 1998)

and circulating prorenin levels may reach as high as 100 times the level of renin under conditions of renal damage and cardiac hypertrophy (Susic et al., 2008).

Veniant and colleagues developed a transgenic rat line that expresses prorenin exclusively in the liver. These rats demonstrated a 400-fold increase in plasma prorenin, but exhibited normal plasma renin levels and blood pressure. However, these animals developed severe liver fibrosis, as well as cardiac and aortic hypertrophy (Veniant et al., 1996). This study gained more attention with the cloning of the PRR (Nguyen et al., 2002). When renin and prorenin are bound to this receptor, a five-fold increase in angiotensinogen to Ang I conversion is noted, and these physiological effects are exerted in a manner completely independent of Ang II generation (Nguyen et al., 2003; Oliver, 2006). In a study on neonatal rat cardiomyocytes, Saris et al. (Saris et al., 2006) demonstrated that prorenin bound to the PRR activated the p38 MAPK/HSP27 pathway; they postulated that this activation is responsible for the severe hypertrophy observed by Veniant et al. Similarly, renin and prorenin have been proven to induce DNA synthesis and to activate the p42/p44 MAPK intracellular pathways and stimulate the release of plasminogen activator inhibitor (PAI)-1, as well as transforming growth factor-β1 (TGF-β1), through binding with the PRR (Cousin et al., 2010). These are profibrotic, inflammatory and hypertrophic signalling pathways that function independent of Ang II generation (Huang et al., 2006; Huang et al., 2007b; Ichihara et al., 2006; Nguyen and Muller, 2010). These pro-hypertrophic signalling cascades are not inhibited by ACE inhibitors, aliskiren or AT_1R blockers (Balakumar and Jagadeesh, 2010a). This and other studies (Methot et al., 1999; Nguyen et al., 1996; Prescott et al., 2002) supports growing evidence that renin and prorenin *per se* exerts hypertrophic cellular effects, independent of Ang II generation, at least some of which involve the PRR.

Furthermore, the "handle region peptide (HRP)", a protein that corresponds to the "handle" region of prorenin, which inhibits the binding of prorenin to the PRR (Paulis and Unger, 2010), has been shown to reduce cardiac hypertrophy and improve left ventricular function in spontaneously hypertensive rats on a high salt diet (Susic et al., 2008). This effect was, however, not replicated in high renin conditions (Ichihara et al., 2010).

The PRR is identical to ATPase-associated protein 2 (encoded by the *ATP6AP2* gene), an accessory protein to a vacuolar proton-transporting ATPase (v-H^+-ATPase). In a study using Xenopus embryos, as well as human cultured cells, Cruciat et al. showed that ATP6AP2 functions in a renin-independent manner as an adaptor between Wnt receptors and the v-H^+-

ATPase complex (Cruciat et al., 2010). Aberrant Wnt signalling has previously been linked to cardiovascular hypertrophy (Balakumar and Jagadeesh, 2010b).

Recently, Connelly et al. corroborated the co-localization of PRR with v-H^+-ATPase in the heart and reported an increased expression of PRR in the hearts of transgenic animals with diabetic cardiomyopathy (Connelly et al., 2011). This increased expression of PRR was associated with diastolic dysfunction, interstitial fibrosis, as well as cardiomyocyte hypertrophy. Direct renin inhibition then reduced cardiac PRR expression in these animals, in association with improved cardiac structure and function (Connelly et al., 2011).

The PRR is, therefore, able to influence hypertrophy development through local RAAS activation, as well as through Wnt signalling, making it an attractive target for antihypertrophic treatment (Finckenberg and Mervaala, 2010). More research is, however, needed to fully elucidate the contribution of the PRR to cardiac hypertrophy in general, as well as to the role of PRR in HCM (Reudelhuber, 2010).

Furthermore, previous studies have shown that M6PR/IGFII is also able to bind prorenin and renin on cardiomyocytes (Van den Eijnden et al., 2001; Van Kesteren et al., 1997a), and to generate renin from prorenin through proteolytic cleavage (Saris et al., 2001a). This binding and activation did not result in Ang II generation in cardiomyocytes (Saris et al., 2002).

Takahashi et al. reported another protein that was capable of forming a complex with renin, which they named RnBP (Takahashi et al., 1983). Further *in vitro* studies showed that this protein is able to form a heterodimer with renin and subsequently to inhibit its activity (Takahashi et al., 1994). This protein was later found to be identical to the enzyme N-acetyl-D-glucosamine 2-epimerase (NAGE) (Takahashi et al., 1999). In a study of RnBP-knockout mice, Schmitz et al. were unable to detect any effect of RnBP-deficiency on renal and circulating RAAS or on blood pressure, leading the authors to speculate that RnBP does not play a role in the regulation of plasma renin and RAAS activity (Schmitz et al., 2000). However, Bohlmeyer and colleagues investigated the expression of RnBP in failing human hearts, with end-stage idiopathic dilated cardiomyopathy. They found that RnBP expression was restricted to endothelial cells in non-failing hearts, while RnBP gene and protein expression was selectively activated in the ventricular cardiomyocytes of failing hearts (Bohlmeyer et al., 2003). Interestingly, they reported that the highest RnBP mRNA levels were detected in a subject with significant LVH. Additionally, RnBP was redistributed from a cytosolic to a

sarcolemmal/sarcomeric fraction, which led the authors to conclude that RnBP may be involved in the modification of cardiac cytoskeletal proteins.

Knoll et al. investigated the effect of a RnBP gene (*RENBP*) T61C variant on several cardiovascular parameters in 505 randomly selected Caucasian individuals (Knoll et al., 1997). Males (n = 293) and females (n = 212) were analysed separately, as the RnBP gene is X-linked, and individuals on antihypertensive medication were excluded. The authors reported an association between this variant and plasma prorenin, as well as the renin/prorenin ratio, but found no significant association with circulating renin, blood pressure, heart rate or LVM in men. These findings were not replicated in females (Knoll et al., 1997). In contrast, Gu et al. reported a strong association between markers rs1557501 and rs2269372 in *RENBP* and systolic blood pressure responses to low-sodium diets in a cohort of 1906 individuals from Han Chinese families who took part in the Genetic Epidemiology Network of Salt Sensitivity (GenSalt) (Gu et al., 2010).

Research on renin and renin-related proteins largely focussed on identifying these proteins in different *in vivo* settings, to pinpoint their physiological function. While association has been detected between *REN* (Ahmad et al., 2005a), *RENBP* (Gu et al., 2010) and the PRR gene, *ATP6AP2*, (Ichihara et al., 2010) variants and blood pressure regulation, to date, no study has detected an effect of polymorphisms in these genes on hypertrophy *per se* or within the context of HCM.

1.6 Angiotensin converting enzyme (ACE)

ACE, encoded by the *ACE* gene, is a transmembrane-ectopeptidase that is responsible for the conversion of Ang I to the active Ang II and the inactivation of bradykinin. This gene remains the most commonly studied candidate modifier gene for HCM, as Ang II and bradykinin exert strong cardiovascular effects in opposing directions. While Ang II promotes hypertension and cardiac hypertrophy, bradykinin exerts cardioprotective effects.

ACE expression is increased in many forms of cardiovascular hypertrophy (Fleming, 2006; Schunkert et al., 1990), while serum ACE activity is significantly related to LVM, independent of systemic blood pressure (Schunkert et al., 1997). ACE inhibitors, together with AT_1R blockers, are currently the gold standard for antihypertensive therapy (Paulis and Unger, 2010). Furthermore, inhibition of ACE with ramipril is associated with LVH regression, independent of its effect on blood pressure (Mathew et al., 2001), while ACE inhibition improved left ventricular function in patients with hypertrophic obstructive cardiomyopathy (Kyriakidis et al.,

1998). These data are complemented by meta-analyses of clinical trials that suggest that ACE inhibitors are effective in causing LVH regression, even after adjustment for treatment duration and change in blood pressure (Dahlof et al., 1992; Klingbeil et al., 2003; Schmieder et al., 1996).

An insertion/deletion (I/D) polymorphism, involving the presence (I) or absence (D) of 287 bp Alu repeats in intron 16 of *ACE*, accounts for 47% of the total variability in serum ACE levels (Rigat et al., 1990). Circulating ACE levels in plasma were nearly 60% higher in DD-homozygotes, compared with II-homozygotes (Rigat et al., 1990). This polymorphism is consequently thought to be functional as it exerts and incremental effect on plasma and tissue ACE levels, where II-homozygotes exhibit the lowest levels of plasma and tissue ACE, heterozygotes the intermediate and DD-homozygotes the highest (Marian, 2002; Tiret et al., 1992).

However, in a meta-analysis of 49 959 individuals (Staessen et al., 1997b), the prevalence of the D-allele was significantly influenced by ethnicity, as the D-allele prevalence was 56.2% in Caucasians, 60.3 % in blacks and 39.1% in Asians. This group added to these findings in a more recent meta-analysis on echocardiographic phenotypes across 38 studies (Jin et al., 2011b) by reporting that both DD-homozygotes and ID-heterozygotes had elevated LVM and an increased mean wall thickness when compared to II-homozygotes. The authors did, however, mention that these results should be interpreted with caution as adjustments for environmental and lifestyle confounders of LVM were inconsistent across studies and they observed a significant publication bias for studies reporting an association between LVM and the I/D polymorphism, which may have lead to an overestimation of the pooled association (Jin et al., 2011b). In contrast, a study on 2439 subjects from the Framingham Heart study did not find a significant association between ACE DD-genotypes and echocardiographically-determined LVM (Lindpaintner et al., 1996).

In the first study of this polymorphism in HCM, Marian et al. reported a significant association between the DD-genotype and an increased risk of sudden cardiac death in HCM patients (Marian et al., 1993). Later, other studies reported a significant association between the D-allele and increased LVH indices, but this association was not replicated in all studies (Table 1).

Table 1.1 Summary of association studies on the influence of ACE I/D polymorphism on HCM phenotypes

Study Cohort	Results
389 unrelated HCM patients	DD-genotype associated with increased left ventricular wall thickness in *MYBPC3* carriers, but not *MYH7* carriers (Perkins et al., 2005)
100 HCM patients and 106 of their unaffected siblings and offspring	DD-genotype associated with increased risk of SCD in HCM (Marian et al., 1993)
114 genetically affected HCM patients	Significant association between D-allele and hypertrophy, which is influenced by HCM-causal mutation (Tesson et al., 1997)
118 Indian HCM Patients and 164 ethnically, age- and gender-matched controls	Prevalence of DD-genotype significantly higher in patients compared to controls and was associated with increased risk of HCM, after adjustment for age, sex, body mass and smoking (Rai et al., 2008)
62 Australian HCM patients	DD-genotype associated with greater progression of LVH, independent of age, body mass and blood pressure (Doolan et al., 2004)
183 Caucasian HCM patients	DD-genotype significantly associated with LVH, independent of age, sex, weight and body surface area (Lechin et al., 1995)
50 unrelated HCM patients and 50 healthy controls	DD-genotype more prevalent in HCM patients (Pfeufer et al., 1996)
80 Japanese HCM patients and 88 of their unaffected children and siblings	DD-genotype more prevalent in HCM patients (Yoneya et al., 1995)
71 Japanese patients with nonfamilial HCM and 122 healthy controls	No association between DD-genotype and indices of hypertrophy (Yamada et al., 1997)
136 Brazilian HCM patients	Serum ACE activity, but not I/D polymorphism affected LVH in HCM (Buck et al., 2009)
104 unrelated Caucasian HCM patients	No association between D-allele and LVM, but variant affected plasma ACE levels (Osterop et al., 1998)
126 genetically diagnosed Japanese HCM patients	D-allele associated with decreased posterior wall thickness, decreased ejection fraction and increased left ventricular end-systolic dimension; no association with septal thickness or maximal left ventricular wall thickness (Funada et al., 2010)
63 HCM patients and 20 healthy controls, all Caucasian	Increased QT dispersion in HCM patients with DD-genotype (Kaya et al., 2010)
245 unrelated HCM patients and 300 healthy controls, all Spanish Caucasians	No association between I/D polymorphism and risk of cardiac hypertrophy (Coto et al., 2010)

One explanation for this discrepancy could be that association between the I/D polymorphism and hypertrophy indices is context-dependent and sensitive to hypertrophy- and environmental confounders in HCM (Sayed-Tabatabaei et al., 2006).

Tesson et al. assessed the effect of the I/D polymorphism in families that harbour HCM causal genes in the cardiac myosin binding protein C (*MYBPC*) or β-myosin heavy chain (*MYH7*) genes and found that the association between this polymorphism and LVH was dependent on the specific HCM mutation (Tesson et al., 1997). When all genetically-affected individuals were analysed together, they found no significant association. A significant association was, however, reported between the ACE D-allele and interventricular septal thickness in individuals with the R403Q mutation in *MYH7* (Tesson et al., 1997). Different HCM mutations show definite variability in phenotypic expression, as some are associated with severe cardiac hypertrophy, while others offer more benign outcomes (Charron et al., 1998; Erdmann et al., 2001). It is, therefore, conceivable that the effect of primary HCM causal mutation cannot be discounted in HCM association studies. Most of the studies in Table 1 did, however, not account for this effect.

Another general difficulty for HCM association studies is shown by the study of Lechin et al. (Lechin et al., 1995). This group used regression analysis to show that ACE I/D genotypes only accounted for 3.7% of the variability in LVM index and 6.5% of a LVH score used to quantify the extent of hypertrophy in unrelated HCM patients (Lechin et al., 1995). This allows for the possibility that ACE variants only make a modest contribution towards hypertrophy in HCM and speaks to the possibility that there might be a number of modifying variants and genes that affect the HCM phenotype.

Buck et al. reported that ACE activity, rather than ACE polymorphisms, affected LVH in HCM, although this study is somewhat hampered by the small sample size (Buck et al., 2009), however, ACE activity is obviously determined by genetic variation within the *ACE* gene. Thus, it remains puzzling that the I/D variant is not located in a coding region and does not exert any clear effect on gene transcription, which points to a very real possibility that it might only be a marker for a functional variant in close proximity (Cox et al., 2002; Keavney et al., 1998). The precise location of this proposed functional variant is still uncertain, although it is thought to be most likely located between intron 18 and the 3' UTR (Sayed-Tabatabaei et al., 2006; Zhu et al., 2000). On the other hand, Kammerer and co-workers recently suggested a QTL on chromosome 4 that impacts on ACE activity (Kammerer et al., 2004), and a yet another possibility for a functional variant exists in the 5' gene region (McKenzie et al., 2005; Villard et al., 1996).

1.7 Angiotensin converting enzyme 2 (ACE2)

A second ACE, ACE2, which is thought to be an essential regulator of cardiovascular function (Crackower et al., 2002) was recently reported. This homologue of *ACE* removes the C-terminal phenylalanine from Ang II, to form Ang-(1-7), which is a ligand of the G-protein-coupled receptor Mas. ACE2-null mice develop progressive Ang II-mediated age-dependent cardiomyopathy, which is associated with increased oxidative stress, as well as pathological hypertrophy (Oudit et al., 2007). ACE2-deficiency also exacerbated adverse cardiovascular remodelling in mice with Ang II-induced hypertrophy, whereas recombinant human ACE2 reduced Ang II-induced hypertrophy in wild type mice and partially prevented the development of dilated cardiomyopathy in pressure-overloaded mice (Zhong et al., 2010). The beneficial effect of ACE2 on adverse cardiac remodelling is further evident from a number of animal models (Huentelman et al., 2005; Tikellis et al., 2011; Trask et al., 2007). In addition, Ang-(1-7) has been shown to attenuate cardiac hypertrophy and dysfunction through a direct local effect in transgenic animal models (Finckenberg and Mervaala, 2010).

Lieb et al. previously reported that the minor alleles of four SNPs in the X-linked *ACE2* gene were associated with LVM_{index} as well as interventricular septal thickness in males (but not females) from the general population in the MONICA (MONitoring trends and determinants in CArdiovascular disease) Augsburg survey (Lieb et al., 2006). These four SNPs (rs4646156, rs879922, rs4240157 and rs233575) showed high pairwise LD and a common haplotype, consisting of the minor alleles of these SNPs, was associated with an increased odds-ratio (OR) for LVH (OR 3.10, p=0.006) in a model adjusted for age, body mass, antihypertensive medications and systolic blood pressure. Males carrying this haplotype displayed modestly increased LVM_{index} and interventricular septal thickness (Lieb et al., 2006).

Subsequently, Wang and co-workers investigated the effect of two other SNPS on HCM in a Chinese population, as the minor alleles of rs879922 and rs4240157 were present at too low frequency, and rs4646156 and rs233575 were not polymorphic, in the Chinese population. They reported that the T-allele of rs2106809 and C-allele of rs6632677 conferred an increased risk for HCM in males, from a study of 261 HCM patients and 600 healthy controls (Wang et al., 2008). These SNPs were in strong LD with each other, and the TC haplotype was associated with an increased risk for HCM and modestly increased interventricular septal thickness, independent of age, body mass and blood pressure. The primary disease-causing HCM mutations of these patients were, however, unidentified and were therefore not adjusted for in the analyses. These findings could also not be replicated in females.

The G-allele of rs879922 was recently reported to be significantly associated with cardiac hypertrophy in a South African HCM family cohort, independent of the primary HCM causal mutation and known hypertrophy covariates, viz. blood pressure, age, sex, body surface area (BSA), heart rate and hypertension diagnosis (Van der Merwe et al., 2008). After adjustment for known hypertrophy covariates, the G-allele significantly increased LVM by 18.7 g, maximum interventricular septal thickness by 1.9 mm and maximum posterior wall thickness by 0.7 mm (van der Merwe et al., 2008b).

Although ACE2 is clearly a plausible hypertrophy modifier in HCM, more studies are needed to fully elucidate the contribution of *ACE2* variants to hypertrophy in HCM as none of these variants have any obvious functional roles.

1.8 Cardiac chymase (CMA)

Ang II production in the heart can also be attributed to a serine protease named cardiac chymase (CMA). Indeed, Urata et al. showed that CMA accounts for 80% of the Ang II generated in the human ventricle (Urata et al., 1993). Chymase is produced from mast cells in the heart and is not inhibited by ACE inhibitors (Guo et al., 2001). Transgenic mice expressing human CMA showed elevated blood pressure and increased LVM (Koga et al., 2003), while cardiac chymase activity was increased in the hearts of naturally-occurring cardiomyopathic hamsters (Shiota et al., 1997; Shiota et al., 1998). Hoshino et al. showed that a CMA-inhibitor significantly increased survival rate, while attenuating cardiac hypertrophy and end-diastolic left ventricular pressure, in a hamster model of myocardial infarction (Hoshino et al., 2003).

Two bi-allelic markers of the cardiac chymase A gene (*CMA*) on chromosome 14q11 have been studied in hypertrophy association studies: a 1625 G/A transition in intron 2, termed CMA/A, and a -1903 G/A transition in the 5' untranslated region, termed CMA/B (He et al., 2005; Pfeufer et al., 1996). He et al. investigated the antihypertrophic properties of the ACE I/D and the CMA/B polymorphisms in a clinical trial on the effect of the ACE inhibitor benazepril on 157 Chinese patients with essential hypertension (He et al., 2005). Benazepril significantly reduced blood pressure and LVH in these patients. Patients treated with benazepril that harboured the ACE DD genotype showed a significantly higher reduction in echocardiographically determined LVM and LVM_{index} when compared to II and ID genotypes. No association was, however, found between the CMA polymorphism and regression of LVH in these patients (He et al., 2005).

Conversely, Gumprecht et al. reported that the CMA/B polymorphism, together with the ACE I/D polymorphism, conferred a genetic predisposition to increased risk of the development of LVH in Caucasian type-2 diabetics (Gumprecht et al., 2002).

The CMA/B was not associated with the extent of LVH in one study of a cohort of 50 unrelated HCM patients and 50 healthy controls (Pfeufer et al., 1996). In a later study, the same group reported that CMA polymorphisms did not affect blood pressure, posterior- or interventricular septal thickness in patients with acute myocardial infarction (Pfeufer et al., 1998). In a family-based association study, the CMA/B polymorphism showed a significant association with LVM in an unadjusted analysis, but the same SNP did not show a significant association with LVM or interventricular septal thickness in a multivariate analysis in which age, sex and the presence of hypertension were adjusted for ($p = 0.06$) (Ortlepp et al., 2002).

1.9 Angiotensin II type 1 Receptor (AT_1R)

The main effector molecule of the RAAS, Ang II, when bound to the AT_1 and AT_2 receptors, influences cardiac hypertrophy, remodelling and contraction in multiple blood pressure independent ways. Binding of Ang II to the AT_1R activates multiple intracellular pathways that involve phospholipids, calcium, reactive oxygen species (ROS) and kinases (Booz, 2004). These AT_1R-mediated pathways elicit cardiovascular hypertrophic effects that are well-documented, which includes vasoconstriction, aldosterone release and growth stimulation (Dostal and Baker, 1992; Hoffmann et al., 2001).

Moreover, overexpression of the AT_1R leads to increased cardiac mass and cardiomyocyte hypertrophy in mice (Hein et al., 1997). It was recently shown that increased cardiac expression of the AT_1R gene (*AGTR1*) in cardiomyocytes of adult transgenic mice caused blood pressure-independent hypertrophy by promoting cardiomyocyte growth, which progressed to pathological remodelling upon further stimulation (Ainscough et al., 2009). Moreover, large clinical trials have concluded that AT_1R antagonists reduce LVH and other cardiac morbidities in humans (Dahlof et al., 2002b; Okin et al., 2003).

AT_1R-antagonists are currently being explored as potential therapeutic options for hypertrophy and associated morbidities in HCM (Force et al., 2010). Four small pilot studies in patients with non-obstructive HCM showed slight improvements in cardiovascular morphology and function (Araujo et al., 2005; Kawano et al., 2005; Penicka et al., 2009; Yamazaki et al., 2007). The most recent of these studies reported that the long-term administration of the AT_1R-antagonist candesartan in HCM patients was associated with significant regression of LVH, improvement of

left ventricular function, and exercise tolerance. This effect was dependent on the specific HCM causal mutation (Penicka et al., 2009). These studies are, however, still too small to allow definitive conclusions to be drawn and more research is needed in this regard.

Castellano et al. studied the effects of an A/C transversion at position 1166 in the 3' untranslated region of *AGTR1* (rs5186) on the regulation of blood pressure and cardiovascular structure in 212 subjects randomly selected from a general population in northern Italy (Castellano et al., 1996). They reported that blood pressure levels were significantly lower in CC-homozygotes, but no association was found with echocardiographically determined LVM after adjustment for potentially confounding variables (age, sex, height, weight, blood pressure and smoking) (Castellano et al., 1996). Similarly, a study on 141 Caucasian patients with normal coronary arteries did not find association between the A1166C, or the *ACE* I/D polymorphism, and left ventricular function or LVM (Hamon et al., 1997).

Wang et al. studied the effects of *AGTR1* C-512T, A1166C and L191L polymorphisms on the progression of blood pressure and LVM in a longitudinal study involving 581 European American and African American youths (Wang et al., 2006). When analysed as a single variant, the L191L polymorphism showed a significant systolic blood pressure-lowering effect in youths with a high socioeconomic status, and a diastolic blood pressure-lowering effect in African American youths. However, haplotype analysis identified a protective haplotype (C-521, 191L and A1166) for LVM in the entire cohort. Individuals homozygous for this haplotype showed a significant decrease of 12.9 g in LVM when compared to the most common reference haplotype (–521T, 191L and A1166) (Wang et al., 2006).

Ishahov et al. reported that this polymorphism did not contribute significantly to hypertrophy development in hypertensive (n = 53) or HCM (n = 96) patients (Ishanov et al., 1998). However, subjects with a family history of HCM, but who did not manifest the disease clinically, had a four-fold increase in C-allele carrier status compared to patients without a family history of HCM (Ishanov et al., 1998). Conversely, the 1166C-allele was associated with increased LVM in 104 unrelated HCM patients, independent of age, gender, peak left ventricular outflow gradient, plasma renin, and the *ACE* I/D polymorphism (Osterop et al., 1998). Coto et al. reported a significantly higher frequency of 1166C-allele carriers among echocardiographically-diagnosed HCM patients compared to controls (OR = 1.56; 95%CI = 1.09-2.23) (Coto et al., 2010). This allele was also associated with higher left ventricular wall thickness in HCM patients (Coto et al., 2010).

In contrast to these studies, in which only echocardiographic criteria were used to diagnose HCM, Funada et al. studied the effect of this polymorphism in 126 genotype-identified HCM patients from 49 Japanese families (Funada et al., 2010). These patients were diagnosed with HCM through standard echocardiographic criteria and the diagnosis was confirmed with genetic testing for sarcomeric gene mutations. While this resulted in an improved diagnosis, the authors did unfortunately not adjust the subsequent association analyses for these sarcomeric mutations, which may have yielded more informative results. They reported that the A1166C alone did not influence LVH, but when analysed in combination with the *ACE* I/D polymorphism, the two SNPs significantly affected adverse cardiac remodelling (Funada et al., 2010). This SNP was additionally found to associate with LVH in a study on 26 individuals from a single family with HCM caused by a previously identified myosin binding protein C mutation (Ortlepp et al., 2002).

A recent study (Sethupathy et al., 2007) proposed a biochemical mechanism of action for the A1166C polymorphism. This SNP is located in the 3' untranslated region in a target site for the hsa-miR-155 micro-RNA, which is able to bind to 3' untranslated regions through complementary base-pairing and thus to repress mRNA expression post-transcriptionally. Using reporter silencing assays, Sethupathy et al. showed that hsa-miR-155 downregulates the expression of only the 1166A, and not the 1166C-allele, which results in increased translation of *AGTR1* in C-allele carriers (Sethupathy et al., 2007). It is, however, unsure at this stage whether *AGTR1* and hsa-miR-155 is concomitantly expressed in the heart and more studies are, therefore, needed to fully explore the functionality of this SNP, while the possibility still exist that it might be in LD with a truly functional mutation. However, this highlights a mechanism by which apparently non-functional polymorphisms can have effects on expression of genes, and thereby may affect biochemical pathways and physiological systems.

1.10 Angiotensin II type 2 Receptor (AT_2 R)

Cardiac expression of the AT_2R is upregulated in heart failure, myocardial infarction and cardiac remodelling (Nio et al., 1995; Ohkubo et al., 1997; Van Kesteren et al., 1997b). Previous studies in adult rat hearts have suggested that AT_2Rs have antihypertrophic effects on the heart that counterbalance the hypertrophic effects of the AT_1Rs (Booz and Baker, 1996; Mukawa et al., 2003). In a study on adult rat hearts, Bartunek et al. demonstrated that AT_2R inhibition amplifies LVH in response to Ang II. They perfused normal and hypertrophied hearts either with only Ang II, or with Ang II together with an AT_2R blocker and measured new protein synthesis within the left ventricles of these hearts. AT_2R blockade in Ang II-treated rats resulted in an amplified left ventricular growth response to Ang II, which was coupled with reduced left

ventricular cGMP content, and enhanced membrane protein kinase C translocation (Bartunek et al., 1999).

The AT_2R gene (*AGTR2*) is located on the X-chromosome and consists of three exons and two introns; the entire open reading frame of the gene is situated in the third exon. Zhang et al. reported that an rs5193/rs5194 haplotype in *AGTR2* is associated with a cardioprotective role in Cantonese patients with essential hypertension (Zhang et al., 2006).

A commonly occurring AT_2R polymorphism, rs1403543, designated as either -1332 G/A or +1675 G/A (Alfakih et al., 2004; Erdmann et al., 2000) is, however, the most frequently studied SNP in hypertrophy association studies. This polymorphism is located at a lariat branch-point in the first intron, 29 bp before exon 2, in a region that is important for transcriptional activity (Erdmann et al., 2000; Warnecke et al., 2005). Nishimura et al. postulated that this polymorphism is functional and may affect pre-mRNA splicing (Nishimura et al., 1999), although a later study provided evidence that it modulates AT_2R protein expression, but not mRNA splicing (Warnecke et al., 2005). Warnecke et al. concluded that +1675 G/A is associated with increased AT_2R protein levels, which may be protective in LVH development (Warnecke et al., 2005).

In a study of 60 normotensive and 60 untreated, mildly hypertensive students at a Bavarian university, Schmieder and colleagues found that the +1675 A-allele is significantly associated with an increase in echocardiographically determined LVM and relative left ventricular wall thickness in young, mildly hypertensive males (Schmieder et al., 2001). The +1675 A-allele was additionally associated with LVH in males aged 55–74 from the Glasgow Heart Scan Old (GLAEOLD) cohort (Herrmann et al., 2002). These findings were, however, not replicated in the similar, but larger Glasgow Heart Scan (GLAECO) cohort, and the authors subsequently concluded that further research into the role of *AGTR2* in LVH was needed (Herrmann et al., 2002).

In contrast, Alfakih et al. reported an association between +1675 G-allele and LVM_{index} as determined with magnetic resonance imaging (MRI) in patients with systemic hypertension (Alfakih et al., 2004). MRI is credited with being a more reliable measure of cardiac hypertrophy than M-mode and 2D-echocardiography, even though echocardiography is still widely used to estimate LVM as it is more readily available as a clinical tool (Myerson et al., 2002).

Deinum et al. investigated the effect of an *AGTR2* +3123 A/C polymorphism (rs11091046) on LVH in 103 unrelated HCM patients (Deinum et al., 2001). Multiple regression analysis showed that the *AGTR2* +3123 C-allele decreased LVM_{index} in female subjects, independent of plasma renin, the +1166 A/C *AGTR1* variant or the I/D *ACE* variant. However, this study of ungenotyped cases did not account for differences in the primary HCM causal mutation, which may have had confounding effects on the association. A later study reported a significant association between the +1675 A-allele and decreased LVH in a HCM family cohort, independent of the primary HCM causal mutation, blood pressure, age, sex, BSA, heart rate and hypertension diagnosis (Carstens et al., 2011).

1.11 Aldosterone synthase (*CYP11B2*)

Recent studies in perfused hearts and cultured cells reported increased cardiomyocyte contractile force and cardiomyocyte hypertrophy in response to aldosterone administration (Barbato et al., 2004; Okoshi et al., 2004; Sato and Funder, 1996). Serum aldosterone was, furthermore, significantly related to LVM, as well as septal and posterior wall thickness in a population based sample of 615 middle-age subjects (Schunkert et al., 1997). Tsybouleva and co-workers showed that myocardial aldosterone levels were increased by 4.5-fold in HCM hearts, when compared to healthy donor hearts, and that mRNA levels from the aldosterone synthase-encoding gene, *CYP11B2,* increased 7-fold in HCM (Tsybouleva et al., 2004).

The -344T/C polymorphism in the 5' promoter region of the *CYP11B2* was significantly associated with levels of plasma aldosterone in a cohort of 216 patients with essential hypertension, after adjustment for age and 24-hour urine Na^+-excretion (Pojoga et al., 1998). This polymorphism also predicted statistically significant variations in left ventricular diameter and LVM, independent of sex, body size, blood pressure, physical activity, smoking, and ethanol consumption in 84 healthy Caucasians (Kupari et al., 1998). The -344T/C polymorphism resides in a putative binding site for steroidogenic transcription factor-1 (SF-1); the -344 C-allele is associated with a four-fold increase in SF-1 binding and could, therefore, possibly influence gene expression (White et al., 1999). Bassett et al. confirmed that the C-allele binds SF-1 more strongly that the T-allele, but added that SF-1 failed to stimulate *CYP11B2* expression *in vivo* (Bassett et al., 2002).

A study on two larger cohorts from the MONICA study did, however, not replicate these associations (Schunkert et al., 1999b). A later meta-analysis (that included data from these, as well as a number of subsequent studies) investigated the effect of this polymorphism on left ventricular structure-related phenotypes (Sookoian et al., 2008). The -344T/C variant was not

significantly associated with LVM or interventricular septal wall thickness in a pooled sample of 2157 unrelated subjects, although hypertensive subjects (n = 332) homozygous for the T-allele showed a 6.9% increase in LVM, compared to CC-homozygous subjects (Sookoian et al., 2008). Posterior wall thickness was 2.4% lower in homozygous CC individuals in a subject pool of 1994 from 10 homogeneous studies; this effect was increased to 11% when only hypertensive individuals were analysed (Sookoian et al., 2008).

While this meta-analysis only included data from unrelated people in case-control study designs, another study investigated the effect of this promoter variant, as well as five other *CYP11B2* polymorphisms (singularly and in combined haplotypes) on heart size, in 955 members from 229 British families (Mayosi et al., 2003). The additional five variants included an intron 2 conversion (I2C) polymorphism, in which intron 2 of the neighbouring 11 beta-hydroxylase gene (*CYP11B1*) gene has been transferred to *CYP11B2,* as well as four other SNPs in the coding region (A2713G, A4550C, T4986C and G5937C). While the authors found no association with the -344T/C SNP, they reported a significant association between G5937C and interventricular septal thickness, whereas the I2C and A4550C polymorphisms associated with left ventricular cavity size. Measured haplotype analyses confirmed the association of I2C and G5937C polymorphisms with interventricular septal thickness, and alleles at the I2C polymorphism with left ventricular cavity size. These *CYP11B2* polymorphisms contributed to 2.4 and 2.0–3.4% of the variability in septal wall thickness and left ventricular cavity size, respectively (Mayosi et al., 2003).

Two independent studies later reported strong LD between *CYP11B2* polymorphisms and variants in the neighbouring *CYP11B1* gene (Ganapathipillai et al., 2005; Keavney et al., 2005). The enzyme encoded by the latter gene catalyses the final step in cortisol biosynthesis. Studies later showed that aldosterone synthesis is highly heritable and is affected by genotypes at *CYP11B2,* as well as *CYP11B1* (Imrie et al., 2006; Alvarez-Madrazo et al., 2009). The association found between *CYP11B2* polymorphisms and cardiovascular phenotypes could, therefore, perhaps be explained (or influenced) by LD across the CYP11B1/B2 locus, but more studies are needed to fully explore this notion.

Patel et al. reported that *CYP11B2* −344T/C genotypes did not influence LVM or interventricular septal thickness in 142 genetically-independent echocardiographically-diagnosed HCM patients (Patel et al., 2000). However, Ortlepp et al. later found that this polymorphism significantly associated with both of these hypertrophy phenotypes after age, sex and the presence of hypertension was controlled for, in a single family with HCM caused by a previously identified

myosin binding protein C (MyBPC) mutation (Ortlepp et al., 2002). In contrast to the study by Tsybouleva et al., Chai et al. reported that plasma and left ventricular tissue levels of aldosterone in 79 genetically-independent subjects with HCM were not significantly different from those in age-matched controls (Chai et al., 2006). They reported a significant association between the *CYP11B2* −344T-allele and LVM_{index}, as well as interventricular septal thickness in men, but not in women. Multiple regression analysis showed that the effect of the −344T-allele on interventricular septal thickness occurred independently of renin or the *ACE* I/D, *AGTR1* +1166 A/C or *AGTR2* +3123 A/C polymorphisms (Chai et al., 2006).

These studies did, however, not investigate the effect of the entire CYP11B1/B2 locus on hypertrophy indices in HCM and more research is thus needed to fully elucidate the contribution of CYP11B1/B2 variants to hypertrophy development in general, an in HCM in particular.

1.12 Mineralocorticoid receptor (MR)

The MR, encoded by *NR3C2*, is responsible for the downstream RAAS functions, as it binds to aldosterone to form an MR/aldosterone complex, which, in turn, activates aldosterone-induced early and late response gene transcription and signalling cascades to mediate cellular Na^+ homeostasis via its downstream effectors. The MR is a member of the steroid/thyroid/retinoid/orphan receptor family of transcription factors and has been identified in cardiac tissue in previous studies (Lombes et al., 1995). Functional polymorphisms in *NR3C2* have been shown to result in a rare condition called pseudohypoaldosteronism type I, which is characterized by unresponsiveness to aldosterone, severe salt wasting, extreme hyperkalaemia and elevated PRA (Edelheit et al., 2005; Geller et al., 1998).

Interestingly, through its binding to the MR, aldosterone is able to directly induce cardiomyocyte hypertrophy (Le Menuet et al., 2004; Yoshida et al., 2010). Nagata et al. investigated the effect of MR-blockade on cardiac hypertrophy in rats with salt-sensitive hypertension and concluded that MR-blockade attenuates LVH, in the absence of an antihypertensive effect (Nagata et al., 2006). Correspondingly, the MR antagonist spironolactone attenuated LVH in uremic rats without a significant reduction in blood pressure (Michea et al., 2008). Several further animal studies, including some using HCM model animals, illustrated beneficial cardiovascular effects with cardiomyocyte-specific MR blockade (Fraccarollo et al., 2011; Lother et al., 2011).

Overexpression of the human MR in mice resulted in mild dilated cardiomyopathy in conjunction with a significant increase in heart rate, while blood pressure levels remained unchanged (Ouvrard-Pascaud et al., 2005). Tsybouleva and colleagues investigated the effect of MR blockade in a transgenic mouse model of human HCM (cTnT-Q92). They were able to demonstrate that MR blockade with spironolactone reduced myocyte disarray and interstitial fibrosis, and also improved diastolic function; they concluded that aldosterone, through the MR, significantly affects the relationship between sarcomeric dysfunction and the cardiac phenotype of HCM (Tsybouleva et al., 2004). Similar approaches were, however, not as effective in feline HCM models (Force et al., 2010; Taillefer and Di Fruscia, 2006). One study speculated that this might be due to inadequate dosage of RAAS inhibitors as more research is needed on the dosage required to adequately disrupt the RAAS in feline models (Taillefer and Di Fruscia, 2006).

In another transgenic mouse model of cardiac hypertrophy, Zhang et al. show that conditional and cardiomyocyte-restricted overexpression of the human MR promoted LVH and diastolic, but not systolic, dysfunction through redox-dependent, blood pressure–independent effects in response to Ang II infusion (Di Zhang et al., 2008). MR blockade with canrenoate was shown to decrease the expression of TGF-β and significantly reduced LVH and cardiac fibrosis in these mice, which is consistent with earlier studies where MR blockade reduced hypertrophy indices determined with MRI in transgenic rats (Stas et al., 2007).

In humans, MR antagonists were shown to reduce ventricular remodelling, sudden cardiac death and myocardial fibrosis in the randomized aldactone evaluation study (RALES) and in the eplerenone post-acute myocardial infarction efficacy and survival study (EPHESUS) clinical trials. These effects were also independent of the antagonist's effect on blood pressure, providing evidence that MR blockade offers cardioprotective effects in patients with heart failure and systolic left ventricular dysfunction (Pitt et al., 1999; Pitt et al., 2003). The MR blocker spironolactone is currently still being investigated as an experimental therapy option in HCM, as small preliminary studies showed beneficial cardiovascular effects, but more research is essential in this regard (Marian, 2009).

Previous reports show a significant association between *NR3C2* polymorphisms and blood pressure control in hypertensive populations (Geller et al., 2000; Martinez et al., 2009; Van Leeuwen et al., 2010), but there is currently a lack of information on the involvement of *NR3C2* variants in both overload-induced and blood pressure-independent hypertrophy. One possible explanation for the lack of knowledge in this regard is the size of the MR gene, as it spans about 360 kb and contains 10 exons. A minimum of about 20 SNPs is needed to at least capture the

main haplotypes for a given population in this gene through linkage disequilibrium unit (LDU)-based density, as well as tag SNP selection.

1.13 11β-HSD2

The MR is able to bind both aldosterone and cortisol, but under normal circumstances it is protected from cortisol occupancy by the short-chain dehydrogenase/reductase 11β-HSD2, which degrades cortisol to corticosterone, which is then unable to bind to the MR (Farman and Bocchi, 2000). Null mutations in this gene causes apparent mineralocorticoid excess (AME), a rare form of congenital hypertension, characterised by severe hypertension, hyperkalaemia and low aldosterone levels, as well as associated end-organ complications such as renal or cardiovascular damage (Dave-Sharma et al., 1998).

Transgenic mice overexpressing 11β-HSD2 in cardiomyocytes were normotensive, but spontaneously developed cardiac hypertrophy, fibrosis, and heart failure and died prematurely on a normal salt diet (Qin et al., 2003). In addition, the MR blocker spironolactone improved adverse left ventricular remodelling in rats by modulating MR and 11β-HSD2 expression levels (Takeda et al., 2007). Recently, Bailey et al. showed that *HSD11B2-null* heterozygote mice, which express only 50% of normal enzyme levels, develop salt-sensitive hypertension (Bailey et al., 2011). These mice exhibited a salt-induced increase in heart:body weight ratio, which was partially reduced by spironolactone (Bailey et al., 2011).

11β-HSD2 is consequently an attractive candidate gene for hypertension in humans and polymorphisms in this gene have been associated with blood pressure regulation in several populations (Ferrari, 2010). However, little is known about the role of this gene in hypertrophy development, although one study reported a correlation between urinary 11β-HSD2 activity and LVM in essential hypertension (Glorioso et al., 2005).

1.14 Downstream RAAS effectors

Various studies have implicated altered intracellular Na^+ in cardiac hypertrophy (Gu et al., 1998; Verdonck et al., 2003). Thus far, we have discussed the indirect (blood pressure-dependent), as well as the direct hypertrophic effect of certain RAAS components on hypertrophy in HCM. Another potential mechanism whereby RAAS genes can alter the HCM hypertrophic phenotype is by altering the balance of intracellular Na^+, which plays a crucial role in the structural, mechanical, and electrical properties of the myocardium. The MR/aldosterone complex exerts its effects on Na^+ homeostasis via the ENaCs, as well as via Na^+/K^+-ATPase.

1.14.1 Na^+/K^+-ATPase

The Na^+/K^+-ATPases catalyze the exchange of Na^+ and K^+ ions across plasma membranes and are essential downstream effectors of the MR/aldosterone complex. The Na^+/K^+-ATPase is a heteromeric protein that consists of α- and β-subunits (Kaplan, 2002) (Figure 1.4). The α-subunit is a polytopic membrane protein that confers the catalytic activity of the enzyme and contains binding sites for Na^+, K^+ and ATP (Shull et al., 1985). The β-subunit modulates the pump function and is important for the efficient translation of the α-subunit on the endoplasmic reticulum, membrane insertion and correct folding of the α-subunit, as well as the expression of the enzyme on the plasma membrane (Rajasekaran et al., 2005).

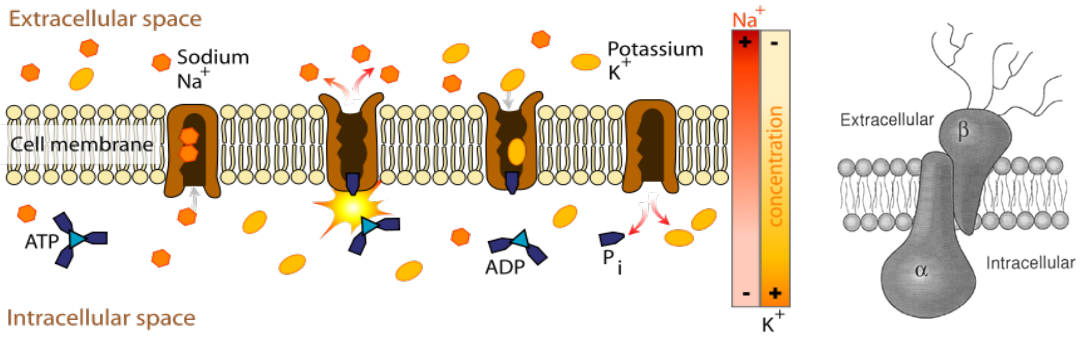

Figure 1.4 Intracellular organisation of Na^+/K^+-ATPase subunits and function. A. *Graphic illustration of Na^+/K^+-ATPase function (http://upload.media.org). **B.** Graphic illustration of Na^+/K^+-ATPase subunits, indicating the intracellular organization of the α- and β-subunits (www.ttuhsc.edu/.../Pressley/Pressley.aspx).*

To date, four isoforms of the α-subunit (α1, α2, α3 and α4) and three isoforms of the β-subunit (β1, β2 and β3) have been described in mammals, which exhibit tissue-specific expression (Blanco and Mercer, 1998). Previous studies have confirmed the expression of the α1, α2, α3, β1, β2 and β3-subunits in human hearts (Malik et al., 1998; Schwinger et al., 1999; Wang et al., 1996). Studies in animal hypertrophy models have additionally demonstrated that the expression of Na^+/K^+-ATPase α- and β-subunit isoforms are altered in hypertrophied ventricles (Baek and Weiss, 2005; Xie et al., 1999; Yamamoto et al., 2009; Zwadlo and Borlak, 2005).

In the first report on Na^+/K^+-ATPase isoform expression in the myocardium of normal and failing human hearts, Allen et al. reported that none of the three α-subunits showed altered expression in the left ventricles of failing hearts (Allen et al., 1992). Later, Shamraj et al. reported that Na^+/K^+-ATPase isoform expression is indeed altered in failing compared to

nonfailing human hearts (Shamraj et al., 1993); this finding was subsequently corroborated by other groups (Muller-Ehmsen et al., 2001; Schwinger et al., 1999).

The Na^+/K^+-ATPase also serves as a receptor for ouabain and other related cardiac glycosides. Previous investigations have established that the positive inotropic effects of cardiac glycosides on the myocardium is due to partial inhibition of the cardiac Na^+/K^+-ATPase, which causes a small increase in intracellular Na^+ and, in turn, affects the Na^+/Ca^{2+} exchanger, ultimately leading to increased intracellular Ca^{2+} and force of contraction (Akera and Ng, 1991; Huang et al., 1997). Fedorova et al. reported that the development of LVH and subsequent transition to heart failure in Dahl salt-sensitive rats on a high Na^+ diet was associated with shifts in left ventricular Na^+/K^+-ATPase isoform composition and sensitivity to ouabain (Fedorova et al., 2004).

Huang et al. demonstrated that partial inhibition of the Na^+/K^+-ATPase by ouabain in cultured neonatal rat cardiomyocytes induced hypertrophic growth, which was coupled with increased expression of TGF-β and other late response markers indicative of cardiac hypertrophy (Huang et al., 1997). Subsequent studies by the same group revealed that the hypertrophic response that follows Na^+/K^+-ATPase inhibition was also associated with p42/44 MAPK and ROS-dependent pathways (Kometiani et al., 1998; Xie et al., 1999).

Animal knockout-models suggest differential roles for α- and β-subunits in the heart. The hearts of heterozygous Na^+/K^+-ATPase α1-knockout mice were hypocontractile, while the hearts of heterozygous α2-knockout mice were hypercontractile as a result of increased Ca^{2+} transients during the contractile cycle (James et al., 1999). In contrast, homozygous Na^+/K^+-ATPase β2-knockout mice exhibited ventricular hypertrophy without any diminution of Na^+/K^+-ATPase enzymatic activity (Magyar et al., 1994). Homozygous knockout mice in which the Na^+/K^+-ATPase β1 gene was inactivated exclusively in the ventricular cardiomyocytes exhibited mild hypertrophy, coupled with reduced contractility and ventricular function in aging mice (Barwe et al., 2009).

Polymorphisms in the α1-subunit gene (*ATP1A1*) (Glorioso et al., 2007; Rice et al., 2000), as well as in the β1-subunit gene (*ATP1B1*) gene (Chang et al., 2007; Xiao et al., 2009), were previously associated with essential hypertension. *ATP1B1* variants have also been associated with QT-intervals in long-QT patients (Pfeufer et al., 2009). However, the effect of genetic variation in the Na^+/K^+-ATPase subunit isoforms on cardiovascular hypertrophy remains unclear.

1.14.2 Amiloride-sensitive epithelial sodium channels (ENaCs)

The amiloride-sensitive epithelial sodium channels (ENaCs) are also important regulators of intracellular Na^+, as they are responsible for the electrodiffusion of Na^+ through epithelial cells. Each ENaC consists of three homologous subunits, i.e. an α-, β-, and a γ-subunit (Canessa et al., 1994), encoded by the *SCNN1A, SCNN1B,* and *SCNN1G* genes, respectively (Figure 1.5).

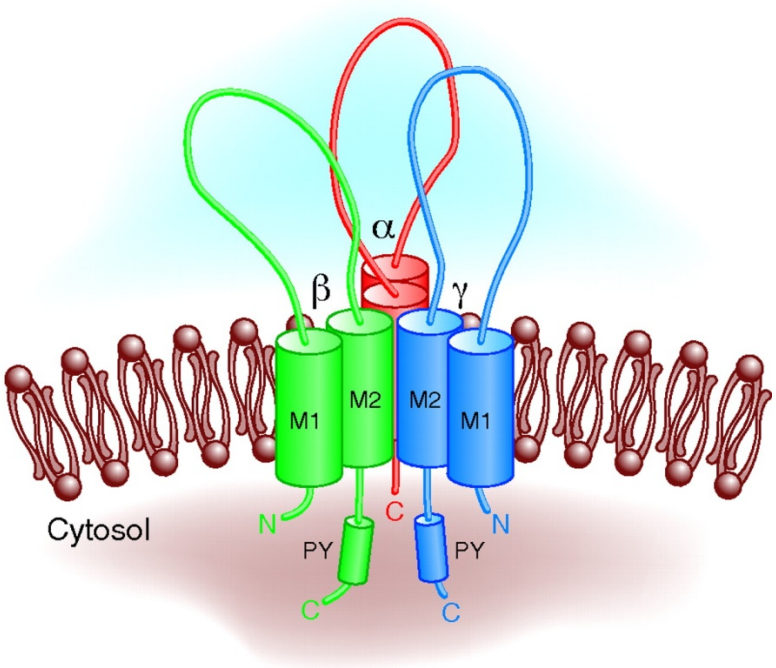

Figure 1.5 Structural features of the Epithelial Sodium Channel (ENaC). *Illustration indicates the three homologous α-, β- and γ- protein subunits that cooperate to form the channel pore via the transmembrane domains. Each subunit has two membrane-spanning domains (M1 and M2) with intracellular N- and C-termini. From Bhalla and Hallows (2008).*

Gain-of-function mutations in ENaC subunit genes can cause a rare condition called Liddle syndrome. These mutations prevent the degradation of ENaCs, resulting in excessive Na^+-absorption, K^+-wasting, systemic hypertension, as well as an elevated incidence of early cardiovascular disease and LVH (Hansson et al., 1995; Jeunemaitre et al., 1997; Rossi et al., 2011).

The association of the ubiquitin ligase Nedd4-2 with an ENaC leads to ubiquitination of the ENaC and its subsequent removal from the plasma membrane. Nedd4-2 is, therefore, vital to the activity and regulation of ENaCs. In a study on Nedd4-2-knockout mice, Shi and colleagues demonstrated that these mice had elevated blood pressure and impaired ENaC activity, which was aggravated by a high salt diet (Shi et al., 2008). Ultimately, these animals developed cardiac hypertrophy and systolic dysfunction (Shi et al., 2008).

The role of ENaC mutations in hypertension is well-documented and the antihypertensive properties of the ENaC inhibitor, amiloride, have long been known (Spence, 2010; Su and Menon, 2001). However, studies in animal models of hypertension have shown that low doses of amiloride can reduce LVH and other cardiovascular pathologies, despite the fact that blood pressure remained high (Ji et al., 2003; Mirkovic et al., 2002). These effects occurred independently of changes on serum K^+. These and other studies highlight the potential cardiovascular benefit of ENaC inhibition (Teiwes and Toto, 2007).

Interestingly, heterozygous α-ENaC-knockout mice appear to have an intact capacity to maintain blood pressure and Na^+ balance despite varying salt diets (Wang et al., 2001). This ability to maintain normal blood pressure margins appears to be caused by increased RAAS activity through a compensatory upregulation of AT_1Rs (Wang et al., 2001). In a study of rats on a salt-restricted diet, the AT_1R antagonist candesartan markedly decreased the quantity of ENaC α-subunits, which have been shown to be rate-limiting for assembly of mature ENaC complexes (Beutler et al., 2003). Candesartan administration was also found to increase the abundance of ENaC β- and γ-subunits in these rats (Beutler et al., 2003).

These studies point towards a definite interplay between the ENaCs and RAAS components. Further studies are, however, needed to fully explore the nature of this interaction in pathological hypertrophy *per se,* as well as within the context of HCM. Studies are additionally needed to investigate the effect of genetic variation in ENaC subunit genes on hypertrophy development.

1.15 Complexity of the RAAS

It is then evident that almost all RAAS components have a plausible impact on hypertrophy development, whether due to hemodynamic changes, or completely independent from systemic blood pressure. However, several gaps exist in our knowledge of the involvement of the RAAS in HCM hypertrophy. For now, HCM modifier studies appear to have converged on a very limited list of "pro-LVH" polymorphisms, however, renin and renin-associated genes, as well as downstream RAAS effectors such as the Na^+/K^+-ATPase and ENaCs, have plausible hypertrophic effects, but their contribution to modifying the hypertrophic phenotype in HCM has received little attention. Moreover, there exists a very real possibility that genetic variation in these genes might not contribute in an equal or even additive manner to hypertrophy in HCM; to complicate matters further, the functionality of most of these polymorphisms remains unclear.

Previous studies have reported modest effect sizes for RAAS variants on hypertrophy (Carstens et al., 2011; Lechin et al., 1995; Mayosi et al., 2003; Van der Merwe et al., 2008), while others reported that RAAS polymorphisms collectively influence the penetrance and extent of hypertrophy in HCM (Kaufman et al., 2007; Ortlepp et al., 2002; Perkins et al., 2005). This suggests that the hypertrophic phenotype of HCM is modulated by the compound effect of a number of hypertrophic RAAS modifier loci, where each polymorphism makes a modest contribution to the eventual phenotype.

However, genes involved in other pathways such as Ca^{2+} dysregulation and myocardial energetics have also been shown to impact on the hypertrophic phenotype of HCM (Ho, 2010c; Tsoutsman et al., 2006), while the contribution of environmental factors to hypertrophy development cannot be ignored. When we consider all the potential hypertrophy modifiers, HCM falls in the region of complex diseases, despite its monogenic origin as shown in Figure 1.6.

Complex phenotypes are the result of interplay between environmental factors and several loci of weak or moderate effect acting in an additive or interactive manner. Bearing in mind the multitude of possible HCM modifiers, the additional contribution of gene-gene and gene-environment interactions to the complex HCM phenotype, single SNP and even single gene association studies that aim to elucidate RAAS involvement in HCM appear to be overly simplistic and difficult to interpret clinically. Considering that RAAS hypertrophy modifier loci probably do not act in isolation, while functional mechanisms are lacking for most variants and more importantly, the frequent lack of adjustment for known hypertrophy confounders in RAAS association results, the question simply remains: “how do we interpret this and what does it imply?”.

However, the clinical relevance of this information might improve significantly if one considers the composite effect of a number of RAAS polymorphisms, where each contributes a modest amount to the eventual hypertrophic phenotype. On the other hand, the compound effect of these RAAS variants on the HCM phenotype might not necessarily be additive in nature due to the influence of epistasis where the effects of one locus is amplified, altered or masked by another locus (Cordell, 2002). It is conceivable that such an interaction should exist between RAAS variants due to their complex biological functions and such epistatic effects have been reported between RAAS variants in cardiovascular phenotypes such as atrial fibrillation (Tsai et al., 2004), coronary atherosclerosis (Ye et al., 2003), hypertension (Williams et al., 2004) and coronary artery disease (Tsai et al., 2007).

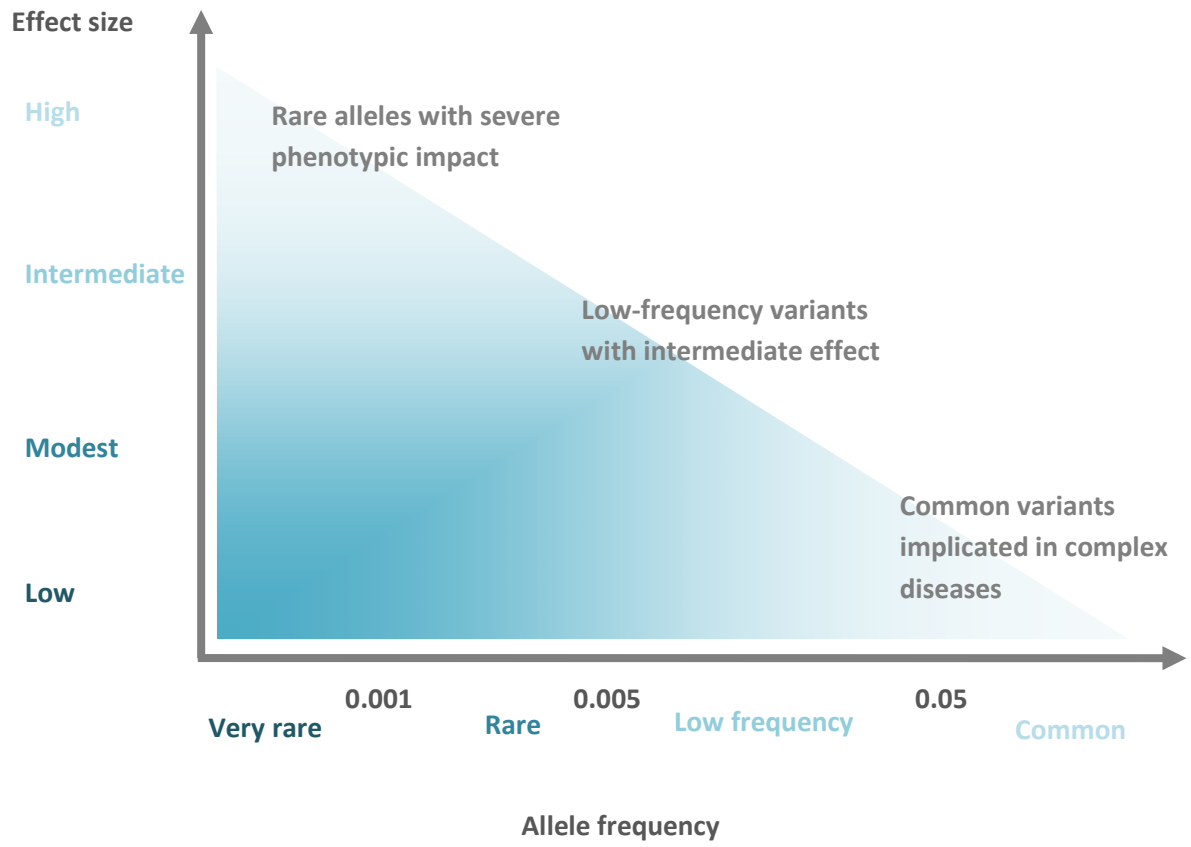

Figure 1.6 The spectrum along which genetic variation contributes to disease phenotypes. *The majority of complex disease susceptibility and modifier loci are likely to be common variants with low impact, while Mendelian (monogenic) disorders are caused by rare variants with high phenotypic impact. Redrawn with modifications from Manolio et al. (2009).*

As there is a complex biological interplay between the multiple components of the RAAS, there is a need for a comprehensive analysis that focuses on the compound effect of multiple hypertrophy modifier loci within the RAAS to gain the most accurate understanding of its role in hypertrophy development in HCM. Comprehensive studies are, furthermore, needed to pinpoint a subset of RAAS variants that confer a measurable biological effect on hypertrophy to improve risk stratification in HCM patients. The use of haplotypes offers an advantage over single SNP association approaches to elucidate the molecular underpinnings of complex phenotypes, such as cardiac hypertrophy, by providing additional power for mapping disease modifier genes while factoring in the interdependency among genetic markers studies as it considers the composite effect of a number of loci (Clark, 2004; Liu et al., 2008).

In summary, it is now clear that there exists an important cross-talk between Ang II/AT_1R and aldosterone/MR, which results in activation of signalling pathways involved in cell growth,

contraction, and collagen deposition (Montezano and Touyz, 2008). These processes are of particular interest in the heart where this interdependency significantly modulates hypertrophy and adverse cardiac remodelling. Considering that a number of RAAS variants contribute significantly to LVH indices, albeit each with modest effect sizes, and considering the growing evidence for epistasis among these variants, it becomes imperative to rethink the way we analyze the involvement of the RAAS in cardiac structure. Indeed, the contribution of all of the components of the RAAS, which often signal in opposite directions, rather than only selected components, should be considered if we want to understand the role of this complex system in cardiac remodelling, as otherwise there remains a risk of overstating, or indeed understating, the effect of this pathway on hypertrophy development.

1.16 The present study

This study constitutes the final component of a larger investigation in which the ultimate aim is to identify hypertrophy-modifying genes within the RAAS using South African HCM founder families. Two substudies were previously reported for this project (Cloete REA, M.Sc and Carstens N, M.Sc). Both these studies reported association between particular variants in RAAS genes and heritable hypertrophy traits in this cohort, while the second study (Carstens N, M.Sc) reported preliminary evidence for epistasis between a further subset of RAAS genes. Since then, five new families were added to the HCM founder cohort during the course of this study due to continued recruiting efforts.

In addition, not all of the genes within the RAAS pathway were covered by these earlier studies. Specifically, the mannose-6-phosphate receptor (*M6PR*), MR (*NR3C2*) and 11-β-hydroxysteroid dehydrogenase (*HSD11B2*) remained to be investigated to ensure comprehensive coverage of the RAAS. The *M6PR* acts as a receptor for renin, which had been shown to be significantly associated with hypertrophy traits in this cohort in a previous study (Carstens N, M.Sc). The MR was previously implicated in hypertrophy development and acts as a receptor for aldosterone, while *HSD11B2* ensures its specificity by inhibiting the binding of cortisol.

Recent studies have reported strong evidence for LD between *CYP11B2* polymorphisms and variants in the neighbouring *CYP11B1* gene (Ganapathipillai et al., 2005; Keavney et al., 2005) and that aldosterone synthesis is influenced by genetic variants in both these genes, as explained in an earlier section. This allows for the possibility that the association previously found between *CYP11B2* polymorphisms and cardiovascular phenotypes could be due to (or influenced by) LD across the CYP11B1/B2 locus (Cloete REA, M.Sc). This possibility was, however, not explored in our previous investigation (Cloete REA, M.Sc).

Moreover, the RAAS represents a perfect example of how the compound effect of genetic variations in each component of a complex enzymatic cascade could have a synergistic and dynamic effect on the eventual activity of a system. More to the point, a need exists for investigations that explore the individual, as well as the cumulative contribution of RAAS variants to hypertrophy in HCM, while adjusting for known hypertrophy confounders.

The present study, therefore, aimed to contribute to the current knowledge of the involvement of RAAS genes in hypertrophy development by investigating the individual, as well as the compound effects of RAAS variants on hypertrophy in a family-based HCM cohort. To this end, we genotyped the entire HCM founder cohort for prioritised variants in the *M6PR*, *NR3C2* and *HSD11B2* genes, as well as additional variants to cover the CYP11B1/B2 locus. Moreover, the 35 additional family members added to the cohort since the end of the previous studies were genotyped for all RAAS variants prioritised in the previous RAAS investigations.

Quantitative measures of the hypertrophic phenotype, such as echocardiographically determined maximal left ventricular wall thickness, a variety of cardiac wall thickness indices and LVM, were tested for association with these single polymorphisms, with adjustment for known hypertrophy covariates and environmental and polygenic variance components. Thereafter, as it is not known what functional variants may exist within these genes in the study population, haplotypes were constructed from all these genotypes in order to capture variability across the genes. Linear mixed-effects models were subsequently used to assess association between these haplotypes and heritable hypertrophy traits at gene-level. Such models allow adjustment for the various known confounders, as well as for the specific relatedness between family members in estimating the various variance components (environment, polygenic, specific genes).

Chapter 2

Materials and Methods

CHAPTER 2

MATERIALS AND METHODS

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2.1 Study Subjects

The University of Stellenbosch 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 cases.

In the process, 27 probands carrying one of three mutations that occur as founder mutations in South Africa, i.e. R92W in *TNNT2*, R403W in *MYH7*, and A797T in *MYH7* were identified. These founder mutations have previously been described within a South African population (Moolman-Smook et al., 1999).

Pedigree tracing was performed for these index individuals and all family members older than 18 years were asked to participate in this modifier gene study. Thus, a panel of 388 individuals from 27 families, which included genetically and clinically affected and unaffected family members, was identified; these individuals were all screened for the presence or absence of all three founder mutations (Table 2.1). Of these, 22 families have been described previously (Carstens et al., 2011; Revera et al., 2008), while 5 new families were added to the cohort since the last report, due to continued recruiting efforts.

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

Pedigree	Ethnic group	Gene	Mutation	HCM mutation-carriers	Non-carriers	Total number of individuals
101	Caucasian	*MYH7*	A797T	12	9	21
104	Mixed Ancestry	*MYH7*	A797T	7	7	14
123	Mixed Ancestry	*MYH7*	A797T	6	10	16
124	Caucasian	*MYH7*	A797T	1	4	5
131	Caucasian	*MYH7*	A797T	12	11	23
138	Caucasian	*MYH7*	A797T	13	17	30
145	Mixed Ancestry	*MYH7*	A797T	3	2	5
147	Mixed Ancestry	*MYH7*	A797T	4	5	9
158	Caucasian	*MYH7*	A797T	2	3	5
159	Mixed Ancestry	*MYH7*	A797T	5	7	12
163	Caucasian	*MYH7*	A797T	6	3	9
172	Caucasian	*MYH7*	A797T	7	10	17
177	Caucasian	*MYH7*	A797T	3	1	4
180	Caucasian	*MYH7*	A797T	4	1	5
190	Caucasian	*MYH7*	A797T	2	1	3
106	Mixed Ancestry	*MYH7*	R403W	29	35	64
134	Mixed Ancestry	*MYH7*	R403W	4	7	11
157	Mixed Ancestry	*MYH7*	R403W	1	3	4
100	Mixed Ancestry	*TNNT2*	R92W	16	30	46
103	Mixed Ancestry	*TNNT2*	R92W	2	3	5
109	Mixed Ancestry	*TNNT2*	R92W	6	4	10
139	Mixed Ancestry	*TNNT2*	R92W	15	22	37
137	Mixed Ancestry	*TNNT2*	R92W	2	5	7
149	Mixed Ancestry	*TNNT2*	R92W	4	6	10
173	Mixed Ancestry	*TNNT2*	R92W	2	1	3
179	Mixed Ancestry	*TNNT2*	R92W	5	4	9
188	Mixed Ancestry	*TNNT2*	R92W	3	1	4
						388

Abbreviations: ***MYH7*:** myosin heavy chain gene 7; ***TNNT2*:** troponin T gene 2

2.2 Blood collection and DNA extraction

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

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

2.3 Clinical Investigations[2]

All participating individuals were clinically characterised by an experienced echocardiographer who was blinded to the mutation status of each subject. A total of 16 2D-echocardiographic measurements of wall thickness were taken at the mitral valve, papillary muscle and apex levels, in accordance with the recommendations of the American Society of Echocardiography (Schiller et al., 1989), as described previously (Revera et al., 2008).

The echocardiographic measurements for the majority of the cohort (353 individuals) were performed by Dr Miriam Revera from Pavia University (Italy) using a GE Healthcare Vivid7 cardiovascular ultrasound system and echocardiographic measurements for the 35 newly recruited individuals were performed by Lenore Naidoo using a portable Siemens Acuson Cypress Ultrasound System. The inter-observer variability between the two echocardiographers was negligible. Analyses included echocardiographic recordings in M-mode, 2D and Doppler blood-flow imaging using a 2.5 Hz transducer in standard parasternal long-axis and short-axis, apical four- and two-chamber views. To determine the maximum LVWT, posterior wall thickness (mPWT) and interventricular septal thickness (mIVST), the heart muscle thickness was measured at three levels, namely, mitral valve, papillary muscle and supra-apex level (Figure 2.1). 2D-echocardiographic measurements were performed in six segments of the LV wall at the mitral valve and papillary muscle levels and in four segments at the smaller supra-apex level, therefore, a total of 16 segments were measured.

[2] These methods were also described in Carstens et al. (2011)

The six measurements at the mitral valve and papillary muscle levels were taken at the anterior IVS, posterior IVS and anterior wall (AW), lateral wall (LW), inferior wall (IW) and posterior wall (PW) of the left ventricle. Assessment of the supra-apex level consisted of segments IVS, AW, LW and PW as per four chamber view.

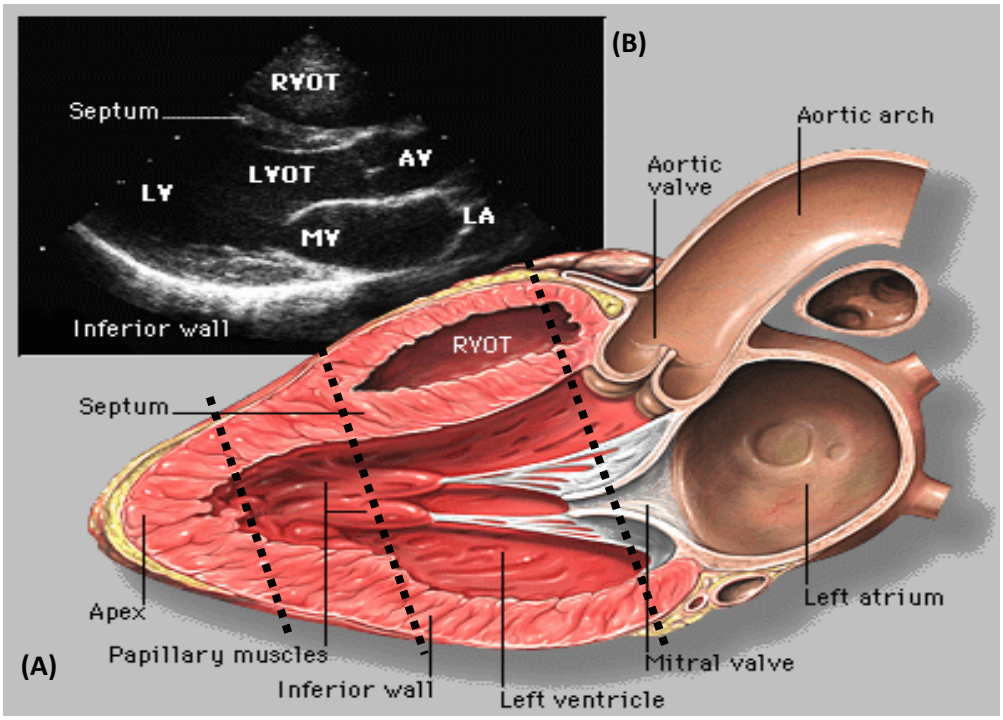

Figure 2.1 Graphical representation of the three levels at which heart muscle thickness was assessed. A) *Long-axis view of left ventricle, taken at level of mitral valve, papillary muscles, as well as just above apex (levels indicated by dotted lines).* ***B)*** *An example of a 2D echocardiographic 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.*

Blood pressure measurements were taken twice in the sitting position, after 5 min of rest, and the second measurement used. Subjects were identified as hypertensive if they were on anti-hypertensive medication or if they had a systolic blood pressure of more than 140 mm Hg or diastolic blood pressure of more than 90 mm Hg. Resting heart rate was derived from standard electrocardiography performed on a MAC1200ST after 5 min of rest. In addition, we recorded medical history and additional covariates of cardiac structure (age, sex and body surface area [BSA]) for each participant.

As both the extent and distribution of hypertrophy in HCM varies greatly, we sought methods of capturing the full variation of LVH in the cohort for use in the association analyses. Echocardiographically determined LVM was calculated to better describe the extent of hypertrophy than single wall-thickness measurements. LVM was calculated using the formula for the estimation of LVM from 2D LV linear dimensions recommended by the American Society of Echocardiography:

$$LVM = 0.8 \times \left(1.04\left[\left(LVIDd + PWTd + SWTd\right)^3 - \left(LVIDd\right)^3\right]\right) + 0.6g.$$

A novel cumulative wall thickness (CWT) score was, furthermore, determined by adding the 16 wall thickness measurements at all three levels of the heart. Furthermore, we used principal component analysis to statistically define a composite hypertrophy score that best described ventricle-wide hypertrophy in the present cohort. To this end, we used the 16 wall thickness measurements to generate the first principal component, which best represented the variability in hypertrophy seen in the present cohort.

2.4 Candidate gene and SNP Selection

SNPbrowser v 4.0.1 software was used to select SNPs to cover the *M6PR*, *NR3C2* and *HSD11B2* genes. SNPs were selected to achieve an even spacing of 0.5 linkage disequilibrium units (LDUs) on the metric LD map for the HapMap CEU and YRI populations (Table 2.4), while prioritising markers with a MAF of at least 0.05 (De La Vega, 2007). LDUs define a metric coordinate system where locations are additive and distances are proportional to the allelic association between markers (Maniatis et al., 2004). These LD maps are analogous to the genetic map expressed in centi-Morgans and can be used to efficiently position markers for population-based disease association studies (Collins et al., 2004).

SNPbrowser uses a set of metric LD maps build from the HapMap NCBI b36 assembly using the LDMAP software (Kuo et al., 2007). The LDMAP software places SNPs on an additive coordinate system, for instance, SNPs in perfect LD have no distance between them, while SNPs with no significant correlation are separated by over three LDUs on this map (De La Vega et al., 2006).

Additional variants were also selected to ensure adequate coverage of *REN*, as well as of the CYP11B1/B2 locus, which had not been fully covered in the previous studies. Table 2.2 depicts the polymorphisms selected for investigation in the present study as well as the respective methods used to obtain genotype data for each variant. Table 2.3 depicts variants that were genotyped during previous studies (Cloete REA, M.Sc; Carstens N, M.Sc) in the HCM founder cohort. TaqMan assays indicated with an asterisk (*) depicts assays used to determine genotypes on the 5 families added during the course of the present study.

Table 2.2 Genetic variants selected for investigation in the present study, as well as the respective methods used to genotype each polymorphism.

Gene	Chromosome	Chromosome position	Polymorphism	Genotyping method/ TaqMan assay ID
NR3C2	4q31	149209810	rs906124	C__1180128_20
		149216852	rs11933380	C__1180132_10
		149233654	rs745019	C___562966_10
		149261743	rs1403142	C__1796075_10
		149284142	rs13150372	C__31208809_20
		149302754	rs7694706	C__29125239_10
		149309538	rs6535584	C__29125233_10
		149352206	rs6535594	C__29125222_10
		149354044	rs7699349	C__26453333_10
		149357075	rs2883930	C__16145331_10
		149365276	rs4835508	C__3203903_10
		149383202	rs11945778	C__3203916_10
		149430127	rs3910047	C__3203934_10
		149448788	rs3846329	C__3203942_10
		149484695	rs2137334	C__1594435_10
		149565037	rs13118022	C__31208669_10
		149569977	rs4635799	C__1594397_10
REN	1q32	202391712	rs3795575	C__27517655_10
		202403564	rs6682082	C__30210733_10
CYP11B2	8q21-q22	143993985	I2C	(White and Slutsker, 1995)
		143990317	rs3097	C__11446379_10
CYP11B1	8q21-q22	143958007	rs6410	C__11609085_10
		143955429	rs6387	Custom designed TaqMan assay
		143953249	rs4310186	C__27915668_10
M6PR	12p13	9097933	rs1805725	C__2665100_10
		9102575	rs987917	C__7554849_10
HSD11B2	16q22	67469733	rs5479	C__11934935_10

Abbreviations: ***CYP11B1:*** 11 beta-hydroxylase gene; ***CYP11B2*****:** aldosterone synthase gene; ***HSD11B2*****:** 11 β-hydroxysteroid-dehydrogenase type 2 gene**;** ***M6PR***: mannose-6-phosphate receptor gene; ***NR3C2*****:** nuclear receptor subfamily 3, group C, member 2 gene; ***REN*****:** renin gene

Table 2.3 Genetic variants genotyped during previous studies (Cloete REA, M.Sc; Carstens N, M.Sc) in the HCM founder cohort, as well as in this study. *TaqMan assays indicated with an asterisk (*) depicts assays used to determine genotypes on the 35 individuals added during the course of the present study.*

Gene	Chromosome	Chromosomal Position	SNP	Genotyping method/ TaqMan assay ID
AGTR2[i]	Xq22-q23	115216220	rs1403543	C__7481825_10*
		115218858	rs5194	C__1841567_20*
		115219154	rs11091046	C__1841568_10*
RENBP[i]	Xq28	152864846	rs762656	C____13880_10*
		152860739	rs2269372	C__15876539_10*
		152849623	rs2269370	C__15876550_10*
ATP6AP2[i]	Xp11.4	40324378	rs2968915	C__15881558_20*
		40327762	rs2968917	C__15881550_10*
		40350712	rs10536	C__8789353_10*
ACE2[ii]	Xp22	15527984	rs1978124	ASREA
		15520269	rs2285666	ASREA
		15500728	rs879922	ASREA
		15494341	rs4646179	ASREA
REN[i]	1q32	202398933	rs10900555	C__31567082_10*
		202397809	rs5705	C__11451777_10*
		202397654	rs11571082	C__31567075_10*
		202395477	rs1464816	C__8687919_1_*
AGT[ii]	1q42-q43	228906719	rs1926723	SNaPshot
		228906892	rs11122575	SNaPshot
		228912417	rs699	SNaPshot
		228912600	rs4762	SNaPshot
		228916495	rs5051	SNaPshot
ATP1A2[i]	1q21-q23	158361751	rs7548116	C__1843215_10*
		158380967	rs11585375	C__31909450_10*
ATP1B1[i]	1q24	167345196	rs1200130	C__8919154_10*
		167346043	rs1358714	C__8919160_10*
		167352845	rs1040503	C__8919179_10*
ATP1A1[i]	1p21	116722542	rs10924074	C__3072256_10*
		116734086	rs850609	C__8696039_10*
ATP1B3[i]	3q23	143106062	rs2068230	C__15861969_10*
AGTR1[ii]	3q21-q25	149899732	rs2640539	ASREA
		149912944	rs3772627	ASREA
		149942085	rs5182	ASREA
CYP11B2[ii]	8q21-q22	143996602	rs1799998	ASREA
		143993541	rs4539	ASREA

Table 2.3 *continued*

Gene	Chromosome	Chromosomal Position	SNP	Genotyping method/ TaqMan assay ID
SCNN1A[i]	12p13	6334123	rs11614164	C__2981241_20*
		6339932	rs3782726	C__2981240_10*
		6346984	rs7973914	C__31787955_10*
		6349553	rs10849446	C__31787949_10*
		6353047	rs2286600	C__1249946_1_*
CMA1[ii]	14q11.2	24045349	rs1885108	SNaPshot
		24049178	rs1800875	SNaPshot
SCNN1B[i]	16p12.2-p12.1	23231527	rs11074555	C__3188761_10*
		23239801	rs9930640	C__30539119_10*
		23253439	rs239345	C__2387896_30*
		23267700	rs238547	C__2387909_1_*
		23269331	rs8044970	C__3280856_10*
		23276591	rs152740	C__2387921_10*
		23286780	rs250563	C__2387939_10*
		23297702	rs2303153	C__15971133_10*
SCNN1G[i]	16p12	23108349	rs5735	C__11894747_10*
		23113158	rs4247210	C__11190190_10*
ACE[ii]	17q23.3	58919624	rs4340	ASREA
		58910932	rs4298	SNaPshot
		58911555	rs4303	SNaPshot
		58925300	rs4356	ASREA

[i] Carstens N, M.Sc; [ii] Cloete REA, M.Sc

Abbreviations: ***ACE*****:** Angiotensin converting enzyme 1 gene; ***ACE2*****:** Angiotensin converting enzyme 2 gene; ***AGT*****:** Angiotensinogen gene; ***AGTR1*****:** angiotensin II receptor, type 1 gene; ***AGTR2*****:** angiotensin II receptor, type 2 gene; **ASREA:** allele-specific restriction enzyme analysis; ***ATP1A1*****:** ATPase, Na^+/K^+ transporting, alpha 1 polypeptide gene; ***ATP1A2*****:** ATPase, Na^+/K^+ transporting, alpha 2 polypeptide gene; ***ATP1B1*****:** ATPase, Na^+/K^+ transporting, beta 1 polypeptide gene; ***ATP1B3*****:** ATPase, Na^+/K^+ transporting, beta 3 polypeptide gene; ***ATP6AP2*****:** ATPase, H^+ transporting, lysosomal accessory protein 2 gene; ***CMA1:*** cardiac chymase gene; ***CYP11B2*****:** aldosterone synthase gene; ***REN*****:** renin gene**;** ***RENBP*****:** renin binding protein gene; ***SCNN1A*****:** sodium channel, non-voltage-gated 1 alpha gene; ***SCNN1B*****:** sodium channel, non-voltage-gated 1, beta gene; ***SCNN1G*****:** sodium channel, non-voltage-gated 1, gamma gene; **SNP:** single nucleotide polymorphism

2.5 TaqMan SNP Genotyping

Genotypes were determined on most DNA samples using TaqMan allelic discrimination technology with ABI TaqMan Validated SNP Genotyping Assays (Applied Biosystems, Foster City CA, USA). Each one of the SNP genotyping assays consist of two primers for amplification of the sequence of interest, as well as two TaqMan MGB probes for allele detection. Every TaqMan probe contains a reporter dye at the 5' end of each allele specific probe (VIC for the Allele 1 probe and FAM for the Allele 2 probe), a minor groove binder (MGB) and a nonfluorescent quencher (NFQ) at the 3' end of each probe. The MGB increases the melting temperature of the probe (T_m) without increasing the length of the probe, which results in greater differences in T_m values between matched and mismatched probes, thus producing more exact allelic discrimination (Kutyavin et al., 2000). Detection is achieved with proven 5' nuclease chemistry by means of exonuclease cleavage of a 5' allele-specific dye label, which generates the permanent assay signal by removing the effect of the 3' quencher (Figure 2.2).

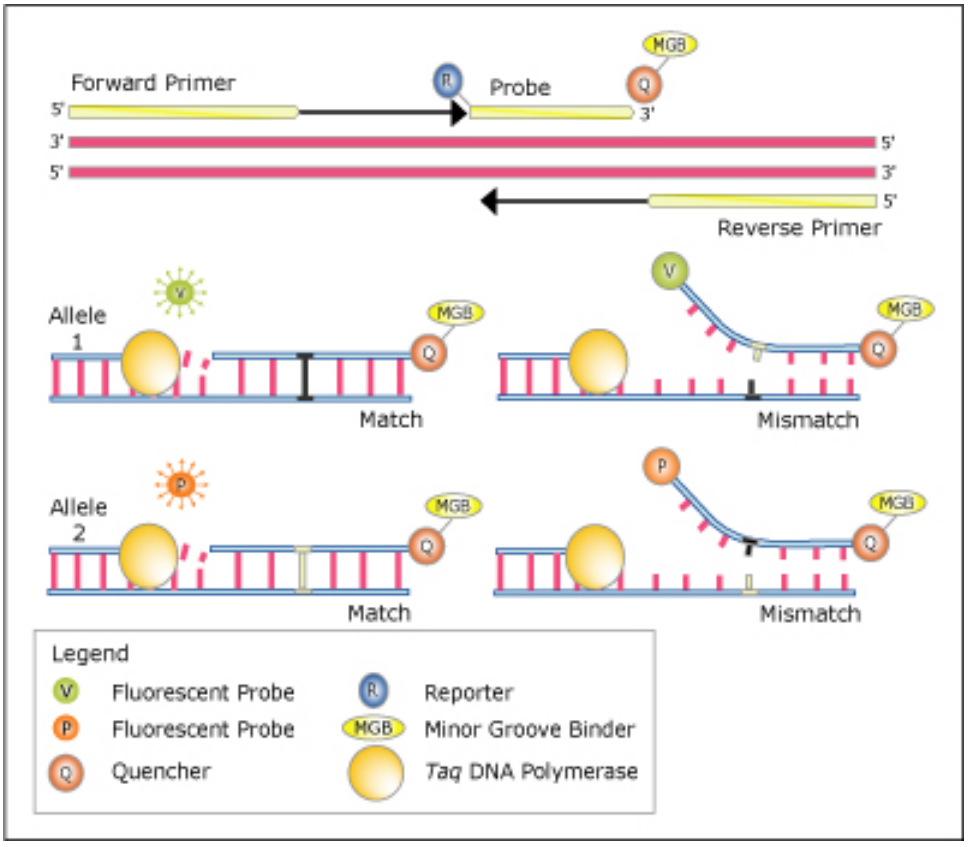

Figure 2.2 Overview of TaqMan allelic discrimination technology. *Allelic discrimination is achieved by the selective annealing of the TaqMan probes and exonuclease cleavage of a 5' allele-specific dye label, which generates the assay signal (Taken from www.servicexs.com/.../TaqMan_AD_SNP_assay.jpg).*

2.5.1. Real-time PCR amplification

Polymerase chain reaction (PCR) amplification for each SNP was performed in a single reaction tube on a thermostable 384-well plate on a Perkin-Elmer 2700 thermal cycler (Applied Biosystems Inc, Foster City CA, USA) following the manufacturer's instructions. All 384-well plates were prepared with an EpMotion pipetting robot (Eppendorf, Hambug, Germany). Figure 2.3 shows a schematic overview of the TaqMan genotyping procedure.

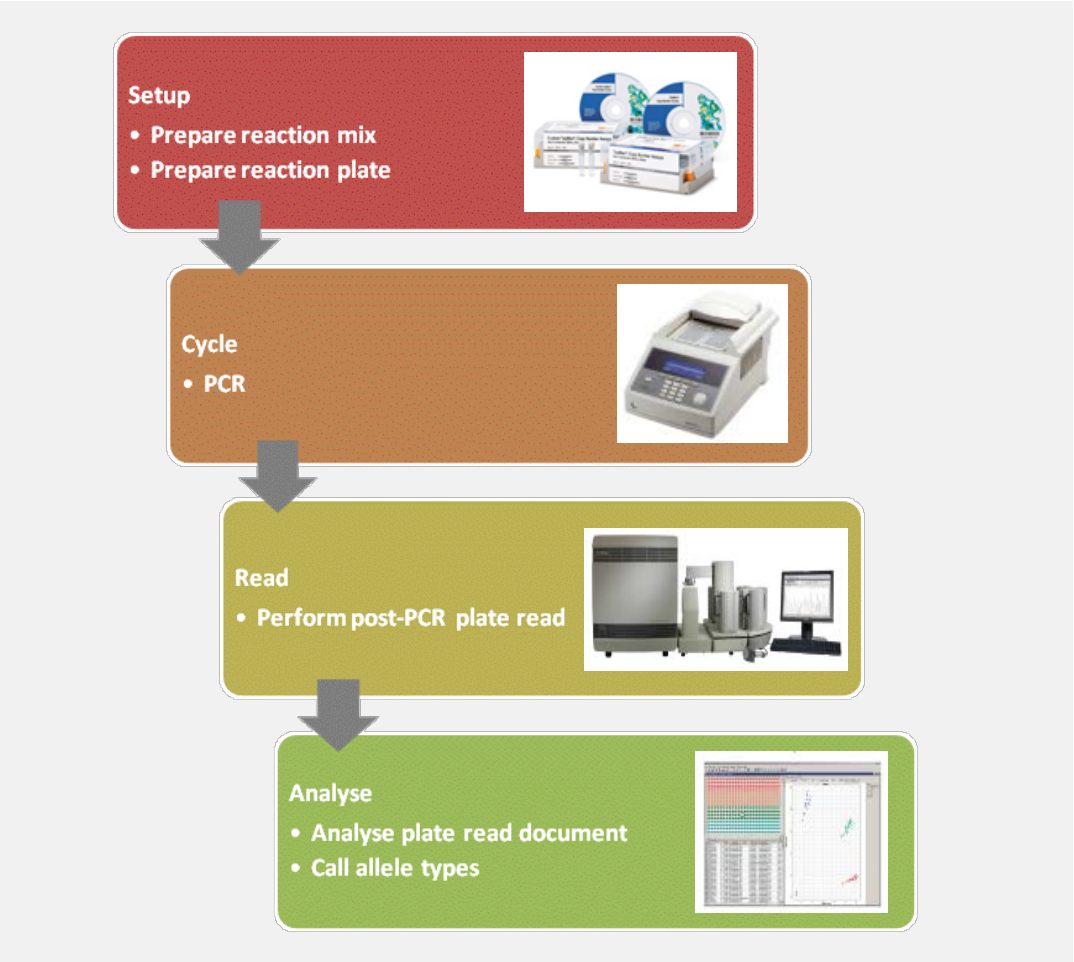

Figure 2.3 Overview of the TaqMan genotyping procedure.

A 5 µl reaction, consisting of 2.5 µl ABI TaqMan Universal PCR Master Mix with the passive reference ROX (Perkin Elmer), 20 ng of genomic DNA, 0.25 µl TaqMan primer and probe dye mix and 1.25 µl DNase-free, sterile-filtered water, was used for all amplifications. At least 10% of all 384-well plates were occupied by non-template control reactions, which contained all the above-mentioned reagents except genomic DNA, to test for contamination. PCR conditions were 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 15 sec at 92˚C and 1 min 30 sec at 60˚C.

2.5.2 Allelic discrimination

Allele discrimination was accomplished by running end point-detection using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City CA, USA) and the Sequence Detection Systems (SDS) 2.3 software (autocaller confidence level 95%). The SDS software reads fluorescence and performs automatic allele-calling by generating allelic discrimination plots. A text file containing the genotyping results was then generated, which was directly incorporated into a database, minimizing errors associated with data transfer. Additionally, all results were confirmed by visual inspection of the real-time PCR multicomponent analysis plots.

2.6 Allele-specific PCR

White and Slutsker (1995) previously reported a conversion variant in intron 2 of the *CYP11B2* gene, where the second intron of the neighbouring *CYP11B1* gene is transferred to *CYP11B2*. As it was not possible to genotype this polymorphism using TaqMan genotyping, we followed the allele-specific PCR genotyping method described by Davies et al. (1999).

2.6.1. PCR amplification

Two separate PCR reactions were used to genotype the I2C variant; the first amplifies the wild type (WT) gene, while the other amplifies the conversion. The two sets of primers, as well as the corresponding PCR conditions are shown in Table 2.4. Each reaction yielded an amplicon of 418 bp.

Table 2.4 Primer sets and PCR conditions used for genotyping the CYP11B2 I2C variant.

Primer	Sequence	PCR Conditions
WT intron 2 sense WT intron 2 antisense	5' TGGAGAAAAGCCCTACCCTGT 3' 5' AGGAACCTCTGCACGGCC 3'	2 min 94°C, (1min 94°C, 30 sec 66°C, 30 sec 72°C, 30 cycles) 72°C, 7 min
Conversion sense Conversion antisense	5' CAGAAAATCCCTCCCCCCTA 3' 5' AGGAACCTCTGCACGGCC 3'	2 min 94°C, (1 min 94°C, 30 sec 66°C, 30 sec 72°C, 30 cycles) 72°C, 7 min

Abbreviations: **A:** adenine; **C:** cytosine; **G:** guanine; **min:** minutes; **sec:** seconds; **T:** thymine; **WT:** wild type

Each PCR amplification was performed in 25 µl reactions, which contained 2x KapaTaq ReadyMix (Kapa Biosystems Inc, RSA), 5 pmol of sense primer, 5 pmol of antisense primer and 20 ng of genomic DNA. A non-template control reaction which contained all the above-mentioned reagents except genomic DNA was included in each PCR amplification run to test for

possible contamination. PCR conditions are shown in Table 2.3. Amplification was performed in a Perkin-Elmer 2700 thermal cycler (Applied Biosystems Inc, Foster City CA, USA). The samples were subsequently analyzed on a 1% agarose gel to determine the presence of PCR product.

2.6.2. Agarose gel electrophoresis

Verification of the PCR-amplification was performed by gel electrophoresis, allowing visualisation of DNA bands on an agarose gel. The agarose gel was prepared by mixing 1 g of agarose powder (Whitehead Scientific, RSA) with 100 ml 1x di-sodium tetraborate-decahydrate buffer (SB) for a 1% agarose gel (Appendix I). The mixture was then heated until the agarose was completely dissolved and 5 µl of (10 mg/ml) ethidium bromide (Whitehead Scientific, RSA) was added to the agarose solution, which was subsequently poured into a casting tray containing a well-forming sample comb and allowed to solidify at room temperature.

After solidification, the gel was placed horizontally into the electrophoresis chamber and covered with 1x SB buffer solution (Appendix I). Electrophoresis was performed as follows: 10 µl of each amplification product was mixed with 3 µl of bromophenol blue loading dye (Appendix I) and then pipetted into the sample wells. A 100 bp DNA ladder (Promega Corp, Madison Wisconsin, USA) was co-electrophoresed with PCR products and used as a molecular size marker.

Samples were electrophoresed at 200 V for 15-20 min in 1x SB buffer solution. Electrophoretically separated 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 using the Syngene gel documentation G-box HR (Frederick, MD, USA).

2.7 Statistical analyses

Genotypic and phenotypic data were captured onto family trees using Cyrillic 2.1 (Cherwell Scientific, UK) and subsequently exported in MLINK format and combined with an Excel sheet containing the echocardiographic and covariate data to create a pedigree file for statistical analyses.

2.7.1 Descriptive statistics and trait distribution

Validation of input files and verification of Mendelian inheritance within families was assessed with Pedstats v. 6.11 (Wigginton and Abecasis, 2005) and genotyping inconsistencies were resolved by re-genotyping. X-chromosome settings were used for the analyses of X-linked genes.

Furthermore, Pedstats was used to test conformance of genotypes with Hardy-Weinberg equilibrium (HWE), as well as the distribution and familial correlations of quantitative traits and covariates. Pedstats selected unrelated individuals from the families for Hardy-Weinberg testing and only females were used to test Hardy-Weinberg equilibrium for the X-linked markers.

Haploview v. 4.2 (Barrett et al., 2005) was used to determine the MAF, as well as the genotyping efficiency for the investigated polymorphisms in the present cohort using X-chromosome settings where appropriate. Haploview reports the genotyping efficiency for the entire cohort after individuals for whom no DNA was available was excluded, while the MAFs were determined on a set of unrelated individuals.

Linear models and variance components analyses are sensitive to kurtosis and skewness in trait distribution and various trait values in the present study were positively skewed. Quantile normalization was therefore used to transform each trait to approximate normality (Pilia et al., 2006) prior to association analysis.

2.7.2 Linkage disequilibrium (LD) determination

Haploview v. 4.2 (Barrett et al., 2005) was used to compute pairwise LD statistics for our study cohort using the genotype data generated in the present study. Haploview provides unique estimates with case-control data and family trios; however, in complex multigenerational family cohorts, such as the present, it attempts to identify a set of maximally informative unrelated individuals to use in subsequent analyses. This is due to the fact that a sample of related individuals will result in an incorrect estimate of LD, as variants in related individuals are in tight LD by definition. The program selects potentially different "informative subsets" with successive runs, but there are sometimes multiple, equally valid unrelated sample sets, which can result in different LD estimates from the same data. This could result in variable LD values when the same data is analysed a number of times. Although these differences are minor (Barrett JC, personal communication), we summarise 100 consecutive Haploview runs by reporting the most frequent, in other words the mode, of the normalised disequilibrium coefficient (D') values.

2.7.3 Principal component score

Since not only the extent, but also the distribution of hypertrophy in HCM is highly variable, it is difficult to distinguish a single echocardiographic measure that accurately quantifies the extent of hypertrophy in all patients. Although echocardiographically derived LVM is most often used

to quantify hypertrophy in HCM, it is known to be an inaccurate measure due to the variable and asymmetric nature of hypertrophy in HCM. Principal component analysis provides a means to derive a single score that comprehensively describes the person-to-person variability in hypertrophy, regardless of the variability in the distribution of the hypertrophy. The outcome variable analysed here, PC1, is the first principal component. It is the linear combination, or in other words, the weighted sum, of the 16 quantile normalised wall thickness values, which explains the largest proportion of the variation in all 16 measurements combined.

2.7.4 Confounders

All analyses were adjusted for covariates that are known to modulate cardiac hypertrophy, namely, whether the individual carries a mutation or not, the identity of the primary HCM-causing mutation ($R92W_{TNNT2}$, $R403W_{MYH7}$, or $A797T_{MYH7}$), hypertension diagnosis and medication, mean arterial blood pressure, sex, age at clinical assessment, ethnicity (as proxy for recent population stratification), BSA and heart rate (as a proxy measure for tachycardia) (Saba et al., 2001)+. These known confounders were included in the models as fixed effects. Such modelling results in the effect of the variant being estimated after the effects of the covariates have been removed from the phenotype.

All analyses were also adjusted for possible clustering of the phenotype in families, as well as the degree of relatedness and possible phenotype correlation between each pair of individuals, by including random effects for family and individual in the models. The coefficients of the random effects were an indicator of family-membership, and a function of the kinship coefficients, respectively.

2.7.5 Heritability

Heritability estimation separates the variability observed in the hypertrophy traits into two components; the contribution of the environment and the contribution of the genetic/inherited factors. The broad sense heritability is the ratio of the inherited variance component to the total variance. The QTDT package (Abecasis et al., 2000) was used to estimate the components of variance of the quantile normalised hypertrophy traits, after adjusting for hypertrophy confounders.

2.7.6 Single polymorphism association

Association between each variant and the six hypertrophy traits chosen to represent the HCM hypertrophic phenotype was assessed with a specialised mixed-effects model (R package kinship, function lmekin, www.r-project.org), which enabled us to adjust for per family, as well

as per individual random effects, so that they included environmental (individual and per family) and polygenic variance components. The genotypes were coded as number of minor alleles (0, 1 or 2) and the mutation groups as a factor with three levels (additive allelic models). For X-linked variants, the (hemizygous) men were coded as having 0 or 2 minor alleles. A significant additive allelic association between a bi-allelic variant and a phenotype means that the mean phenotype value differs significantly between those with no minor alleles (wild type homozygotes, 0 minor alleles) and heterozygotes (1 minor allele), and that the same significant difference exists between heterozygotes and minor homozygotes (2 minor alleles). This difference in the mean phenotype associated with a specific allele is the effect size (see section 2.7.7).

As previous studies reported HCM mutation-specific effects for RAAS variants (Tesson et al., 1997), we also explored the differential effects of RAAS variants in the three HCM mutation groups, viz. $A797T_{MYH7}$, $R403W_{MYH7}$ and $R92W_{TNNT2}$, by testing the statistical interaction between these HCM mutation groups and the genotyped RAAS variants. This means that each model yields three p-values and three effect sizes, one for comparison of each pair of HCM mutation groups.

2.7.7 Effect sizes

When a significant effect is detected (p-value < 0.05), we report the details of the separate effects. Modelling quantile normalised phenotypes are necessary and appropriate for statistical inference (i.e. they deliver valid p-values), but they provide effect estimates in terms of the change in quantile-normalised hypertrophy measures, which are not clinically meaningful. To provide effect estimates in the original units of measurement, we estimated effect sizes, by modelling the raw, untransformed, cardiac wall thickness measures in exactly the same way as described for quantile normalised phenotypes.

A graphical representation and detailed discussion on the interpretation of a significant interaction is discussed in section 3.7. If the interaction was not significant, and the variant effect was significant, then this effect is reported.

2.6.8 Haplotype association analysis

We used Simwalk v. 2.91 (Sobel and Lange, 1996) to infer a most likely pair of haplotypes for each individual, for the genes in the autosomal chromosomes. This program uses so-called simulated annealing to estimate the most likely set of fully-typed maternal and paternal haplotypes of the marker loci at each individual in the pedigree. Simwalk v. 2.91 does not

necessarily converge to the best answer on the first run, but it provides the user with detailed information as to how to run it several times, with different (randomly selected) starting points, in order to be assured of finding the optimal haplotype configuration. The haplotype configuration for this particular cohort was, therefore, determined from multiple, successive Simwalk runs. Each haplotype was subsequently coded as number of that haplotype (0, 1 or 2) inferred for each individual. The haplotype-phenotype pairs were modelled with the R function lmekin in exactly the same way as described for genotypes.

2.6.9 Optimal selection

Finally, we attempted to identify a subset of RAAS variants that together explain most of the variation in hypertrophy traits in the present cohort. First, we used backwards stepwise-selection on the models for 4 traits; LVM, mIVST, mLVWT and PC1, in an attempt to identify a subset of RAAS variants that significantly affected each of these hypertrophy traits, independent of the other variants in the model. We chose LVM as it is most commonly used in HCM modifier studies as an overall indication of the extent of hypertrophy, while PC1 best described the variability in the 16 cardiac wall thickness measurements in the present cohort. We also did these analyses for mIVST as this cardiac region was most commonly affected in this cohort, while mLVWT provides an appraisal of the extent of left ventricular wall thickening.

Each of the four models initially included the eight hypertrophy covariates used for the association analyses, as well as the 12 RAAS polymorphisms that showed significant association with at least one hypertrophy trait. The other components were retained in the models throughout, while the least significant RAAS variants were removed in successive steps, until the final model only retained SNPs that impacted significantly on the respective hypertrophy traits. The backwards stepwise-selection yielded optimal models containing between 2 and 6 variants each, and 9 variants were in at least one model.

Secondly, we modelled the nine selected SNPs on the quantile normalised traits, to obtain p-values, and on the untransformed traits to obtain estimated effect sizes, which are adjusted for the eight hypertrophy covariates and the eight other variants in each model. For discussion purposes, we also modelled the 5 “pro-LVH” polymorphisms (Kaufman et al., 2007; Ortlepp et al., 2002; Perkins et al., 2005) in the same way, even though these variants did not associate with hypertrophy traits in the present cohort.

Chapter 3

Results

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CHAPTER 3: Results

3.1 Basic characteristics of the HCM cohort

The cohort consists of 353 individuals, described in two previous studies (Cloete REA, M.Sc and Carstens N, M.Sc), as well as 35 newly recruited individuals, which amounted to a total cohort size of 388. DNA was available for all 388 individuals, although only 256 consented to clinical evaluation. The basic characteristics of these individuals, as well as selected hypertrophy traits, stratified into mutation carrier (MC) and non-carrier (NC) groups for the three HCM causal mutations present in the study cohort, are presented in Table 3.1. Data are summarised here as median (interquartile range) due to the skewness of some of the trait distributions.

3.2 Candidate gene selection and polymorphism prioritisation

Candidate genes were selected to represent the different steps in the RAAS and polymorphisms were selected to cover these genes, as explained in section 2.3. A total of 84 polymorphisms from 20 RAAS genes were consequently prioritised for investigation.

Diagrams to depict the chromosomal locations of these genes, as well as the intragenic location of the respective prioritised variants, were drawn to scale using the FancyGene v. 1.4 software (Rambaldi and Ciccarelli, 2009).

3.3 Genotyping results

3.3.1 TaqMan allelic discrimination

All initial PCR amplification runs were completed successfully, while no amplification was observed in the non-template controls, ruling out the possibility of contamination. Figure 3.1 depicts the allelic discrimination results for the rs4635799 polymorphism in *NR3C2,* as a representative example. Results from the end-point allelic discrimination analyses were exported and incorporated into the main RAAS genotype database for statistical analyses.

3.3.2 Allele-specific PCR

The I2C polymorphism was genotyped using a previously described allele-specific PCR method (Davies et al., 1999). No amplification was observed in the non-template controls, ruling out the possibility of contamination.

Table 3.1 Basic characteristics of the entire study cohort, stratified into mutation carrier (MC) and non-carrier (NC) groups according to HCM mutation status. *Data are summarised here as median (interquartile range) due to the skewness of some of the trait distributions.*

	A797T*MYH7*		**R92W*TNNT2***		**R403W*MYH7***	
	MC	**NC**	**MC**	**NC**	**MC**	**NC**
n*	68	50	48	34	32	24
age	43.0 (26.8-56.8)	41.5 (29.3-53.0)	36.0 (21.0-47.0)	42.0 (25.5-49.0)	36.5 (25.0-46.8)	38.0 (31.3-50.8)
BSA (m^2)	1.90 (1.60-2.00)	1.90 (1.67-2.03)	1.70 (1.60-1.80)	1.80 (1.60-1.90)	1.80 (1.70-2.00)	1.95 (1.70-2.05)
SBP (mm Hg)	120 (115-134)	120 (110-130)	115 (110-130)	120 (110-120)	120 (115-130)	120 (114-143)
DBP (mm Hg)	80 (70-87)	80 (80-89)	73 (70-80)	80 (70-80)	80 (79-80)	80 (78-90)
HR (bpm)	68 (60-75)	68 (62-76)	68 (61-76)	67 (60-73)	65 (60-76)	72 (65-84)
LVM (g)	191 (135-237)	135 (109-159)	146 (104-185)	116 (100-144)	170 (134-205)	154 (115-202)
mIVST (mm)	14.5 (11.0-20.9)	10.5 (9.2-11.3)	14.0 (9.6-18.3)	10.0 (8.6-10.9)	13.6 (11.6-16.0)	11.0 (10.2-12.6)
mLVWT (mm)	14.0 (11.0-20.7)	10.5 (9.5-11.3)	13.4 (9.9-18.1)	10.0 (9.2-10.9)	13.6 (11.6-17.8)	11.2 (10.2-12.4)
mPWT (mm)	10.2 (9.1-11.8)	9.0 (8.3-10.0)	10.2 (8.1-11.5)	8.1 (7.5-9.1)	10.1 (9.1-11.5)	9.9 (8.8-10.4)
CWT score (mm)	196 (151-239)	139 (127-148)	159 (131-192)	131 (117-142)	172 (148-190)	153 (136-166)
PC1	2.12 (-0.14-4.88)	-1.31 (-2.52--0.01)	0.86 (-2.62-2.72)	-2.56 (-4.26--1.01)	1.42 (-0.34-2.42)	0.1 (-2.02-1.44)

*n- number of individuals with available clinical data

Abbreviations: **BSA:** body surface area; **CWT score:** cumulative wall thickness score; **DBP:** diastolic blood pressure; **HR:** heart rate; **LVM:** left ventricular mass; **MC:** HCM mutation carrier; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **NC:** non-carrier; **PC1:** first principal component; **SBP:** systolic blood pressure

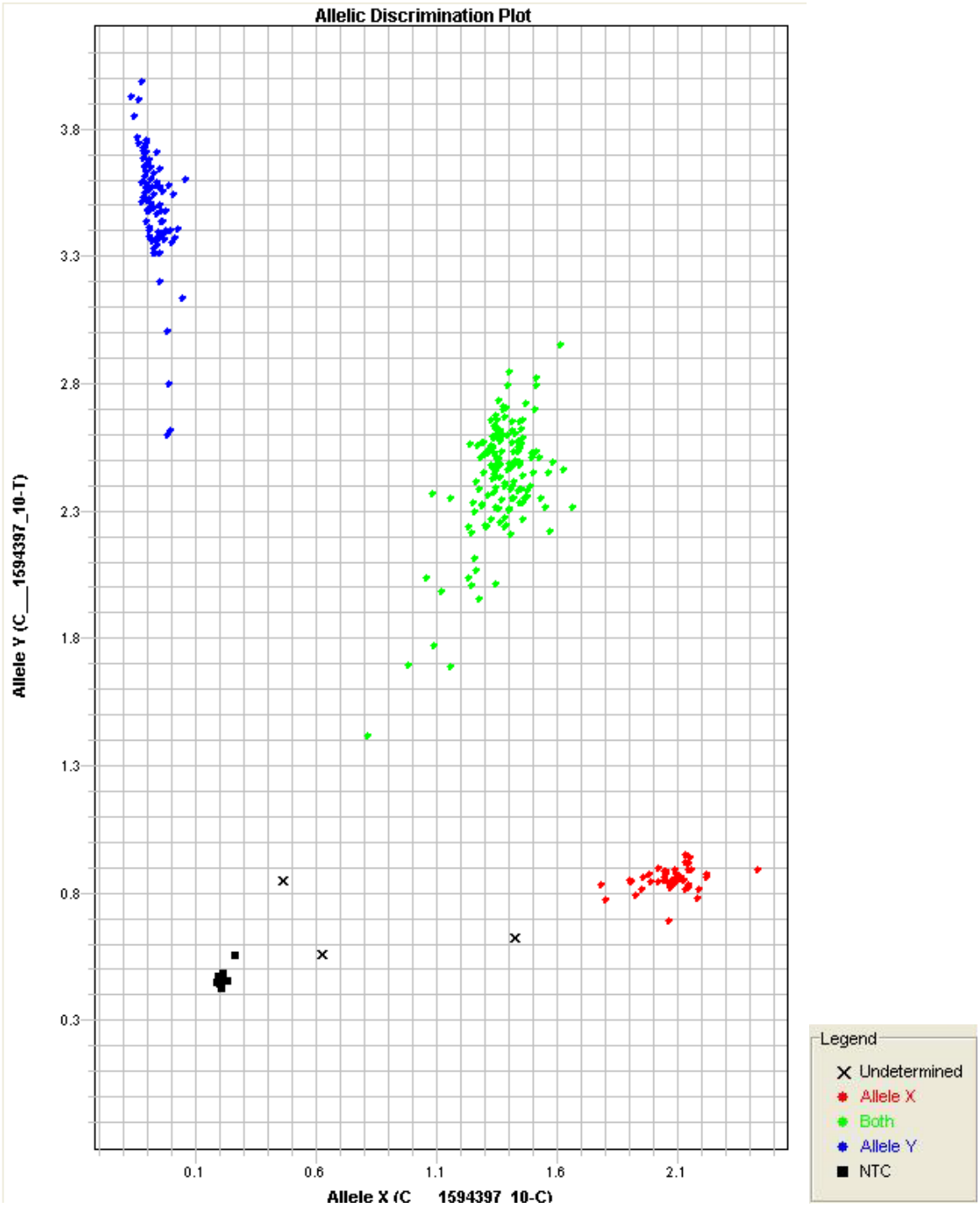


Figure 3.1 Representative genotyping result for the TaqMan allelic discrimination analyses. *This figure depicts the genotyping results for the rs4635799 polymorphism as a representative example of the allelic discrimination plots obtained with the SDS software during end-point analyses. Allele X = C; Allele Y = T*

3.4 Descriptive statistics

3.4.1 Descriptive statistics for investigated polymorphisms

All genotype data obtained from the present study was combined with genotype data from the two substudies previously reported in the greater project (Cloete REA, M.Sc and Carstens N, M.Sc). Table 3.2 summarises basic descriptive statistics for the complete dataset. Genotype frequencies for all markers were in agreement with Hardy-Weinberg equilibrium as the cut-off value of $p = 0.01$.

3.4.2 LD assessment

Pairwise LD statistics for the candidate genes for which two or more variants were genotyped, were obtained with Haploview v. 4.2 (Barrett et al., 2005). We provide the modal D' values, which were determined from 100 consecutive Haploview runs, as an estimate of LD. A pair of polymorphisms is regarded as being in complete LD if $D' = 1$, as this indicates a complete lack of evidence of recombination between the two variants. Pairwise LD statistics will be discussed with the association analysis results for ease of interpretation.

Table 3.2 Minor allele frequencies (MAFs), genotyping efficiency, as well as p-values for tests of Hardy-Weinberg equilibrium (HWE) for markers in the study*. MAF as determined in a set of unrelated individuals.*

Gene	Polymorphism	Genotyping Efficiency (%)	a_2:a_1	MAF	HWE
AGTR2	rs1403543	99.2	G:A	0.353	0.06
	rs5194	95.5	A:G	0.395	0.02
	rs11091046	100	A:C	0.404	0.02
RENBP	rs2269370	100	C:A	0.353	0.36
	rs2269372	96.4	G:A	0.456	0.01
	rs762656	99.7	G:A	0.468	0.01
ATP6AP2	rs2968915	99.4	A:G	0.264	1.00
	rs2968917	99.7	T:C	0.190	1.00
	rs10536	98.6	A:G	0.211	1.00
ACE2	rs4646179	96.5	T:C	0.020	0.08
	rs879922	86.5	C:G	0.231	0.28
	rs2285666	98.1	C:T	0.263	0.38
	rs1978124	84.0	T:C	0.312	1.00
ATP1A2	rs7548116	98.9	A:T	0.337	0.11
	rs6695366	92.7	A:G	0.097	0.18
	rs11585375	96.6	A:G	0.340	0.73
ATP1B1	rs1200130	95.5	C:T	0.262	1.00
	rs1358714	99.7	G:A	0.459	0.34
	rs1040503	97.8	G:A	0.470	0.35
REN	rs3795575	94.6	G:A	0.120	1.00
	rs1464816	97.0	G:T	0.301	0.50
	rs11571082	97.0	C:T	0.192	0.31
	rs5705	97.0	T:G	0.286	0.70
	rs10900555	96.5	A:G	0.497	0.54
	rs6682082	81.7	C:T	0.183	0.57
AGT	rs1926723	93.7	A:C	0.091	1.00
	rs11122575	93.7	T:C	0.094	1.00
	rs699	96.8	C:T	0.316	0.15
	rs4762	97.2	C:T	0.060	1.00
	rs5051	97.2	T:C	0.300	0.26
ATP1A1	rs10924074	97.8	A:G	0.145	0.08
	rs850609	98.3	A:T	0.141	1.00
ATP1B3	rs2068230	100	A:T	0.439	0.01
AGTR1	rs2640539	98.3	A:C	0.184	0.38
	rs3772627	94.3	C:A	0.454	0.10
	rs5182	98.3	C:A	0.455	0.73

Table 3.2 *Continued*

Gene	Polymorphism	Genotyping Efficiency (%)	a_2:a_1	MAF	HWE
	rs906124	91.2	T:C	0.466	0.50
	rs11933380	99.2	T:C	0.439	0.51
	rs745019	88.5	A:G	0.283	1.00
	rs1403142	94.2	A:G	0.389	0.77
	rs13150372	97.8	G:A	0.221	0.44
	rs7694706	99.5	A:G	0.340	0.70
	rs6535584	98.6	C:T	0.374	0.52
	rs6535594	98.6	A:G	0.497	1.00
NR3C2	rs7699349	98.6	C:T	0.298	0.20
	rs2883930	98.4	C:G	0.296	0.70
	rs4835508	99.5	C:T	0.265	0.13
	rs11945778	99.7	C:T	0.444	1.00
	rs3910047	97.8	T:C	0.278	0.19
	rs3846329	99.2	G:T	0.263	0.19
	rs2137334	84.9	C:T	0.399	0.28
	rs13118022	95.9	G:T	0.476	0.12
	rs4635799	97.0	T:C	0.423	0.07
CYP11B1	rs4310186	98.9	C:G	0.432	0.22
	rs6387	97.5	G:A	0.470	0.06
	rs6410	98.9	C:T	0.494	0.07
CYP11B2	rs3097	100	C:T	0.165	0.61
	rs4539	79.1	A:G	0.277	1.00
	I2C	93.6	I:C	0.376	0.15
	rs1799998	85.2	C:T	0.241	0.07
	rs11614164	97.1	A:G	0.284	0.75
	rs3782726	94.8	T:G	0.294	0.48
SCNN1A	rs7973914	97.4	C:T	0.407	0.50
	rs10849446	98.0	A:C	0.292	0.52
	rs2286600	96.5	G:A	0.284	0.74
M6PR	rs1805725	94.8	A:C	0.161	0.57
	rs987917	96.7	C:A	0.293	0.28
CMA1	rs1885108	96.3	A:G	0.335	0.64
	rs1800875	91.0	A:G	0.317	0.13
SCNN1G	rs5735	97.2	T:C	0.273	0.09
	rs4247210	99.4	G:C	0.272	0.50
	rs11074555	100	T:C	0.424	0.16
	rs9930640	98.0	G:A	0.092	1.00
SCNN1B	rs239345	99.2	T:A	0.393	0.46
	rs238547	96.4	C:T	0.276	0.20
	rs8044970	99.2	T:G	0.247	0.07

Table 3.2 *Continued*

Gene	Polymorphism	Genotyping Efficiency (%)	a_2:a_1	MAF	HWE
	rs152740	95.0	A:T	0.456	0.34
SCNN1B	rs250563	98.6	C:T	0.077	1.00
	rs2303153	94.4	G:C	0.267	0.66
HSD11B2	rs5479	100	C:A	0.087	0.31
ACE1	rs4303	90.9	G:T	0.040	1.00
	rs4298	89.1	C:T	0.129	1.00
	rs4340	94.5	D:I	0.384	1.00
	rs4356	92.7	T:C	0.161	1.00

*a_2:a_1 major: minor allele

Abbreviations: ***ACE*****:** Angiotensin-converting enzyme 1 gene; ***ACE2*****:** Angiotensin converting enzyme 2 gene; ***AGT*****:** Angiotensinogen gene; ***AGTR1*****:** angiotensin II receptor, type 1 gene; ***AGTR2*****:** angiotensin II receptor, type 2gene; ***ATP1A1*****:** ATPase, Na^+/K^+ transporting, alpha 1 polypeptide gene; ***ATP1A2*****:** ATPase, Na^+/K^+ transporting, alpha 2 polypeptide gene; ***ATP1B1*****:** ATPase, Na^+/K^+ transporting, beta 1 polypeptide gene; ***ATP1B3*****:** ATPase, Na^+/K^+ transporting, beta 3 polypeptide gene; ***ATP6AP2*****:** ATPase, H^+ transporting, lysosomal accessory protein 2 gene; ***CMA1:*** cardiac chymase gene; ***CYP11B1:*** 11 beta-hydroxylase gene; ***CYP11B2*****:** aldosterone synthase gene; ***HSD11B2*****:** 11 β-hydroxysteroid-dehydrogenase type 2 gene**; HWE:** Hardy-Weinberg Equilibrium; ***M6PR***: mannose-6-phosphate receptor gene; ***NR3C2*****:** nuclear receptor subfamily 3, group C, member 2 gene; ***REN*****:** renin gene**;** ***RENBP*****:** renin binding protein gene; ***SCNN1A*****:** sodium channel, non-voltage-gated 1 alpha gene; ***SCNN1B*****:** sodium channel, non-voltage-gated 1, beta gene; ***SCNN1G*****:** sodium channel, non-voltage-gated 1, gamma gene

3.5 Principal component analysis

Principal component analysis was used to generate the first principal component (PC1) of the 16 quantile-normalised cardiac wall thickness measurements. This PC1 score is a weighted average of these wall thickness measurements, and explained 75% of the overall variability in these measurements in the present cohort. The respective weights (loadings) ranged from 0.221 to 0.269 (Table 3.3).

Table 3.3 Weights of wall thickness measures in the PC1 hypertrophy score (loadings for the first principal component)

Hypertrophy trait	Loading in PC1
pIVS at mitral valve	0.253
aIVS at mitral valve	0.261
AW at mitral valve	0.256
LW at mitral valve	0.249
IW at mitral valve	0.221
PW at mitral valve	0.221
pIVS at papillary muscle	0.262
aIVS at papillary muscle	0.269
AW at papillary muscle	0.267
LW at papillary muscle	0.254
IW at papillary muscle	0.234
PW at papillary muscle	0.238
IVS at apex	0.263
AW at apex	0.259
LW at apex	0.239
PW at apex	0.248

Abbreviations: **aIVS:** anterior interventricular septal thickness; **AW:** anterior wall thickness; **IVS:** interventricular septal thickness; **IW:** inferior wall thickness; **LW:** lateral wall thickness; **PC1:** first principal component; **pIVS:** posterior interventricular septal thickness; **PW:** posterior wall thickness

3.6 Heritability estimation

Table 3.4 reports the estimated percentage variance attributable to environment (E) and inherited or genetic factors (H), as well as p-values for the test of heritability for the six hypertrophy traits chosen for investigation. The heritability values were adjusted for the known hypertrophy covariates. In other words, these values are independent of the primary HCM-causal mutation, hypertension diagnosis, ethnicity, age, sex, BSA, blood pressure and heart rate. After adjustment for these covariates, a strong heritable component was found for all six hypertrophy traits used in this study.

Table 3.4 Estimated percentage variance attributable to environment (E) and genetic factors (H), as well as the p-values for heritability. *All tests were adjusted for the HCM-causal mutation as well as other known hypertrophy covariates.*

Hypertrophy trait	E (%)	H (%)	p-value
LVM	41	59	< 0.0001
mIVST	56	44	< 0.0001
mLVWT	44	55	0.0001
mPWT	44	56	0.0002
CWT score	59	41	0.0018
PC1	48	52	0.0009

Abbreviations: **CWT score:** cumulative wall thickness score; **E:** environment; **G:** polygenetic factors; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7 Association analyses

In this section, the results of our statistical analyses will be presented per gene, starting with a schematic of the locations of the variants in the gene. This will be followed by single SNP and haplotype analysis results, from both association and interaction models. Next, we plot the negative logarithm base 10 from the association analyses as an indication of the relative magnitude of the association effect sizes for the variants, as well as the haplotypes, for the six HCM phenotypes; in these plots, a red broken line indicates the value corresponding to a p-value of 0.05. We also present tables of joint p-values, for each of the three possible pairs of mutation groups, from the interaction models, for each variant-phenotype and also for each haplotype-phenotype pair.

As interaction can be a somewhat complex concept, a representative example of a significant interaction between HCM mutation group and a RAAS variant on a trait is shown below in Figure 3.2. This example illustrates a significant interaction between HCM mutation group and the rs2068230 SNP (*ATP1B3*) on mIVST, corresponding to the p-values discussed later in this chapter. The estimated differences in effect between the HCM mutation groups were as follows. The addition of a T-allele was associated with a 3.56 mm higher mIVST in the $R92W_{TNNT2}$ compared to the $A797T_{MYH7}$ group (p= 0.002) and a 2.68 mm higher mIVST in the $R403W_{MYH7}$ compared to the $A797T_{MYH7}$ group (p = 0.033). The difference in effect of the T-allele was, however, not significant between the $R92W_{TNNT2}$ and $R403W_{MYH7}$ groups (p = 0.563).

The graph depicts estimated values of mIVST for 9 hypothetical "average" individuals in the present cohort, from each of the three mutation groups, with each possible rs2068230 genotype (Figure 3.2). An "average" individual in this cohort would be a 41 year-old male, on hypertensive medication, with a BSA of 1.93 m^2, mean arterial pressure of 94 mm Hg and a heart rate of 69 beats per minute (bpm), who does not carry the specific HCM mutation. For "real" individuals with different values for these hypertrophy covariates, the modelled graph will simply shift up or down, but the effect sizes (differences between the HCM mutation groups and RAAS genotypes) will remain the same. In mutation carriers, 4.98 units would be added to the average.

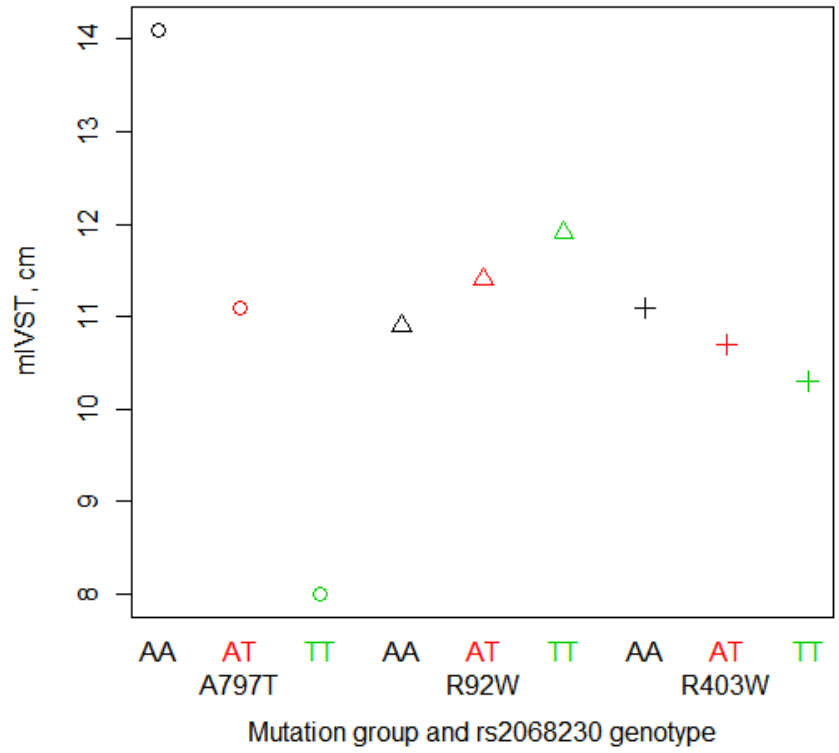


Figure 3.2 Graph of estimated mIVST by mutation group and rs2068230 genotype.

It is then evident from the graph that the average effect of the addition of a T-allele in the *ATP1B3* gene would result in significantly different outcomes, i.e. different interventricular wall thickness measurements, depending on the particular HCM causal mutation of that individual. More specifically, mIVST will decrease by 3.05 mm in $A797T_{MYH7}$ carriers, while each T-allele will increase mIVST in $R92W_{TNNT2}$ carriers by 0.51 mm, whereas each T-allele decreases mIVST with 0.37mm in $R403W_{MYH7}$ carriers (Figure 3.2). As the effect of this polymorphism is significantly different in $A797T_{MYH7}$ carriers compared to both other groups, we conclude that

this polymorphism has a significant effect in A797T$_{MYH7}$ carriers. Note that if one allele is associated with a decrease in phenotype, the other allele at that locus will automatically be associated with a corresponding increase in that phenotype.

Haplotypes could unfortunately not be estimated for the X-linked variants as we are not aware of software to assign haplotypes for X-linked genes in extended families.

3.7.1 Angiotensinogen (AGT)

Figure 3.3 depicts the chromosomal location of *AGT,* as well as the intragenic location of the investigated *AGT* polymorphisms. Complete LD (D' = 1) was observed between all investigated SNPs in *AGT.*

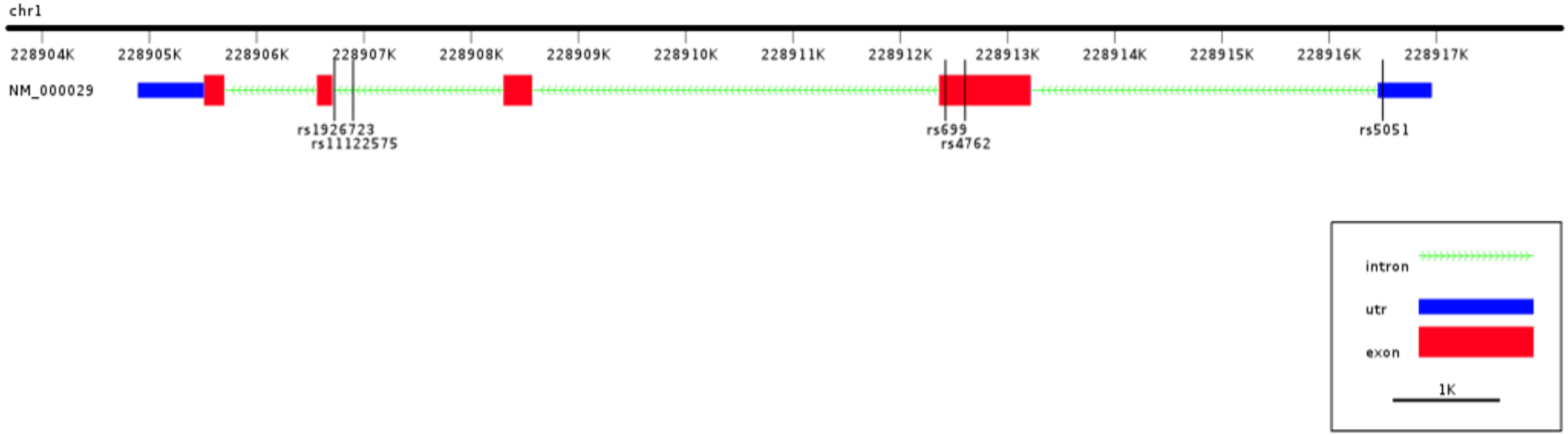
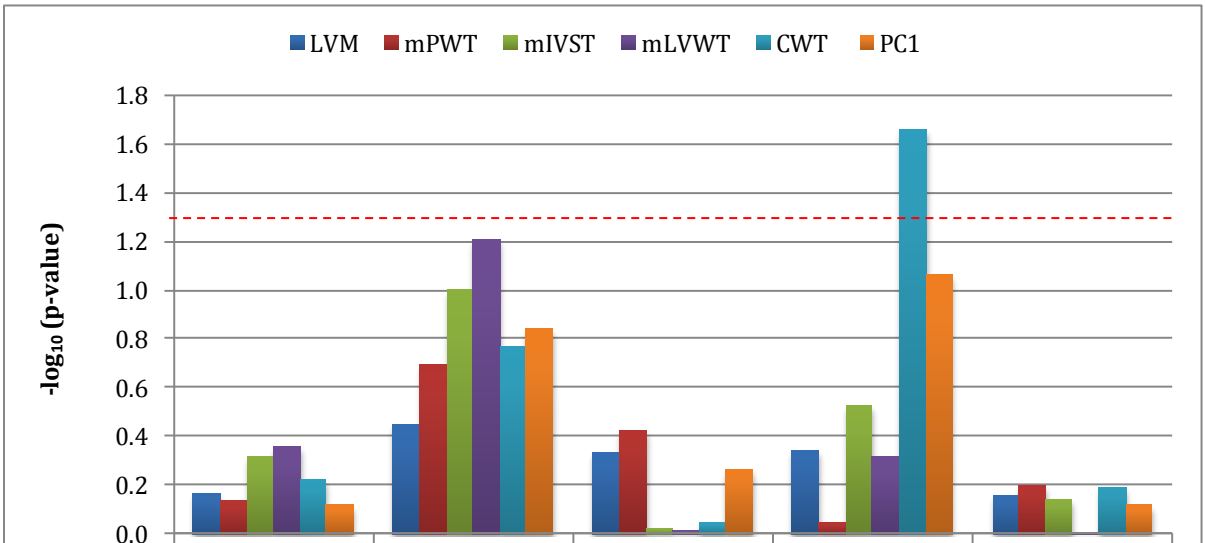


Figure 3.3 Scale diagram depicting chromosomal location and structure of the AGT gene, as well as intragenic location of target polymorphisms.

Arrows indicate direction of transcription.

P-values for tests of additive allelic association are illustrated in Figure 3.4, independent of specific HCM mutation group and of whether a mutation is present, as well as the hypertrophy confounders.



	rs1926723	rs11122575	rs699	rs4762	rs5051
LVM	0.680	0.358	0.460	0.452	0.694
mPWT	0.738	0.200	0.380	0.895	0.637
mIVST	0.481	0.100	0.946	0.294	0.713
mLVWT	0.438	0.062	0.961	0.483	0.977
CWT	0.597	0.169	0.898	0.022	0.641
PC1	0.762	0.145	0.548	0.086	0.754

Figure 3.4 Single polymorphism association results for AGT. *Graph indicates –log 10 transformed p-values for the tests of association between AGT variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Table 3.5 contains the p-values for simultaneous testing of differences in additive allelic association between all pairs of HCM mutation groups and *AGT* genotype, as explained in section 2.6.4. Significantly different allelic effects were observed on LVM and mLVWT (Table 3.5). The effect of the addition of each r699 T-allele on LVM was 25.6 g higher in the R403W$_{MYH7}$ group, when compared to the A797T$_{MYH7}$ group (Table 3.5). Similarly, the rs5051 C-allele is associated with a 27.8 g higher effect in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group. On the other hand, the effect of C-allele of rs1926723 is 5.15 mm lower in the R403W$_{MYH7}$ group, when compared to the A797T$_{MYH7}$ group, while the effect of the C-allele of rs11122575 is 5.80 mm lower in the R403W$_{MYH7}$ group, when compared to the A797T$_{MYH7}$ group.

Table 3.5 The p-values for interaction between HCM mutation group and AGT genotype, illustrating the differences in allelic effect of the particular AGT variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
AGT	**rs1926723**	0.703	0.142	0.250	0.242	0.643	0.179	0.13	0.166	**0.031**	0.374	0.333	0.181	0.310	0.328	0.139	0.396	0.251	0.157
	rs11122575	0.672	0.096	0.209	0.281	0.356	0.137	0.177	0.060	**0.025**	0.482	0.158	0.171	0.373	0.166	0.12	0.487	0.118	0.145
	rs699	0.347	0.258	**0.030**	0.237	0.776	0.113	0.229	0.767	0.105	0.993	0.413	0.408	0.748	0.326	0.191	0.915	0.358	0.310
	rs4762	0.333	0.925	0.243	0.570	0.533	0.926	0.888	0.766	0.901	0.961	0.131	0.142	0.886	0.820	0.675	0.814	0.761	0.538
	rs5051	0.214	0.398	**0.023**	0.257	0.808	0.127	0.227	0.683	0.075	0.946	0.373	0.395	0.648	0.261	0.105	0.819	0.318	0.212

Abbreviations: ***AGT*****:** Angiotensinogen gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Figure 3.5 summarises the *AGT* haplotype association analyses results, while Table 3.6 depicts the observed haplotype distribution as well as the exact p-values for the tests of association between the *AGT* haplotypes and the investigated hypertrophy traits. No statistically significant association was found between any of the *AGT* haplotypes and the investigated hypertrophy traits.

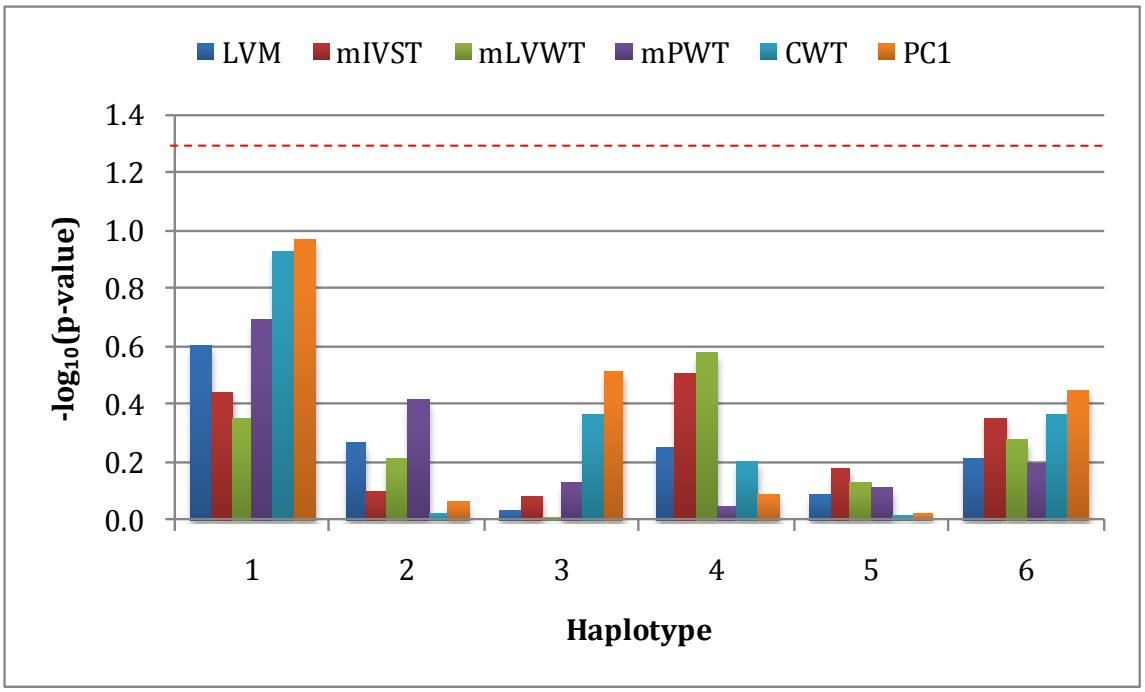


Figure 3.5 Summary of haplotype association results for AGT. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of $p < 0.05$.*

Table 3.6 Haplotype distribution within AGT, as well as the exact p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs1926723	rs11122575	rs699	rs4762	rs5051	freq.	p-value for test of association					
							LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	T	C	C	T	0.275	0.252	0.367	0.449	0.203	0.118	0.108
2	A	T	T	C	C	0.214	0.541	0.804	0.614	0.386	0.942	0.868
3	A	T	C	T	T	0.062	0.930	0.833	0.987	0.739	0.432	0.306
4	C	C	C	C	T	0.050	0.564	0.314	0.263	0.896	0.632	0.812
5	A	T	T	C	T	0.021	0.813	0.667	0.743	0.778	0.961	0.956
6	A	T	C	C	C	0.008	0.610	0.449	0.531	0.636	0.431	0.356

Haplotypes with a frequency < 0.008 not indicated

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.7 shows the p-values for interaction between the HCM mutation group and the first four *AGT* haplotypes, to illustrate the differences in allelic effect of these haplotypes between the mutation groups. Haplotypes with a frequency of less than 0.050 were not tested. Haplotype 2 was significantly associated with a 0.97 mm higher effect in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (p = 0.044).

Table 3.7 The p-values for interaction between HCM mutation group and AGT haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.538	0.327	0.680	0.625	0.394	0.184	0.555	0.554	0.248	0.427	0.655	0.759	0.553	0.115	0.318	0.386	0.190	0.628
2	0.766	0.078	0.118	0.818	0.125	0.164	0.964	0.102	0.089	0.602	0.057	0.153	0.522	**0.044**	0.190	0.386	0.054	0.331
3	0.384	0.652	0.092	0.551	0.302	0.757	0.790	0.976	0.740	0.922	0.086	0.081	0.868	0.370	0.231	0.837	0.337	0.186
4	0.742	0.085	0.231	0.278	0.577	0.191	0.227	0.105	0.049	0.359	0.156	0.112	0.432	0.162	0.145	0.518	0.073	0.125

Haplotypes with a frequency < 0.050 not tested

Abbreviations: ***AGT*:** Angiotensinogen gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.2 Renin and renin–associated genes

Figure 3.6 depicts the chromosomal location of *REN,* as well as the intragenic location of the investigated polymorphisms. A very high degree of LD was observed in the region extending from rs5705 to rs3795575 in *REN* (Table 3.8). Although rs1464816 appears to be in LD with all the other prioritised variants in *REN* (D' values ranging from 0.8 to 1), we found intermediate to low D' values for the variants covered between rs5705 and rs6682082.

All three investigated SNPs in *RENBP* were in tight LD (Figure 3.7). Rs296895 and rs2968917 were found to be in complete LD, while weaker evidence for pairwise LD exists between rs10536 and both of these variants in *ATP6AP2* (Figure 3.8). The two SNPs investigated in *M6PR* were found to be in complete LD (D' = 1) (Figure 3.9).

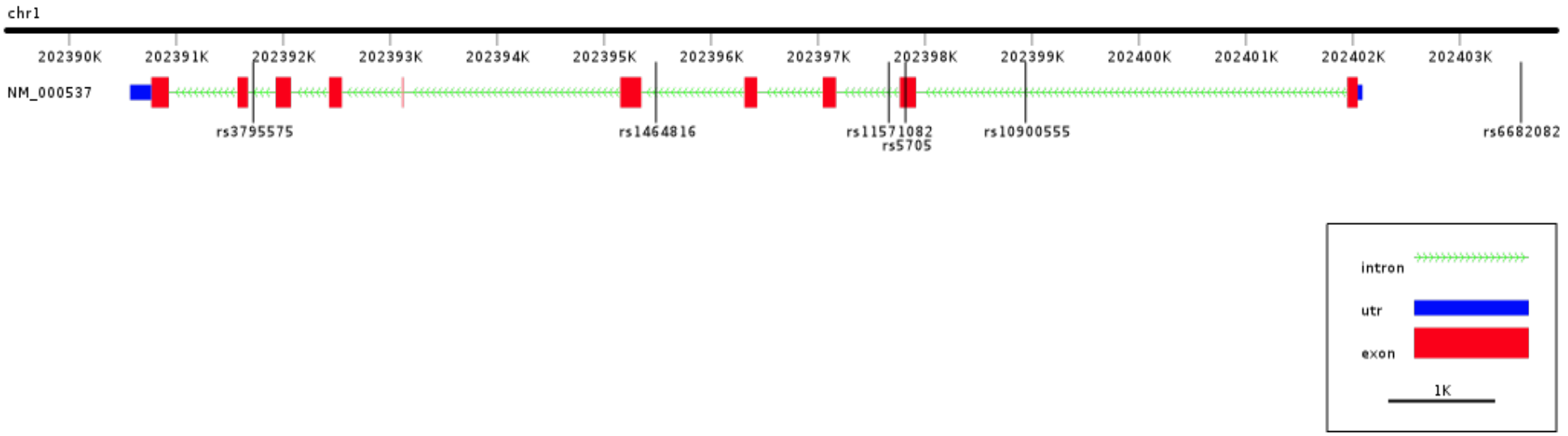


Figure 3.6 Scale diagram depicting chromosomal location and structure of the REN gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.8 Pairwise D' values as a representation of the observed LD structure within REN in the present cohort.

	rs3795575	rs1464816	rs11571082	rs5705	rs10900555	rs6682082
rs3795575		1	0.90	1	0.60	0.19
rs1464816			0.80	1	0.95	1
rs11571082				0.94	0.57	0.20
rs5705					0.72	0.60
rs10900555						0.60
rs6682082						

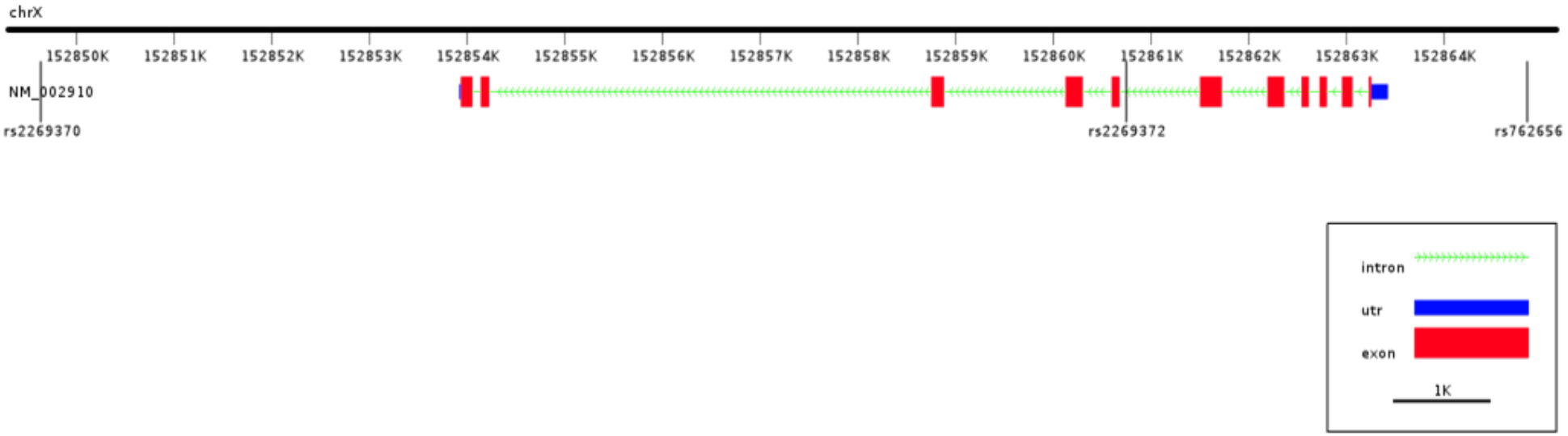


Figure 3.7 Scale diagram depicting chromosomal location and structure of the RENBP gene, as well as intragenic location of target polymorphisms. *LD values were D' = 0.94 between rs762656 and rs2269372, and D' = 1 for rs2269370 with both other SNPs. Arrows indicate direction of transcription.*

Figure 3.8 Scale diagram depicting chromosomal location and structure of the ATP6AP2 gene, as well as intragenic location of target polymorphisms. *LD values were D' = 1 between rs2968917 and rs2968915, D' = 0.34 between rs10536 and rs2968915 and D' = 0.48 between rs10538 and rs2968917. Arrows indicate direction of transcription.*

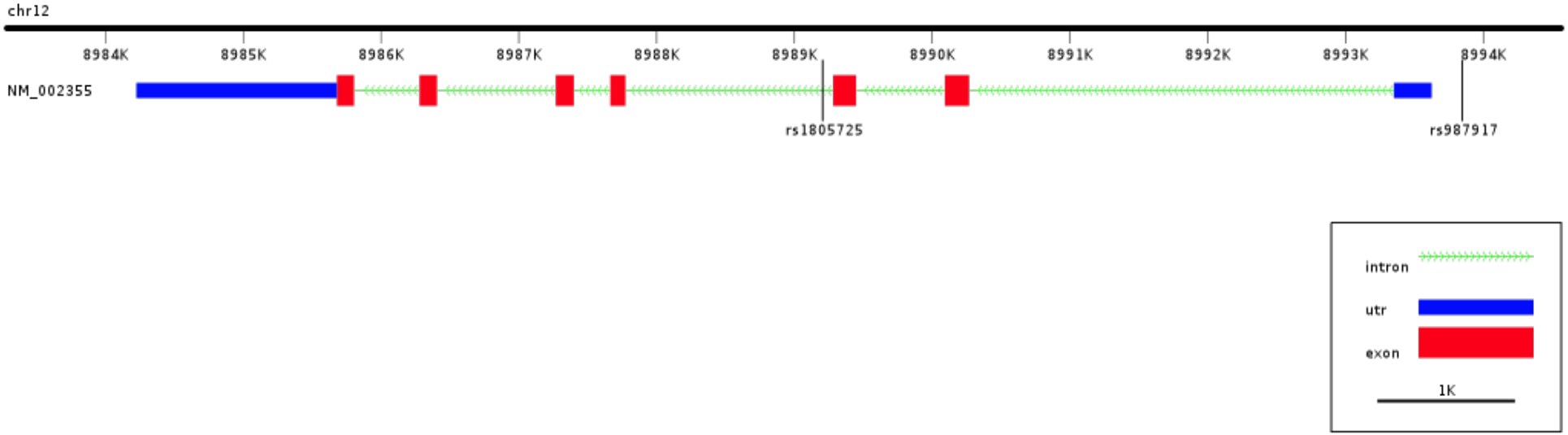
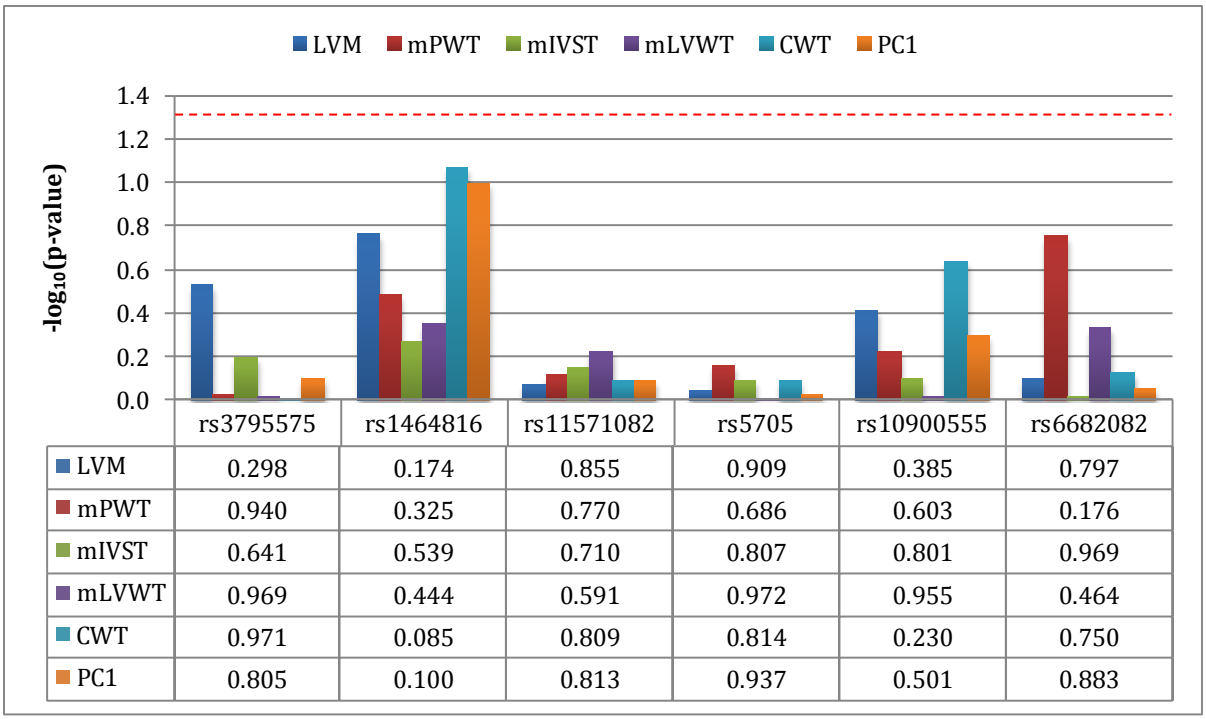


Figure 3.9 Scale diagram depicting chromosomal location and structure of the M6PR gene, as well as intragenic location of target polymorphisms. *Both SNPs were in complete LD (D' = 1). Arrows indicate direction of transcription.*

3.7.2.1 Renin (*REN*)

A summary of the *REN* single SNP association analysis in the entire cohort, as well as the exact p-values for tests of additive allelic association are shown in Figure 3.10. No evidence for association was found between the investigated *REN* SNPs and hypertrophy traits in this analysis.



	rs3795575	rs1464816	rs11571082	rs5705	rs10900555	rs6682082
LVM	0.298	0.174	0.855	0.909	0.385	0.797
mPWT	0.940	0.325	0.770	0.686	0.603	0.176
mIVST	0.641	0.539	0.710	0.807	0.801	0.969
mLVWT	0.969	0.444	0.591	0.972	0.955	0.464
CWT	0.971	0.085	0.809	0.814	0.230	0.750
PC1	0.805	0.100	0.813	0.937	0.501	0.883

Figure 3.10 Single polymorphism association results for REN. *Bar graph indicates –log10 transformed p-values for the tests of association between REN variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Table 3.9 contains the p-values for interaction between the HCM mutation group and the respective genotypes for the variants investigated in *REN*, *RENBP*, *ATP6AP2* and *M6PR*, illustrating the differences in allelic effect of the particular variants between these groups.

The T-allele of rs1464816 in *REN* was associated with a 42.55 g lower effect on LVM in the R92W$_{TNNT2}$ group versus the R403W$_{MYH7}$ group (p = 0.049) (Table 3.9). Similarly, the G-allele of rs10900555 (*REN*) was found to result in a 0.05 higher PC1 in the R403W$_{MYH7}$ group versus the A797T$_{MYH7}$ group (p = 0.046).

Table 3.9 The p-values for interaction between the HCM mutation group and REN, RENBP, ATP6AP2 or M6PR genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
REN	**rs3795575**	0.267	0.500	0.631	0.833	0.774	0.943	0.757	0.636	0.414	0.969	0.947	0.979	0.736	0.486	0.732	0.544	0.498	0.965
	rs1464816	**0.049**	0.153	0.264	0.172	0.523	0.270	0.245	0.999	0.156	0.367	0.186	0.919	0.346	0.356	0.746	0.444	0.340	0.931
	rs11571082	0.846	0.550	0.491	0.356	0.350	0.955	0.309	0.959	0.328	0.350	0.570	0.699	0.948	0.641	0.662	0.827	0.657	0.871
	rs5705	0.845	0.989	0.838	0.390	0.846	0.499	0.205	0.686	0.107	0.966	0.424	0.448	0.437	0.549	0.204	0.569	0.637	0.338
	rs10900555	0.976	0.225	0.265	0.990	0.692	0.733	0.812	0.734	0.577	0.508	0.463	0.174	0.508	0.163	0.060	0.431	0.168	**0.046**
	rs6682082	0.588	0.364	0.183	0.360	0.187	0.747	0.942	0.787	0.864	0.897	0.613	0.574	0.533	0.837	0.703	0.410	0.598	0.774
RENBP	**rs2269370**	0.578	0.428	0.804	0.945	0.432	0.428	0.758	0.066	0.149	0.384	0.494	0.866	0.902	0.079	0.089	0.609	0.198	0.106
	rs2269372	0.218	0.680	0.394	0.201	0.330	0.656	0.203	0.207	0.880	0.125	0.730	0.214	0.347	0.121	0.725	0.452	0.187	0.730
	rs762656	0.819	0.738	0.944	0.985	0.650	0.682	0.858	0.351	0.522	0.429	0.691	0.658	0.816	0.240	0.240	0.699	0.276	0.208
ATP6AP2	**rs2968915**	0.718	0.823	0.562	0.955	0.290	0.299	0.697	0.131	0.319	0.630	0.287	0.630	0.916	0.286	0.388	0.946	0.321	0.333
	rs2968917	0.239	0.760	0.146	0.331	0.077	**0.020**	0.362	0.097	**0.029**	0.780	0.227	0.234	0.293	0.166	**0.039**	0.225	0.210	**0.033**
	rs10536	0.591	0.756	0.537	0.664	0.963	0.833	0.869	0.642	0.577	0.982	0.691	0.682	0.817	0.715	0.820	0.915	0.773	0.824
M6PR	**rs1805725**	0.558	0.232	0.573	0.173	0.071	0.742	0.097	**0.020**	0.536	0.151	0.464	0.460	0.340	0.067	0.370	0.308	0.072	0.420
	rs987917	0.599	0.955	0.574	0.152	0.121	0.964	0.130	0.079	0.920	0.502	0.512	0.214	0.431	0.147	0.566	0.517	0.271	0.697

Abbreviations: ***ATP6AP2***: ATPase, H+ transporting, lysosomal accessory protein 2 gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; ***M6PR***: mannose-6-phosphate receptor gene; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; ***REN*****:** renin gene; ***RENBP*****:** renin binding protein gene

Figure 3.11 depicts a graphical summary of the haplotype association results. When the *REN* variants were analysed as a haplotype, we found a significant association between haplotype 4 (GGTGGC) and LVM, despite the fact that the single SNP association analysis yielded no significant results (Figure 3.11).

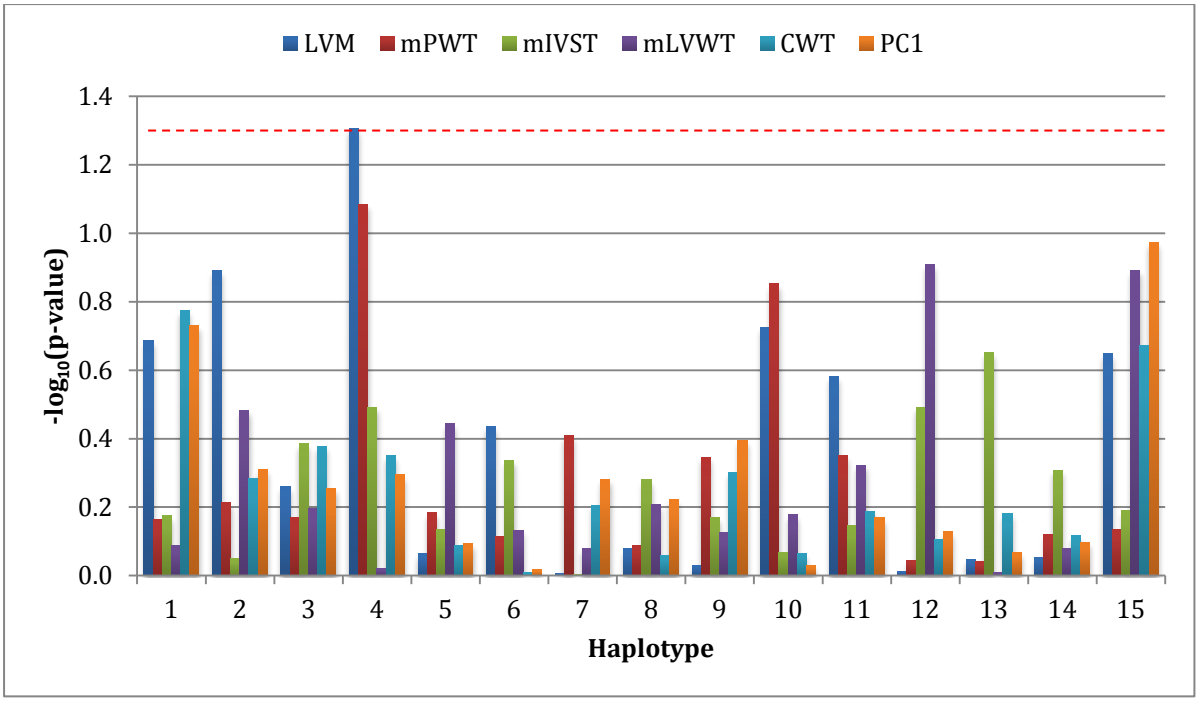


Figure 3.11 Summary of haplotype association results for REN. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of $p < 0.05$.*

Table 3.10 depicts the haplotype distribution observed for the *REN* gene, as well as the respective p-values for tests of association for these haplotypes. Haplotype 4 (GGTGGC) was observed in 6.3% of the entire cohort and associated significantly with a 29.10 g decrease in LVM ($p = 0.049$).

Table 3.10 Haplotype distribution within REN, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs3795575	rs1464816	rs11571082	rs5705	rs10900555	rs6682082	freq.	p-value for test of association					
								LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	G	T	C	T	A	C	0.157	0.206	0.686	0.669	0.819	0.168	0.185
2	G	G	C	T	G	C	0.155	0.128	0.610	0.894	0.329	0.520	0.489
3	G	G	C	T	A	C	0.132	0.549	0.676	0.411	0.635	0.419	0.557
4	G	G	T	G	G	C	0.063	**0.049**	0.082	0.323	0.951	0.447	0.508
5	G	G	C	T	A	T	0.059	0.864	0.653	0.735	0.360	0.818	0.803
6	A	G	T	G	G	C	0.051	0.367	0.767	0.462	0.739	0.983	0.962
7	G	T	C	T	G	C	0.026	0.985	0.389	0.993	0.834	0.623	0.525
8	G	G	C	G	G	T	0.022	0.835	0.818	0.526	0.620	0.873	0.600
9	G	G	C	G	A	T	0.015	0.934	0.453	0.676	0.746	0.498	0.402
10	G	G	C	G	G	C	0.013	0.188	0.141	0.853	0.664	0.861	0.935
11	A	G	C	G	G	C	0.011	0.262	0.446	0.716	0.477	0.650	0.679
12	G	T	C	T	A	T	0.009	0.972	0.900	0.324	0.123	0.785	0.746
13	A	G	C	G	G	T	0.009	0.897	0.910	0.223	0.978	0.658	0.856
14	G	T	T	G	G	C	0.009	0.887	0.758	0.493	0.831	0.764	0.799
15	G	T	C	G	G	T	0.008	0.224	0.734	0.643	0.129	0.212	0.106

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.11 contains the p-values for the interaction between the *REN* haplotypes and the three different HCM mutation groups as an indication of the difference in effect of the particular haplotypes between the groups. Haplotypes 4, as well as 9 to 15 could unfortunately not be tested due to unequal distribution of these haplotypes in the three mutation groups, which resulted in stratified sample groups that were too small to test.

Interestingly, we found that when the T-allele of rs146816 was present in a haplotype, the differential effect between HCM mutation groups persisted (Table 3.11). Haplotype 1 was associated with a 50.33 g lower effect on LVM in the R92W$_{TNNT2}$ group compared to the R403W$_{MYH7}$ group (p = 0.025). On the other hand, haplotype 7 associated with a significantly higher effect of 4.05 mm on the CWT score (p = 0.050) and a 0.25 higher PC1 (p = 0.049) in the R92W$_{TNNT2}$ group compared to the R403W$_{MYH7}$ group.

Table 3.11 The p-values for interaction between the HCM mutation group and REN haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

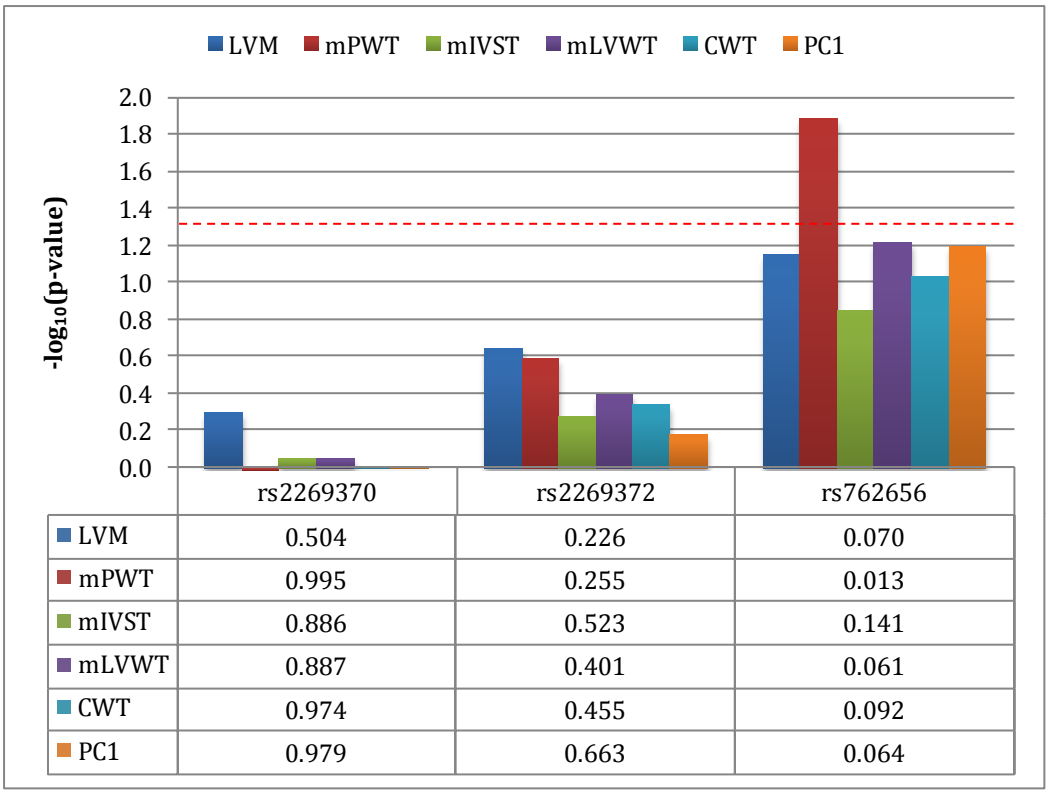
Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	**0.025**	0.266	0.070	0.507	0.921	0.374	0.358	0.787	0.186	0.269	0.051	0.819	0.318	0.259	0.762	0.388	0.213	0.958
2	0.729	0.212	0.395	0.124	0.214	0.539	0.144	0.320	0.438	0.929	0.711	0.788	0.515	0.190	0.544	0.707	0.181	0.325
3	0.464	0.767	0.270	0.595	0.919	0.485	0.903	0.363	0.288	0.632	0.705	0.358	0.628	0.412	0.186	0.500	0.553	0.196
5	0.441	0.689	0.399	0.127	0.719	0.565	0.481	0.842	0.815	0.343	0.511	0.232	0.300	0.825	0.708	0.216	0.866	0.592
6	0.910	0.090	0.112	0.293	0.972	0.266	0.258	0.403	0.060	0.717	0.422	0.286	0.794	0.339	0.299	0.893	0.349	0.370
7	0.219	0.842	0.105	0.539	0.405	0.119	0.358	0.699	0.149	0.838	0.579	0.420	**0.050**	0.602	0.101	**0.049**	0.585	0.104
8	0.393	0.064	0.402	0.242	0.141	0.909	0.131	0.090	0.957	0.799	0.339	0.526	0.265	0.086	0.681	0.565	0.391	0.856

*Haplotypes 4 and 9-15 not tested

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.2.2 Renin-binding protein (*RENBP*)

There was no statistically significant difference between the HCM mutations groups with regards to the effect of *RENBP* polymorphisms on the investigated hypertrophy traits (Table 3.9). However, the A-allele of rs762656 in *RENBP* was found to significantly decrease mPWT by 0.511 mm (p = 0.013) as shown in Figure 3.12.



	rs2269370	rs2269372	rs762656
LVM	0.504	0.226	0.070
mPWT	0.995	0.255	0.013
mIVST	0.886	0.523	0.141
mLVWT	0.887	0.401	0.061
CWT	0.974	0.455	0.092
PC1	0.979	0.663	0.064

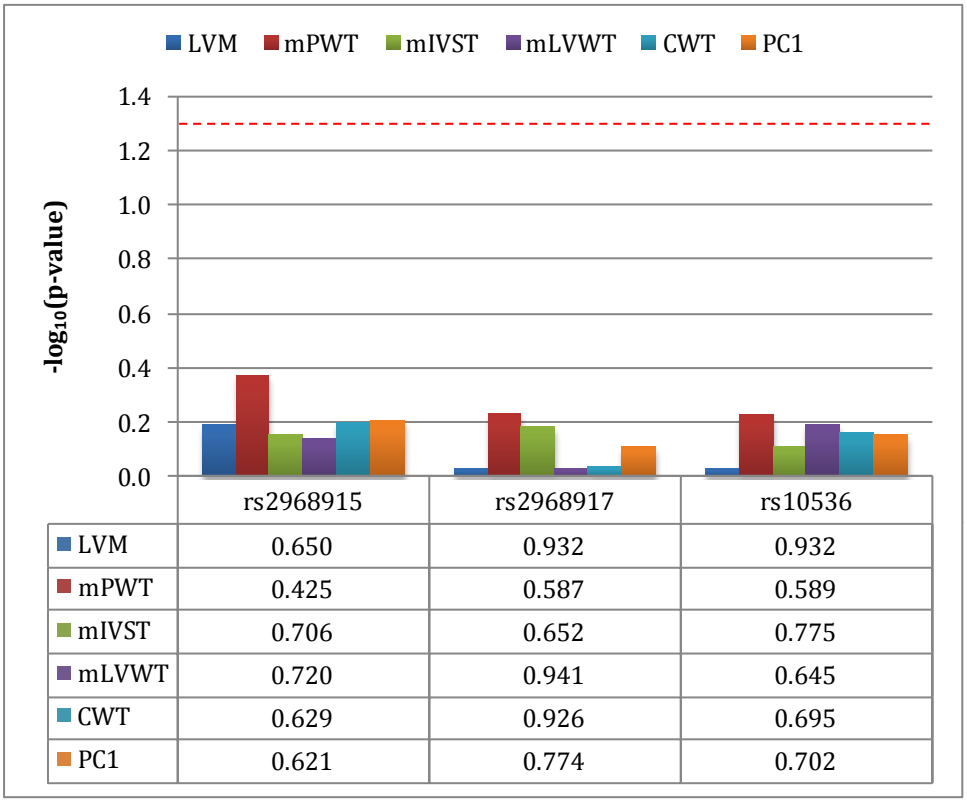
Figure 3.12 Single polymorphism association results for RENBP. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between RENBP variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

3.7.2.3 (Pro)renin receptor (*ATP6AP2*)

We found no statistically significant evidence for association between the investigated *ATP6AP2* variants and any of the hypertrophy traits (Figure 3.13).

However, we observed a difference in effect size caused by the rs2968917 polymorphism in *ATP6AP2* (Table 3.9). The C-allele of this polymorphism was associated with a significantly increased effect of 4.19 mm on mIVST (p = 0.020), 4.18 mm on mLVWT (p = 0.029), 1.81 mm on

the CWT score (p = 0.039), as well as 0.11 on PC1 (p = 0.033) in the R403W$_{MYH7}$ group compared to the A797T$_{MYH7}$ group.

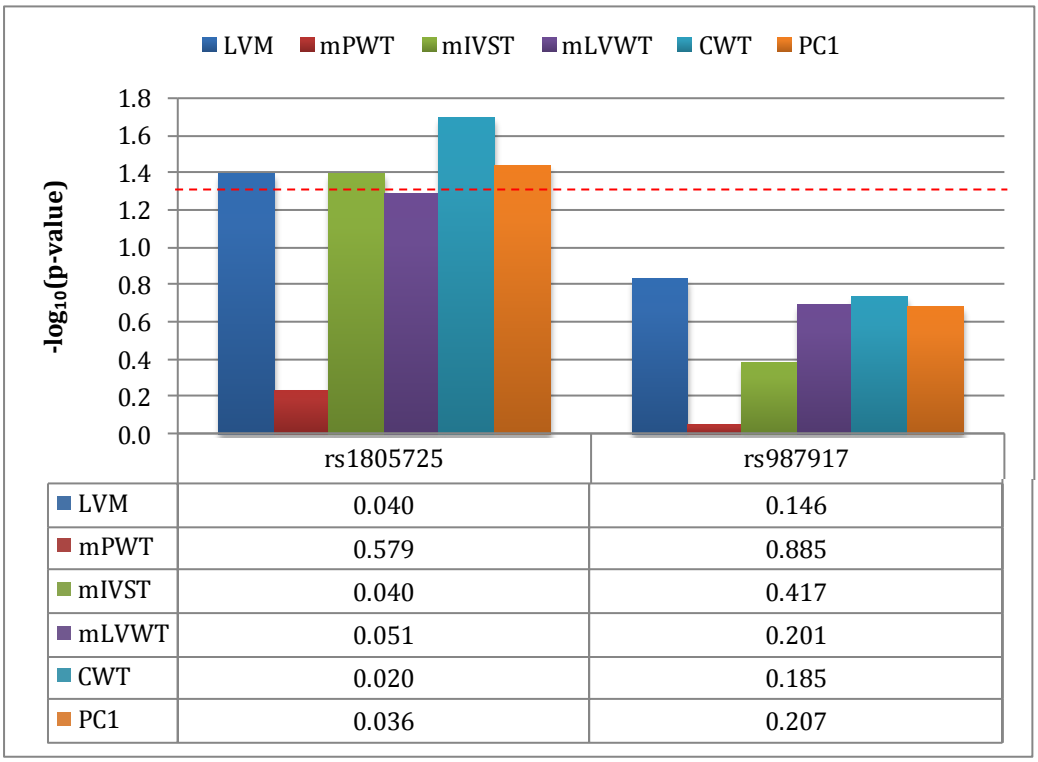


	rs2968915	rs2968917	rs10536
LVM	0.650	0.932	0.932
mPWT	0.425	0.587	0.589
mIVST	0.706	0.652	0.775
mLVWT	0.720	0.941	0.645
CWT	0.629	0.926	0.695
PC1	0.621	0.774	0.702

Figure 3.13 Single polymorphism association results for ATP6AP2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between ATP6AP2 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

3.7.2.4 Mannose-6-Phosphate Receptor (*M6PR*)

The C-allele of rs1805725 in *M6PR* was significantly associated with a 15.1 g decrease in LVM (p = 0.040), a 1.28 mm decrease in mIVST (p = 0.040), a 0.62 mm decrease in CWT score (p = 0.020), as well as a 0.04 mm decrease in PC1 (p = 0.036) as indicated in Figure 3.14. However, this allele was associated with a 2.99 mm lower effect on mLVWT in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (Table 3.9).



	rs1805725	rs987917
LVM	0.040	0.146
mPWT	0.579	0.885
mIVST	0.040	0.417
mLVWT	0.051	0.201
CWT	0.020	0.185
PC1	0.036	0.207

Figure 3.14 Single polymorphism association results for M6PR. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between M6PR variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Two haplotypes in *M6PR* were found to associate significantly with hypertrophy traits (Figure 3.15). Interestingly, both of these contain a C-allele for the rs987917 SNP (Table 3.12). When this C-allele is accompanied by an A-allele at rs1805725, the resulting haplotype is associated with a 0.67 mm increase in mLVWT ($p = 0.049$). However, when the rs987917 C-allele is accompanied by a C-allele at rs1805725, the resulting haplotype is associated with a 44.90 g decrease in LVM ($p = 0.013$), a 3.73 mm decrease in mIVST ($p = 0.002$), a 3.39 mm decrease in mLVWT ($p = 0.006$), a 1.59 mm decrease in CWT score ($p = 0.009$), as well as a 0.10 decrease in PC1 ($p = 0.020$). This haplotype was, however, only observed in 1.7% of the cohort.

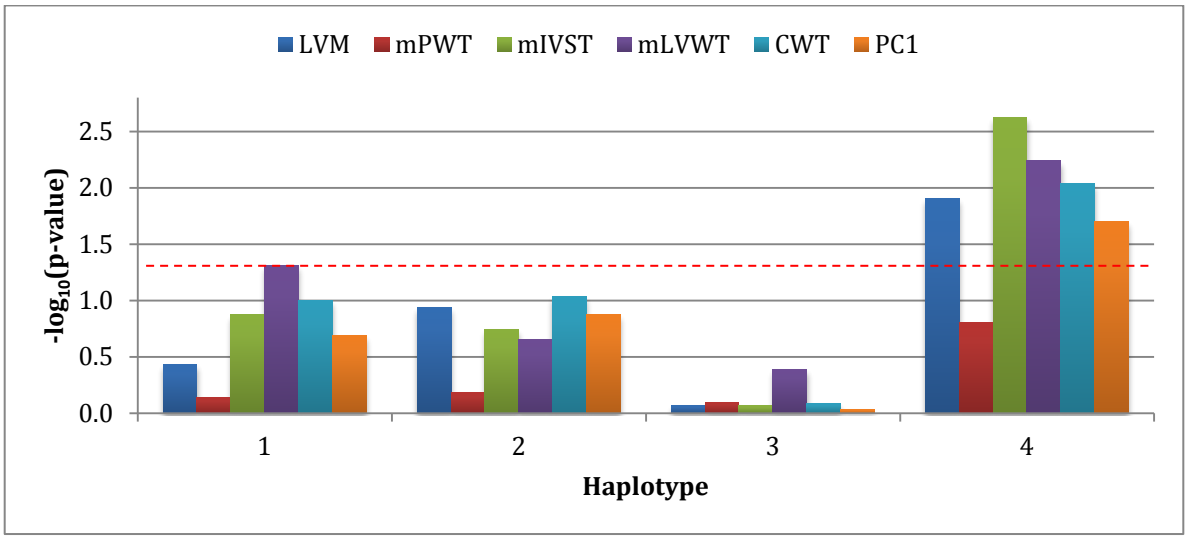


Figure 3.15 Summary of haplotype association results for M6PR. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of $p < 0.05$ and effect sizes for significant associations are indicated in the text.*

Table 3.12 Haplotype distribution within M6PR, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Significant p-values indicated in bold red font and effect sizes discussed in the text.*

	rs1805725	rs987917	freq.	p-value for test of association					
				LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	C	0.422	0.368	0.134	**0.049**	0.725	0.100	0.205
2	C	A	0.117	0.115	0.180	0.220	0.655	0.092	0.132
3	A	A	0.109	0.852	0.856	0.405	0.798	0.815	0.929
4	C	C	0.017	**0.013**	**0.002**	**0.006**	0.158	**0.009**	**0.020**

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

A haplotype containing the C-allele of rs1805725 and the A-allele of rs987917, i.e. haplotype 2, showed a significant difference in effect on three hypertrophy traits in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (Table 3.13). This haplotype was associated with a 3.23 mm lower effect on mLVWT (p = 0.012), a 1.8 mm lower effect on the CWT score (p = 0.016), as well as a 0.11 lower effect on PC1 (p = 0.027). Haplotype 4 (which also contains the rs1805725 C-allele) was unfortunately not tested due to the low allele frequency of this haplotype.

Table 3.13 The p-values for interaction between HCM mutation group and M6PR haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.615	0.811	0.773	0.126	0.207	0.647	0.142	0.256	0.629	0.564	0.282	0.144	0.688	0.618	0.981	0.773	0.779	0.963
2	0.907	0.463	0.546	0.375	0.105	0.498	0.166	**0.012**	0.255	0.221	0.417	0.663	0.311	**0.016**	0.126	0.302	**0.027**	0.191
3	0.533	0.192	0.099	0.267	0.801	0.367	0.476	0.823	0.375	0.928	0.095	0.160	0.513	0.692	0.347	0.694	0.527	0.385

*Haplotype 4 not tested

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.3 Angiotensin converting enzymes

Figure 3.16 depicts the chromosomal location and structure of the *ACE* gene, as well as intragenic location of target polymorphisms. Table 3.14 shows the D' values determined for *ACE*. Complete LD was observed between rs4340 and rs4298, rs4340 and rs4303, as well as rs4340 and rs4356, while incomplete LD exists between rs4298 and rs4303. Very little evidence for LD was observed between the remaining variants genotyped in *ACE*.

Insufficient evidence for LD was observed between rs1978124 and rs879922 (D' = 0.08), while the other polymorphisms investigated in *ACE2* appear to be in complete LD as D' = 1 for the remaining SNP pairs (Table 3.15).

Figure 3.18 depicts the chromosomal location and structure for *CMA1,* as well as intragenic location of the investigated polymorphisms. The two SNPs investigated in *CMA1* were in complete LD (D' = 1).

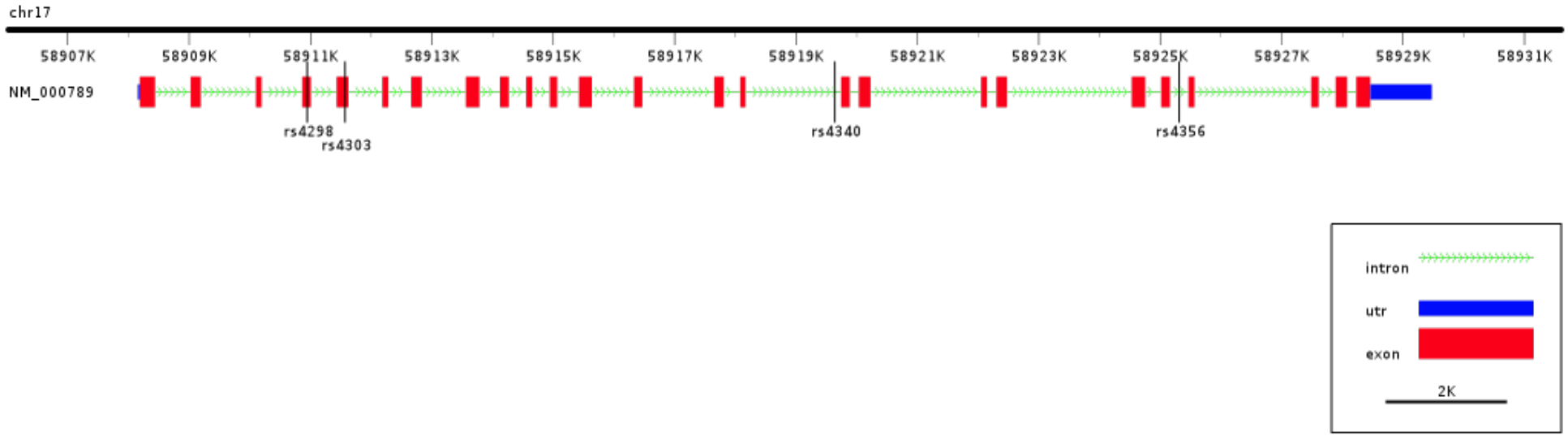


Figure 3.16 Scale diagram depicting chromosomal location and structure of the ACE gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.14 Pairwise D' values as a representation of the observed LD structure within ACE in the present cohort.

	rs4298	rs4303	rs4340	rs4356
rs4298		0.75	1	0.24
rs4303			1	0.26
rs4340				1
rs4356				

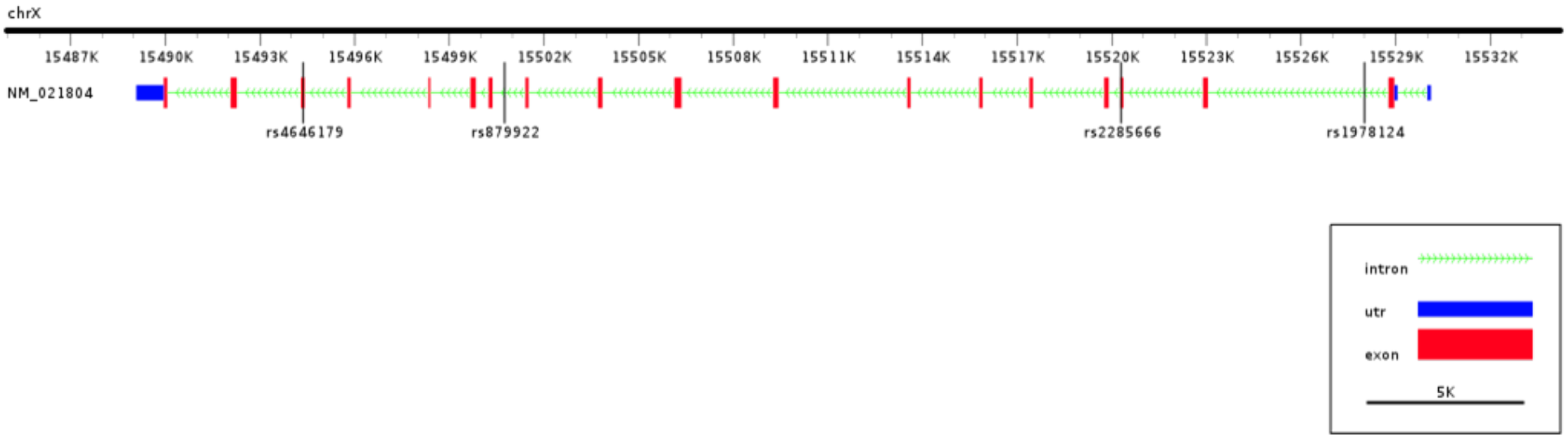


Figure 3.17 Scale diagram depicting chromosomal location and structure of the ACE2 gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.15 Pairwise D' values as a representation of the observed LD structure within ACE2 in the present cohort.

	rs1978124	rs2285666	rs879922	rs4646179
rs1978124		1	0.08	1
rs2285666			1	1
rs879922				1
rs4646179				

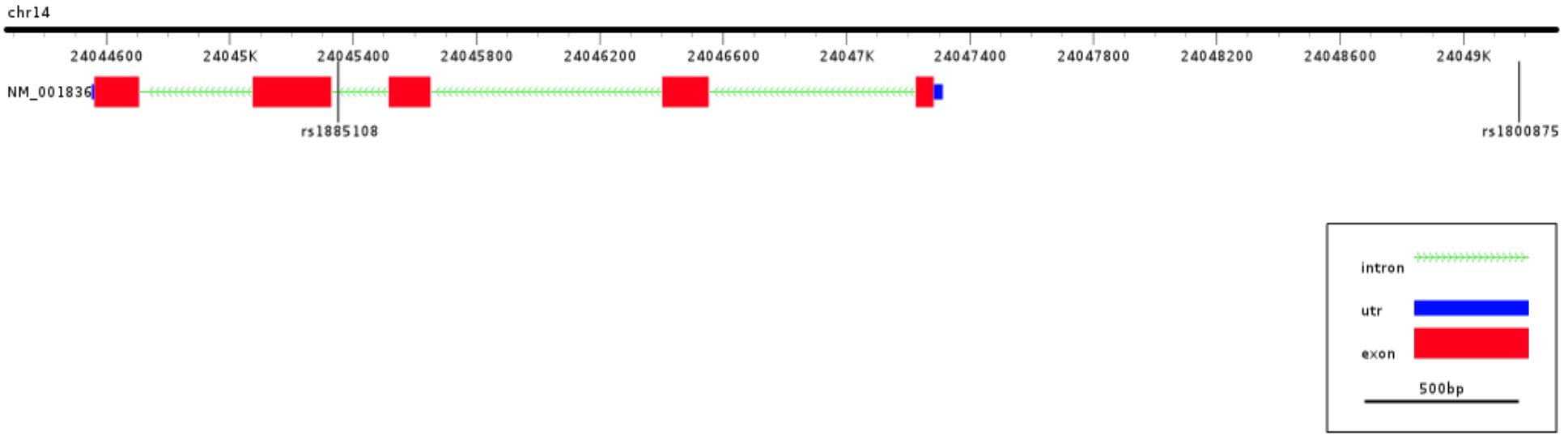
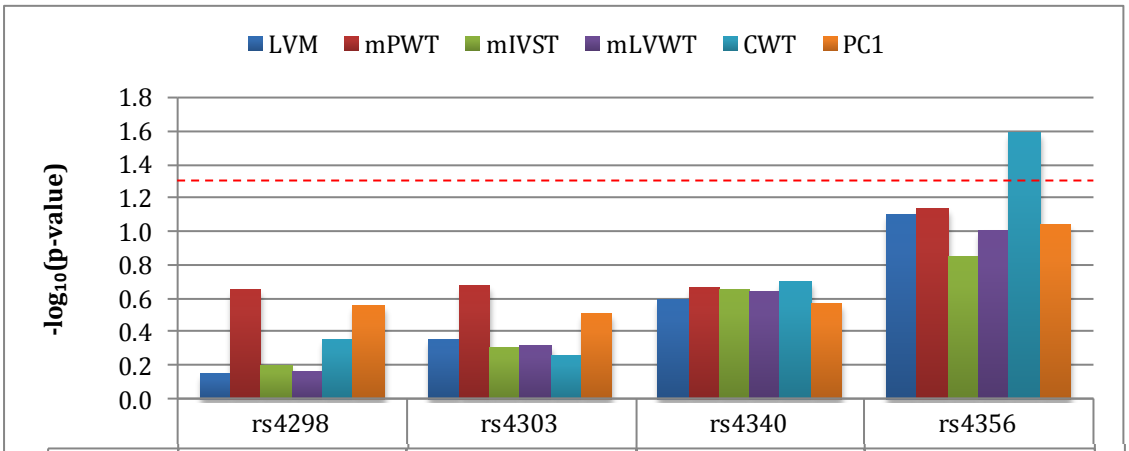


Figure 3.18 Scale diagram depicting chromosomal location and structure of the CMA1 gene, as well as intragenic location of target polymorphisms. *The two investigated SNPs were in complete LD (D' = 1). Arrows indicate direction of transcription.*

3.7.3.1 Angiotensin converting enzyme 1 (*ACE*)

The C-allele of one SNP in *ACE*, rs4356, was associated with a significant CWT score increase of 0.743 mm (Figure 3.19).



	rs4298	rs4303	rs4340	rs4356
LVM	0.700	0.442	0.254	0.079
mPWT	0.226	0.211	0.220	0.073
mIVST	0.638	0.498	0.225	0.141
mLVWT	0.682	0.483	0.227	0.098
CWT	0.443	0.544	0.198	0.026
PC1	0.280	0.313	0.268	0.091

Figure 3.19 Single polymorphism association results for ACE. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between ACE variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Table 3.16 depicts the p-values for interaction between HCM mutation group and *ACE*, *ACE2* or *CMA1* genotypes, illustrating the differences in allelic effect of the particular variants between these groups.

The T-allele of rs4303 was found to differentially affect the HCM mutation groups (Table 3.16). The effect of the addition of this T-allele on mPWT was 0.93 mm higher in the R92W$_{TNNT2}$ group, when compared to the R403W$_{MYH7}$ group, but 2.22 mm lower in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group. There was, however, no statistically significant difference between the effect of this allele on the R92W$_{TNNT2}$ and A797T$_{MYH7}$ groups.

Table 3.16 The p-values for interaction between HCM mutation group and ACE, ACE2 or CMA1 genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			**mIVST**			**mLVWT**			**mPWT**			**CWT score**			**PC1**		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
ACE	rs4340	0.556	0.773	0.416	0.640	0.471	0.282	0.896	0.553	0.520	0.512	0.870	0.632	0.676	0.484	0.852	0.712	0.642	0.970
	rs4298	0.771	0.646	0.754	0.367	0.704	0.555	0.399	0.704	0.602	0.297	0.337	0.933	0.184	0.255	0.983	0.120	0.131	0.805
	rs4303	0.255	0.652	0.165	0.556	0.591	0.297	0.308	0.957	0.448	**0.050**	0.406	**0.015**	0.088	0.724	0.085	0.071	0.687	0.064
	rs4356	0.986	0.183	0.191	0.488	0.291	0.746	0.460	0.388	0.943	0.380	0.628	0.649	0.721	0.198	0.452	0.722	0.234	0.502
ACE2*	rs1978124	0.142	0.056	0.978	0.548	0.733	0.691	0.825	0.828	0.949	0.642	0.468	0.915	0.426	0.490	0.764	0.464	0.517	0.791
	rs2285666	0.306	0.111	0.648	0.283	0.316	0.886	0.369	0.164	0.680	**0.046**	**0.027**	0.958	0.329	0.079	0.468	0.291	0.059	0.438
	rs879922	0.476	0.339	0.989	0.154	0.129	0.768	0.102	0.155	0.546	0.139	**0.041**	0.936	0.148	0.169	0.630	0.329	0.122	0.870
CMA1	rs1885108	0.983	0.960	0.943	0.950	0.958	0.991	0.846	0.817	0.670	0.958	0.792	0.835	0.905	0.702	0.803	0.890	0.744	0.654
	rs1800875	0.523	0.404	0.170	0.630	0.528	0.300	0.681	0.496	0.318	0.497	0.997	0.459	0.597	0.675	0.355	0.723	0.753	0.516

* rs4646179 not tested due to low allele frequency

Abbreviations: ***ACE*****:** Angiotensin converting enzyme 1 gene; ***ACE2*****:** Angiotensin converting enzyme 2 gene; ***CMA1:*** cardiac chymase gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

No statistically significant evidence for association was observed between the identified haplotypes and the respective hypertrophy traits (Figure 3.20). Table 3.17 contains the exact p-values for the tests of association, as well as the haplotype distribution observed for *ACE*.

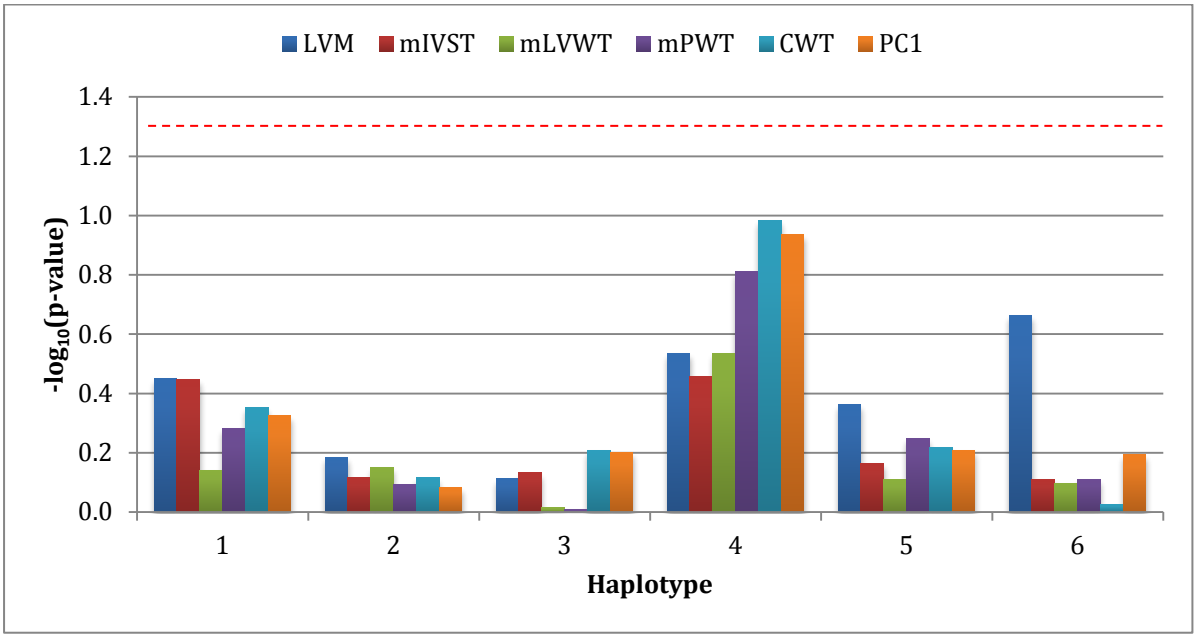


Figure 3.20 Summary of haplotype association results for ACE. *The bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of $p < 0.05$.*

Table 3.17 Haplotype distribution within ACE, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs4298	rs4303	rs4340	rs4356	freq.	p-value for test of association					
						LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	C	G	D	T	0.279	0.354	0.357	0.723	0.523	0.443	0.474
2	C	G	I	T	0.195	0.654	0.768	0.706	0.810	0.768	0.827
3	C	T	D	T	0.064	0.774	0.738	0.968	0.980	0.620	0.630
4	C	G	I	C	0.052	0.292	0.349	0.292	0.155	0.104	0.116
5	C	G	D	C	0.051	0.435	0.685	0.780	0.566	0.605	0.619
6	T	T	D	T	0.020	0.217	0.779	0.799	0.775	0.945	0.641

Abbreviations: **C:** cytosine; **CWT:** cumulative wall thickness score; **D:** *Alu* deletion; **G:** guanine; **I:** *Alu* insertion; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.18 contains the p-values for interaction between the different HCM mutation groups and *ACE* haplotypes to illustrate the differences in allelic effect of the different haplotypes between these groups. Haplotype 6 was not tested due to low allele frequencies.

Haplotype 3 was associated with a 2.52 mm lower effect on mPWT (p = 0.010), a 2.29 mm lower effect on the CWT score (p = 0.029), as well as a 0.14 lower effect on PC1 in the $R403W_{MYH7}$ group, compared to the $A797T_{MYH7}$ group. Conversely, this haplotype had a 1.80 mm increased effect on the CWT score (p = 0.033), as well as a 0.11 increased effect on PC1 (p = 0.033) in the $R92W_{TNNT2}$ group, compared to the $R403W_{MYH7}$ group. Haplotype 4 was associated with a 2.38 mm increased effect on mPWT (p = 0.017) in the $R92W_{TNNT2}$ group, compared to the $R403W_{MYH7}$ group.

Table 3.18 The p-values for interaction between the HCM mutation group and ACE haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

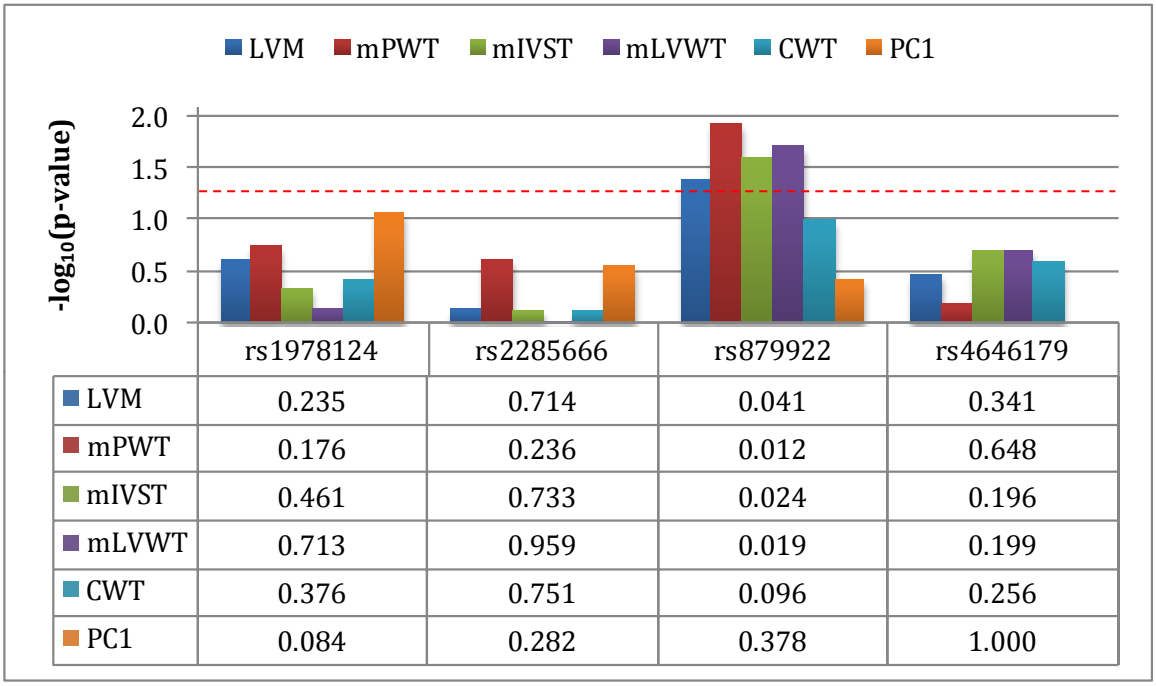
Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.060	0.287	0.282	0.123	0.414	0.352	0.175	0.158	0.806	0.633	0.925	0.568	0.066	0.273	0.283	0.109	0.290	0.390
2	0.619	0.954	0.591	0.283	0.556	0.111	0.436	0.582	0.203	0.328	0.646	0.601	0.691	0.534	0.345	0.943	0.512	0.508
3	0.110	0.715	0.080	0.327	0.473	0.119	0.227	0.850	0.219	0.180	0.152	**0.010**	**0.033**	0.694	**0.029**	**0.033**	0.539	**0.017**
4	0.785	0.496	0.627	0.728	0.900	0.548	0.542	0.919	0.364	**0.017**	0.085	0.327	0.637	0.675	0.885	0.412	0.445	0.835
5	0.614	0.532	0.420	0.989	0.286	0.603	0.895	0.359	0.569	0.545	0.981	0.534	0.978	0.233	0.581	0.835	0.348	0.507

*Haplotype 6 not tested due to low allele frequency

Abbreviations: ***CMA1:*** cardiac chymase gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.3.2 Angiotensin converting enzyme 2 (*ACE2*)

The rs879922 SNP in *ACE2* was significantly associated with four hypertrophy traits, viz. LVM, mPWT, mIVST and mLVWT (Figure 3.21). The G-allele of this polymorphism was found to increase LVM by 13.70 g (p = 0.041), mPWT by 0.62 mm (p = 0.012), mIVST by 1.59 mm (p = 0.024) and mLVWT by 1.68 mm (p = 0.019).



	rs1978124	rs2285666	rs879922	rs4646179
LVM	0.235	0.714	0.041	0.341
mPWT	0.176	0.236	0.012	0.648
mIVST	0.461	0.733	0.024	0.196
mLVWT	0.713	0.959	0.019	0.199
CWT	0.376	0.751	0.096	0.256
PC1	0.084	0.282	0.378	1.000

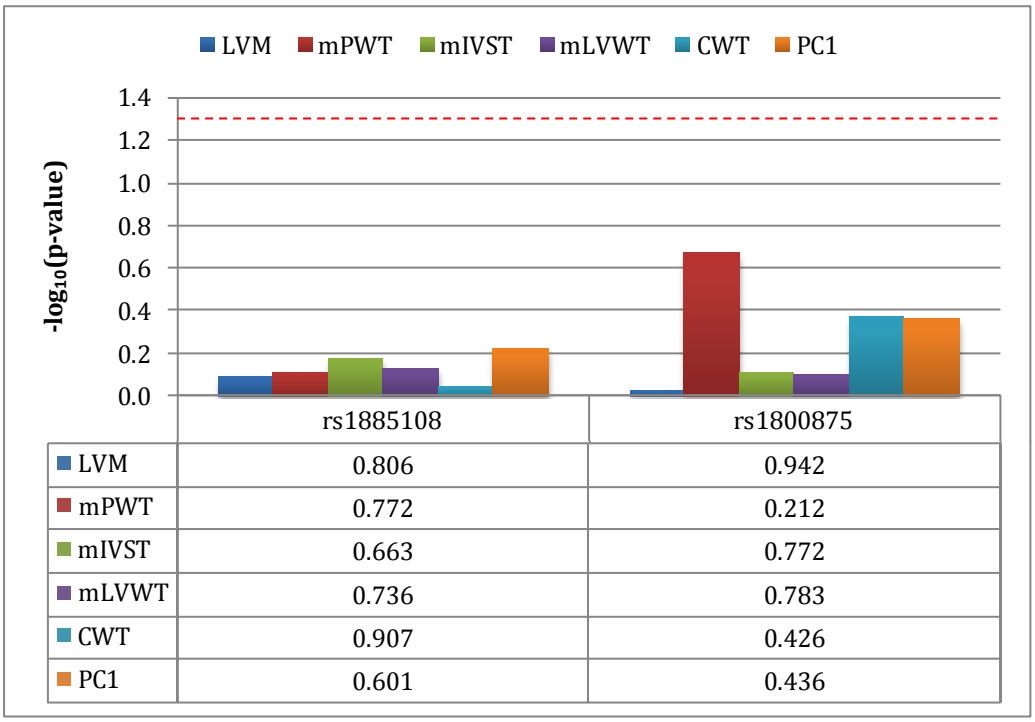
Figure 3.21 Single polymorphism association results for ACE2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between ACE2 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

The effect of the G-allele of rs879922 in *ACE2* on mPWT was 1.20 mm lower in the R92W$_{TNNT2}$ group when compared to the A797T$_{MYH7}$ group (p = 0.041), while the effect of the rs2285666 T-allele on mPWT was 1.02 mm lower in the R92W$_{TNNT2}$ group when compared to the R403W$_{MYH7}$ group (p = 0.046) and 0.99 mm lower when compared to the A797T$_{MYH7}$ group (p = 0.027) (Table 3.16).

3.7.3.3 Cardiac chymase (*CMA1*)

No statistically significant differences in effect were observed for the variants investigated in *CMA1* (Table 3.16). Similarly, we did not find any statistically significant evidence for

association between *CMA1* variants any of the heritable hypertrophy traits investigated (Figure 3.22).



	rs1885108	rs1800875
LVM	0.806	0.942
mPWT	0.772	0.212
mIVST	0.663	0.772
mLVWT	0.736	0.783
CWT	0.907	0.426
PC1	0.601	0.436

Figure 3.22 Single polymorphism association results for CMA1. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between CMA1 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

We also did not find any significant evidence for association between the identified *CMA1* haplotypes and the investigated hypertrophy traits (Figure 3.23 and Table 3.19).

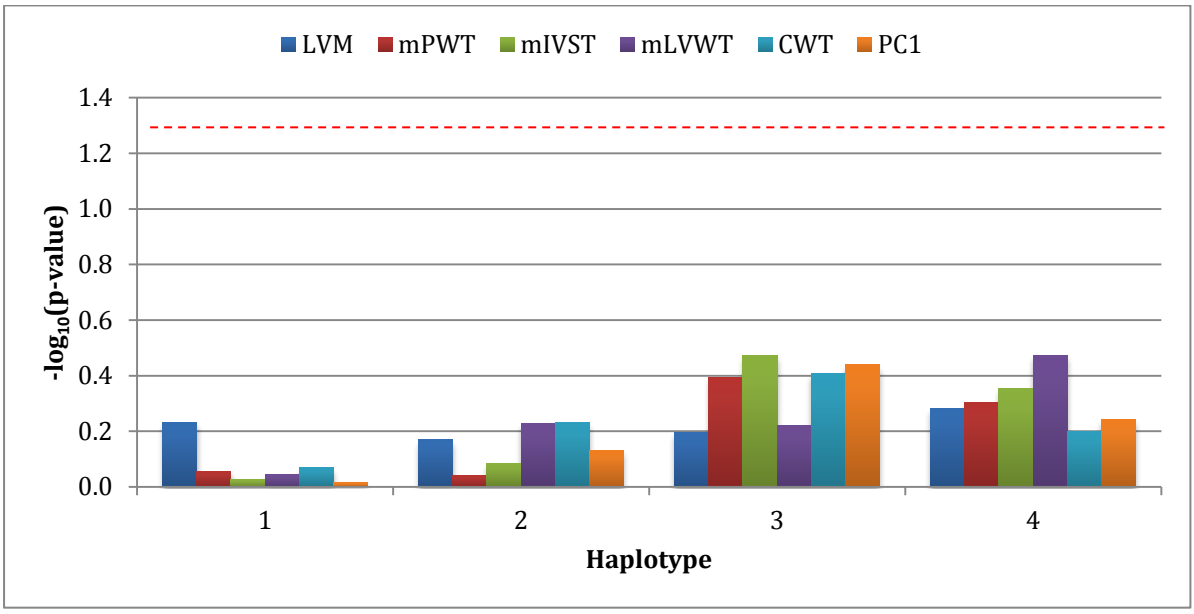


Figure 3.23 Summary of haplotype association results for CMA1. *Bar graph indicates –log 10 transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of p < 0.05.*

Table 3.19 Haplotype distribution within CMA1, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs1885108	rs1800875	freq.	p-value for test of association					
				LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	A	0.268	0.586	0.880	0.941	0.900	0.854	0.964
2	T	A	0.223	0.677	0.909	0.822	0.593	0.586	0.739
3	A	T	0.210	0.636	0.404	0.336	0.601	0.390	0.364
4	T	T	0.011	0.521	0.495	0.442	0.337	0.633	0.570

Abbreviations: **A:** adenine; **CWT:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.20 contains the p-values for interaction between the different HCM mutation groups and *CMA1* haplotypes to illustrate the differences in allelic effect of the different haplotypes between these groups. There were no statistically significant differences in effect of identified *CMA1* haplotypes between the three HCM mutation groups.

Table 3.20 The p-values for interaction between the HCM mutation group and CMA1 haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.446	0.280	0.778	0.668	0.348	0.628	0.961	0.343	0.378	0.860	0.828	0.692	0.790	0.257	0.406	0.865	0.234	0.325
2	0.829	0.766	0.945	0.651	0.655	0.973	0.762	0.717	0.969	0.730	0.775	0.933	0.664	0.367	0.664	0.828	0.420	0.571
3	0.713	0.474	0.326	0.902	0.493	0.488	0.716	0.695	0.476	0.200	0.839	0.116	0.627	0.775	0.445	0.773	0.632	0.483

*Haplotype 4 not tested due to low allele frequency

Abbreviations: ***CMA1:*** cardiac chymase gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.4 Angiotensin II Receptors

Figures 3.24 and 3.25 depict the chromosomal location and structure of the *AGTR1* and *AGTR2* genes, as well as intragenic location of target polymorphisms within these genes.

Very little evidence for LD exists between rs5182 and rs2640539 (D' = 0.28), as well as between rs5182 and rs3772627 (D' = 0.02) within *AGTR1*, while an intermediate D' value was observed between rs3772627 and rs2640539 (D' = 0.78) (Figure 3.24).

Complete LD was observed between rs5194 and rs11091046 in *AGTR2* (D' = 1), while a very high degree of LD was observed between rs1403543 and rs5194, as well as rs11091046 (Figure 3.25).

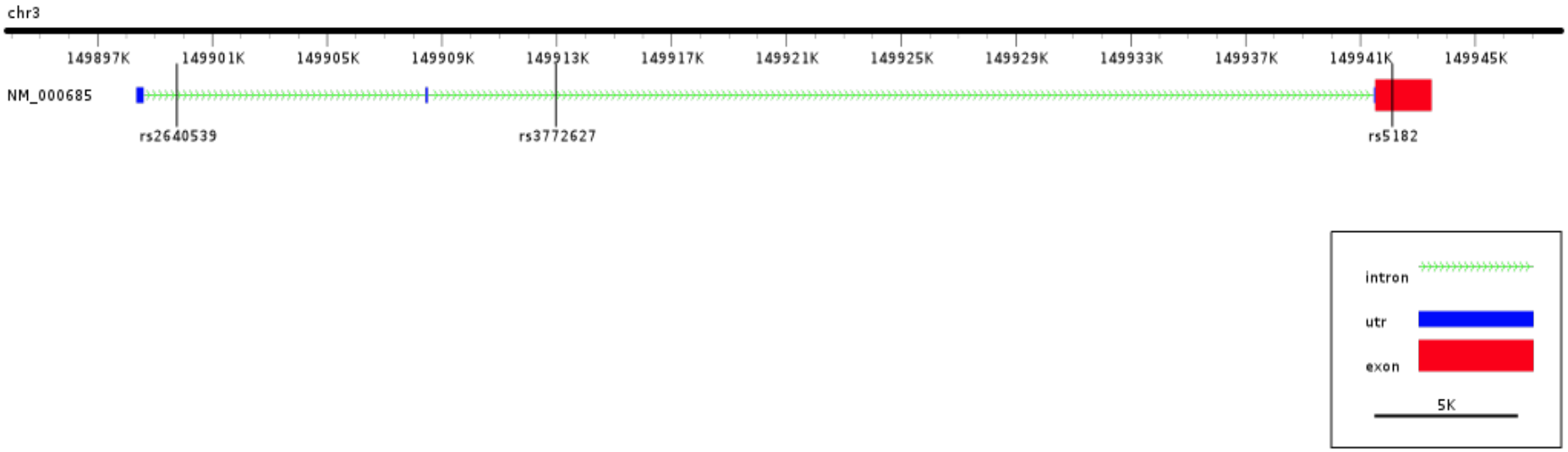


Figure 3.24 Scale diagram depicting chromosomal location and structure of the AGTR1 gene, as well as intragenic location of target polymorphisms. *LD values were D' = 0.78 between rs2640539 and rs3772627, D' = 0.28 between rs2640539 and rs5182 and D' = 0.02 between rs3772627 and rs5182. Arrows indicate direction of transcription.*

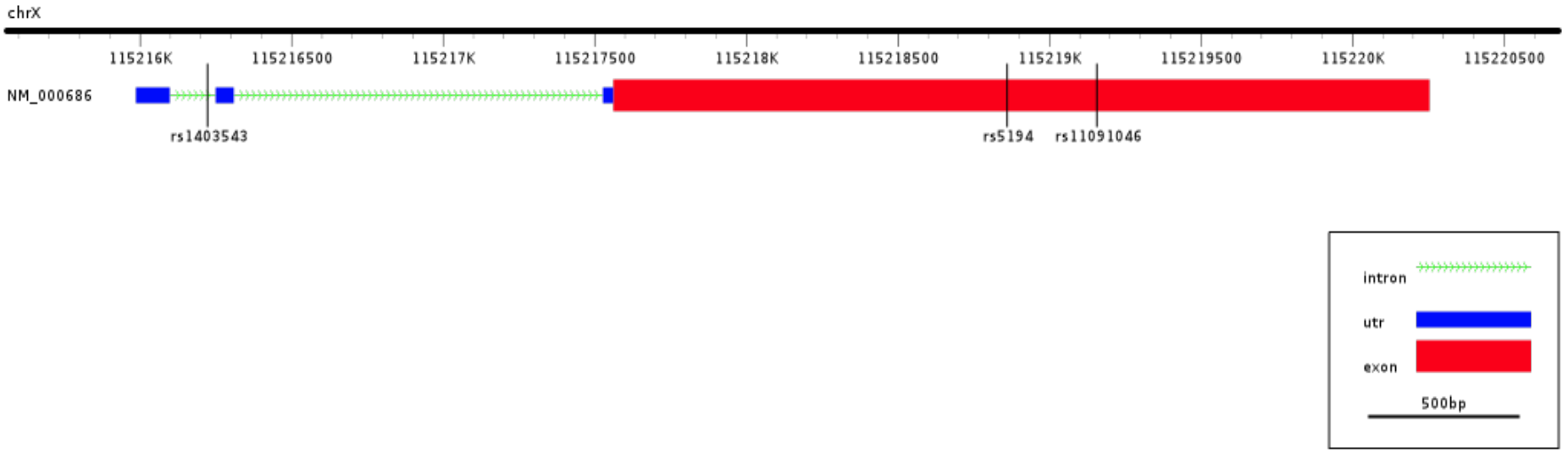
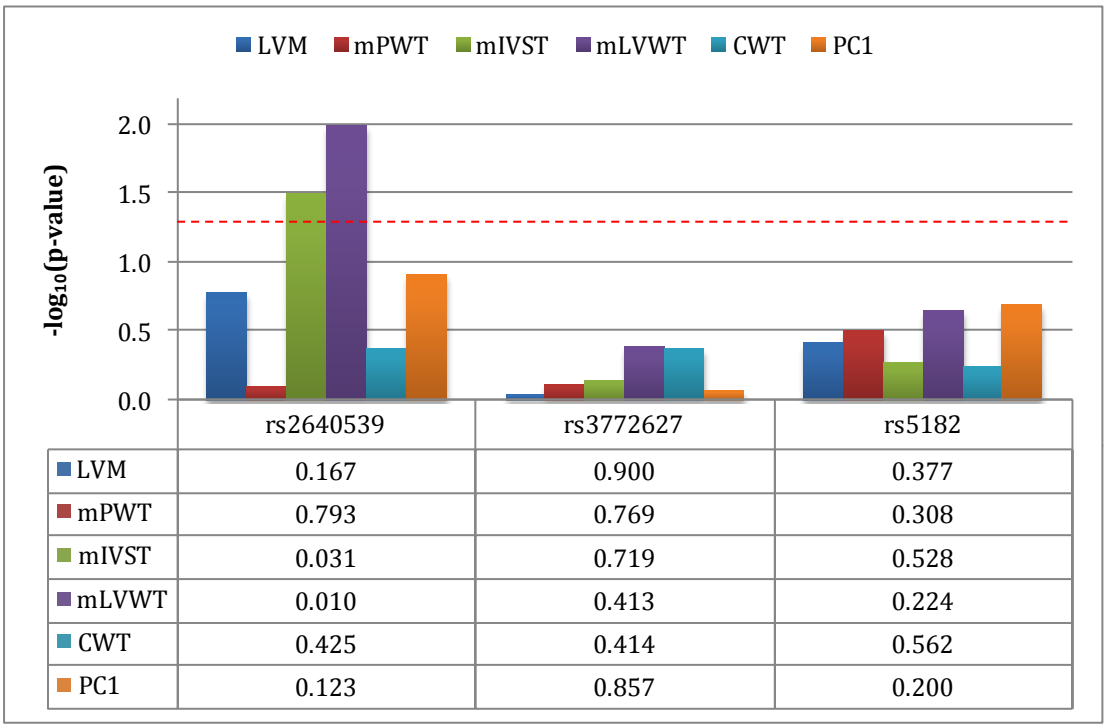


Figure 3.25 Scale diagram depicting chromosomal location and structure of the AGTR2 gene, as well as intragenic location of target polymorphisms. *LD values were D' = 0.86 between rs14035439 and rs5194, D' = 0.86 between rs14035439 and rs11091046 and D' = 1 between rs5194 and rs11091046. Arrows indicate direction of transcription.*

3.7.4.1 Angiotensin II Receptor Type 1 (*AGTR1*)

The C-allele of rs2640539 in *AGTR1* was significantly associated with an increase in mIVST of 1.15 mm (p = 0.031), as well as an increase of 1.22 mm in mLVWT (p = 0.010) (Figure 3.26).



	rs2640539	rs3772627	rs5182
LVM	0.167	0.900	0.377
mPWT	0.793	0.769	0.308
mIVST	0.031	0.719	0.528
mLVWT	0.010	0.413	0.224
CWT	0.425	0.414	0.562
PC1	0.123	0.857	0.200

Figure 3.26 Single polymorphism association results for AGTR1. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between AGTR1 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Table 3.21 depicts the p-values for interaction between HCM mutation groups and *AGTR1* or *AGTR2* genotypes, illustrating the differences in allelic effect of the particular variants between these groups.

Rs3772627 within *AGTR1* showed significantly different effect sizes in the R92W$_{TNNT2}$ group compared to the A797T$_{MYH7}$ group (Table 3.21). The effect of the A-allele of this polymorphism was 19.11 g higher on LVM (p = 0.049), 2.32 mm higher on mLVWT (p = 0.041), 1.59 mm higher on CWT score (p = 0.003) and 0.10 higher on PC1 (p = 0.003) in the R92W$_{TNNT2}$ group compared to the A797T$_{MYH7}$ group (Table 3.21).

Table 3.21 The p-values for interaction between HCM mutation groups and AGTR1 or AGTR2 genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			**mIVST**			**mLVWT**			**mPWT**			**CWT score**			**PC1**		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
AGTR1	rs2640539	0.603	0.494	0.292	0.625	0.897	0.551	0.450	0.783	0.578	0.474	0.277	0.908	0.618	0.912	0.554	0.609	0.772	0.772
	rs3772627	0.472	**0.049**	0.312	0.457	0.062	0.376	0.317	**0.041**	0.446	0.278	0.176	0.972	0.369	**0.003**	0.077	0.546	**0.003**	**0.043**
	rs5182	0.905	0.945	0.843	0.627	0.221	0.539	0.539	0.124	0.440	0.971	0.291	0.343	0.975	0.272	0.331	0.918	0.225	0.317
AGTR2	rs1403543	0.863	0.203	0.194	0.635	0.132	0.071	0.458	0.912	0.380	0.625	0.344	0.762	0.604	0.643	0.359	0.892	0.549	0.517
	rs5194	0.836	0.115	0.240	0.754	0.061	**0.048**	0.480	0.413	0.139	0.409	0.889	0.316	0.323	0.730	0.189	0.432	0.796	0.302
	rs11091046	0.893	0.076	0.085	0.646	0.061	**0.031**	0.560	0.434	0.189	0.615	0.518	0.268	0.406	0.663	0.224	0.578	0.696	0.369

Abbreviations: ***AGTR1*****:** Angiotensin II receptor type 1 gene; ***AGTR2*****:** Angiotensin II receptor type 2 gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Haplotype 8 was associated with a significant increase in mLVWT of 2.29 mm (p = 0.021), as well as an average increase of 1.16 mm in the CWT score (p = 0.042) (Figure 3.27 and Table 3.22).

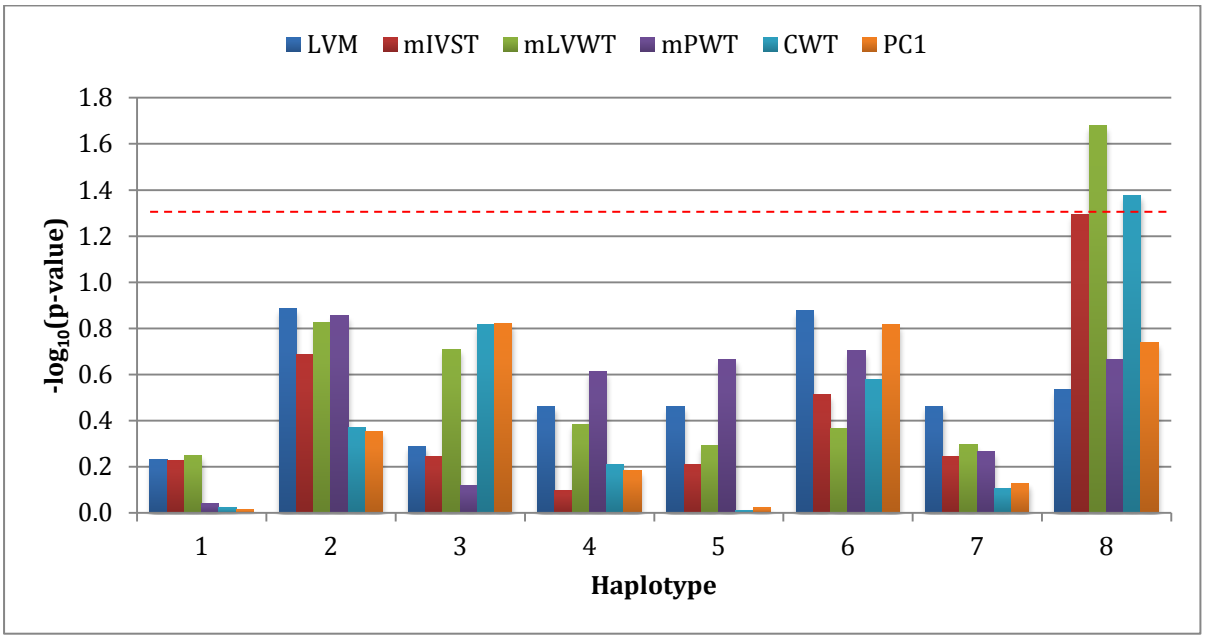


Figure 3.27 Summary of haplotype association results for AGTR1. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.22 Haplotype distribution within AGTR1, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs2640539	rs3772627	rs5182	freq.	p-value for test of association					
					LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	C	C	0.191	0.585	0.591	0.564	0.913	0.948	0.968
2	A	A	A	0.162	0.130	0.206	0.150	0.139	0.428	0.442
3	A	C	A	0.142	0.515	0.569	0.195	0.762	0.153	0.151
4	A	A	C	0.134	0.345	0.800	0.413	0.244	0.618	0.652
5	C	A	C	0.084	0.344	0.615	0.511	0.216	0.973	0.948
6	C	C	C	0.055	0.133	0.308	0.431	0.198	0.264	0.152
7	C	C	A	0.025	0.346	0.567	0.507	0.540	0.783	0.746
8	C	A	A	0.021	0.293	0.051	**0.021**	0.216	**0.042**	0.182

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Table 3.23 contains the p-values for interaction between the different HCM mutation groups and *AGTR1* haplotypes to illustrate the differences in allelic effect of the different haplotypes between these groups.

Haplotypes 1 and 4 caused different effects in the different HCM mutation groups (Table 3.23). Haplotype 1 caused a 27.12 g lower LVM (p = 0.029), a 2.71 mm lower mIVST (p = 0.029), 2.82 mm lower mLVWT (p = 0.017), a 1.65 mm lower CWT score (p = 0.009), as well as a 0.1 lower effect on PC1 (p = 0.013) in the $R92W_{TNNT2}$ group, compared to the $A797T_{MYH7}$ group. Conversely, haplotype 4 was associated with an increased effect on LVM of 28.72 g (p = 0.031), mLVWT of 2.54 mm (p = 0.043), CWT score of 1.43 mm (p = 0.033) and PC1 of 0.09 (p = 0.029) (Table 3.23). Interestingly, haplotype 8 had a 14.80 mm higher effect on mIVST (p = 0.027), as well as a 6.20 mm higher effect on the CWT score in the $R403W_{MYH7}$ group, compared to the $A797T_{MYH7}$ group (p = 0.044) (Table 3.23).

Table 3.23 The p-values for interaction between HCM mutation groups and AGTR1 haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

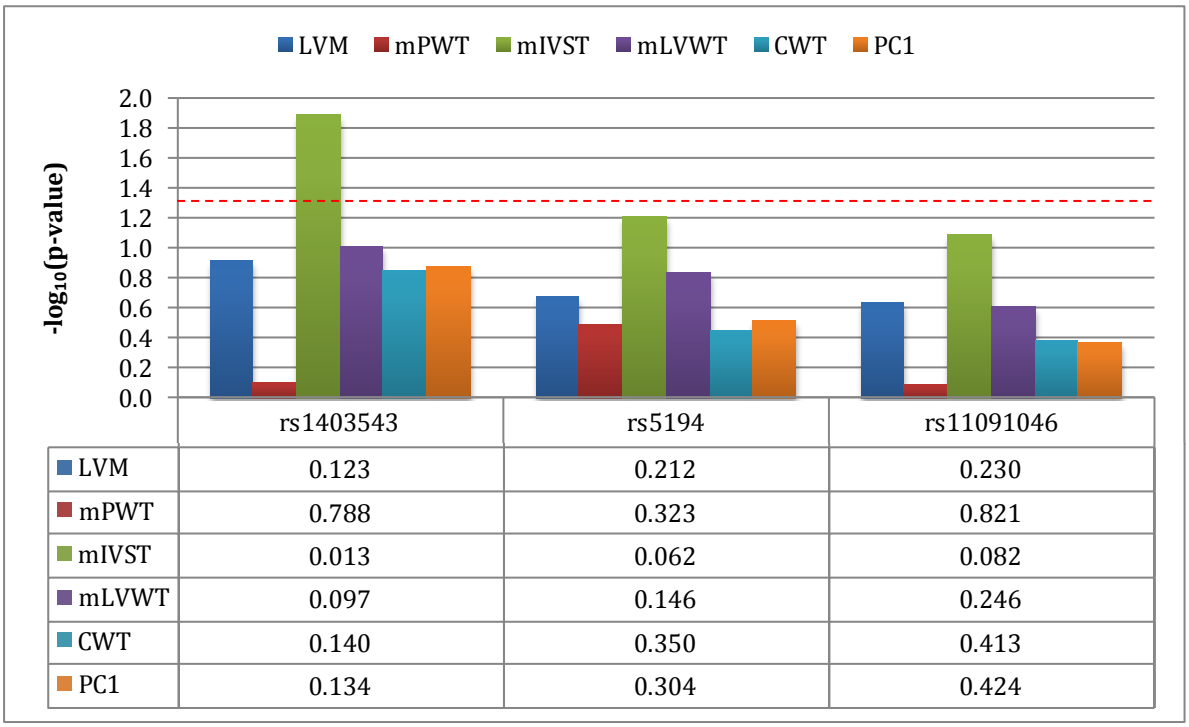
Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.580	**0.029**	0.210	0.684	**0.029**	0.157	0.456	**0.017**	0.223	0.895	0.197	0.339	0.701	**0.009**	0.068	0.930	**0.013**	**0.044**
2	0.800	0.673	0.909	0.446	0.659	0.654	0.396	0.697	0.550	0.457	0.333	0.938	0.711	0.242	0.499	0.765	0.152	0.315
3	0.397	0.978	0.393	0.429	0.548	0.170	0.566	0.258	0.104	0.406	0.381	0.095	0.496	0.825	0.610	0.506	0.822	0.625
4	0.598	**0.031**	0.170	0.872	0.065	0.070	0.827	**0.043**	0.113	0.966	0.109	0.166	0.788	**0.033**	0.105	0.937	**0.029**	0.062
5	0.847	0.471	0.296	0.897	0.463	0.501	0.960	0.483	0.467	0.379	0.497	0.773	0.994	0.492	0.445	0.707	0.717	0.398
6	0.710	0.316	0.884	0.711	0.201	0.772	0.536	0.169	0.921	0.379	0.293	0.744	0.346	0.269	0.754	0.308	0.184	0.788
7	0.303	0.432	0.561	0.610	0.702	0.771	0.554	0.656	0.736	0.910	0.304	0.568	0.396	0.830	0.296	0.368	0.786	0.256
8	0.136	0.305	**0.014**	0.354	0.129	**0.027**	0.423	0.196	0.062	0.397	0.570	0.171	0.486	0.106	**0.044**	0.856	0.199	0.225

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.4.2 Angiotensin II Receptor Type 2 (*AGTR2*)

The A-allele of rs1403543 in *AGTR2* was significantly associated with a decrease of 0.9 mm in mIVST (Figure 3.28).

The effects of both the rs5194 G-allele (p = 0.048) and the rs11091046 C-allele (p = 0.031) on mIVST were 2.69 mm higher in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group (Table 3.21).



	rs1403543	rs5194	rs11091046
LVM	0.123	0.212	0.230
mPWT	0.788	0.323	0.821
mIVST	0.013	0.062	0.082
mLVWT	0.097	0.146	0.246
CWT	0.140	0.350	0.413
PC1	0.134	0.304	0.424

Figure 3.28 Single polymorphism association results for AGTR2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between AGTR2 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

3.7.5 CYP11B1/B2 locus

Figure 3.29 shows the chromosomal location and structure of the CYP11B1/B2 locus, as well as intragenic location of target polymorphisms, while Table 3.24 indicates the observed LD pattern across this locus in the present cohort. A very high degree of LD existed within the *CYP11B1* gene, which extends from rs4310186 within *CYP11B1* to the first variant in CYP11B2, rs3097, as high D' values were observed across these loci (Table 3.24). Intermediate D' values were, however, observed between the remaining *CYP11B2* and *CYP11B1* variants, which points toward incomplete LD between these two genes in our study population. This is in contrast to previous studies that reported complete LD across the entire locus in a Caucasian population.

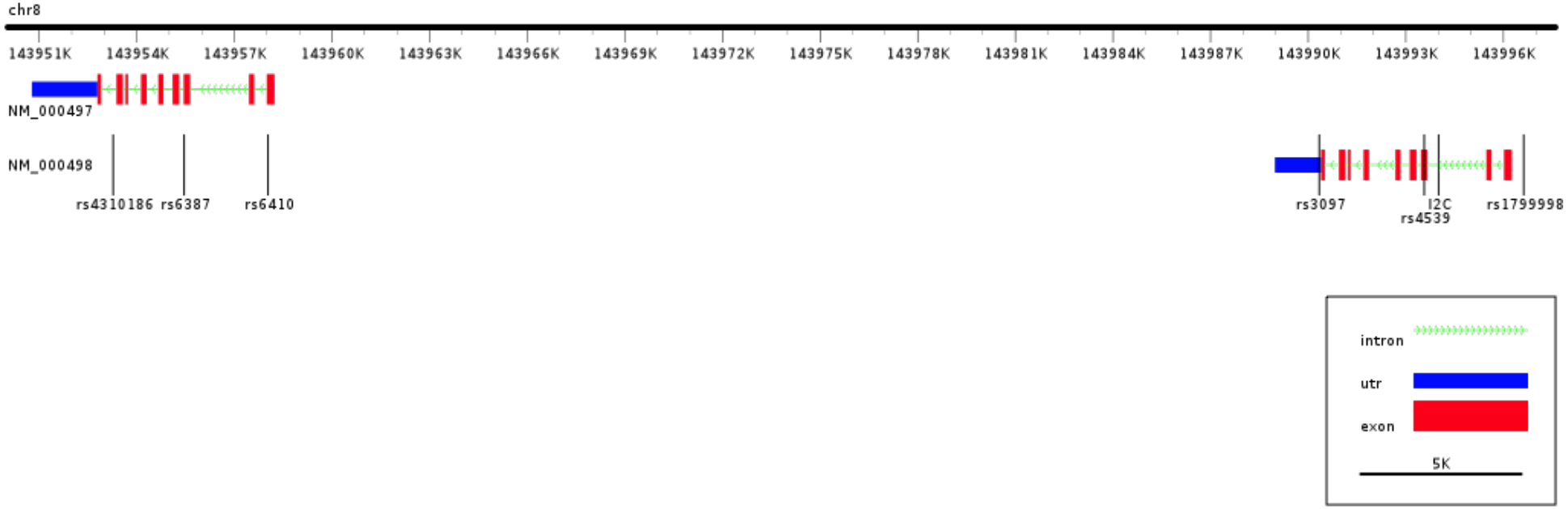
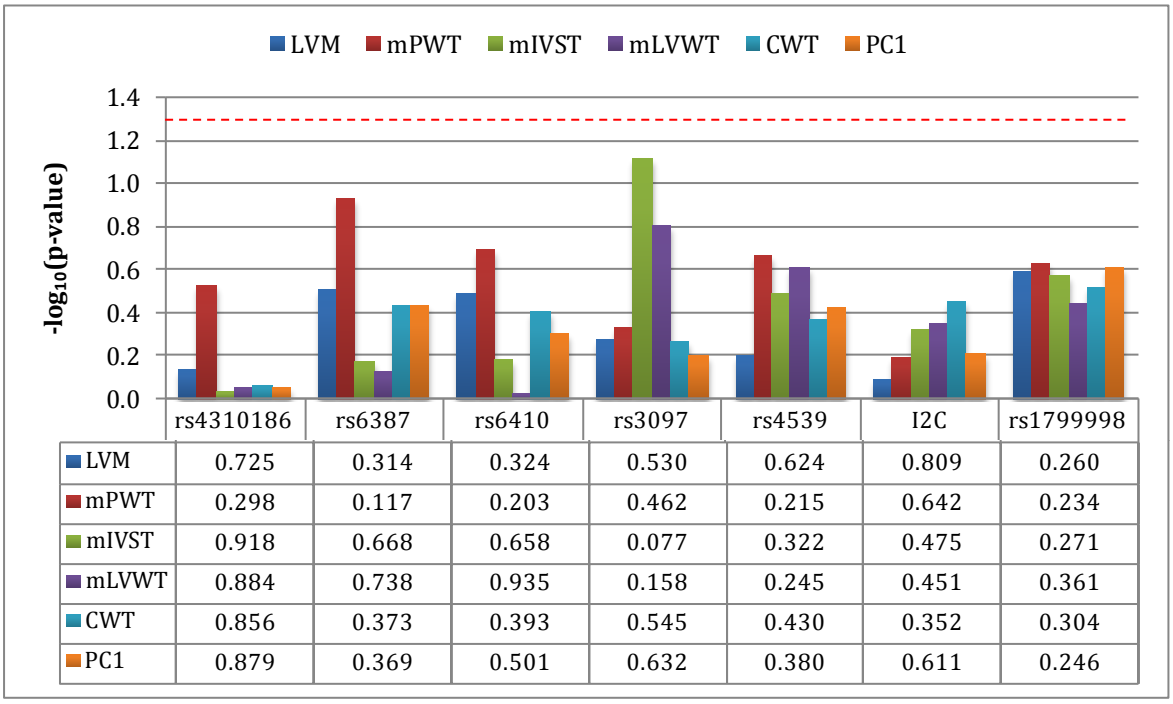


Figure 3.29 Scale diagram depicting chromosomal location and structure of the CYP11B1/B2 locus, as well as intragenic location of target polymorphisms. *The CYP11B1 (drawn from NCBI accession number NM_000497) and CYP11B2 (drawn from NCBI accession number NM_000498) genes, as well as the intragenic location of target polymorphisms are shown. Arrows indicate direction of transcription.*

Table 3.24 Pairwise D' values as a representation of the observed LD structure across the CYP11B1/B2 locus in the present cohort.

	rs4310186	rs6387	rs6410	rs3097	rs4539	I2C	rs1799998
rs4310186		1	0.94	1	0.57	0.62	0.74
rs6387			1	1	0.61	0.46	0.70
rs6410				1	0.66	0.47	0.83
rs3097					1	0.59	0.75
rs4539						0.52	0.71
I2C							0.51
rs1799998							

However, we did not find any statistically significant evidence for association between any of the investigated SNPs at the CYP11B1/B2 locus and the heritable hypertrophy traits (Figure 3.30).



	rs4310186	rs6387	rs6410	rs3097	rs4539	I2C	rs1799998
LVM	0.725	0.314	0.324	0.530	0.624	0.809	0.260
mPWT	0.298	0.117	0.203	0.462	0.215	0.642	0.234
mIVST	0.918	0.668	0.658	0.077	0.322	0.475	0.271
mLVWT	0.884	0.738	0.935	0.158	0.245	0.451	0.361
CWT	0.856	0.373	0.393	0.545	0.430	0.352	0.304
PC1	0.879	0.369	0.501	0.632	0.380	0.611	0.246

Figure 3.30 Single polymorphism association results for CYP11B1 and CYP11B2. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Table 3.25 contains the p-values for interaction between HCM mutation group and *CYP11B1* or *CYP11B2* genotype, illustrating the differences in allelic effect of the particular variants between these groups.

We found that the G-allele of rs4310186 (*CYP11B1*) and the T-allele of rs3097 (*CYP11B2*) caused significantly different effects in the R92W$_{TNNT2}$ group, when compared to the A797T$_{MYH7}$ group. The R92W$_{TNNT2}$ group showed a 1.82 mm higher increase in mIVST due to the G-allele of rs4310186 (p = 0.044) and a 2.68 mm higher increase in mIVST due to the T-allele of rs3097 (p = 0.039) (Table 3.25). Also, the rs4310186 G-allele was associated with a significantly higher mLVWT in the R92W$_{TNNT2}$ group when compared to the R403W$_{MYH7}$ (2.80 mm) and A797T$_{MYH7}$ groups (1.83 mm).

Table 3.25 The p-values for interaction between HCM mutation groups and CYP11B1 or CYP11B2 genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
CYP11B1	**rs4310186**	0.334	0.625	0.524	0.065	**0.044**	0.715	**0.017**	**0.028**	0.419	0.983	0.608	0.700	0.337	0.126	0.875	0.533	0.127	0.610
	rs6387	0.889	0.966	0.912	0.620	0.221	0.601	0.481	0.303	0.895	0.382	0.918	0.405	0.745	0.430	0.744	0.812	0.425	0.672
	rs6410	0.343	0.843	0.416	0.289	0.089	0.692	0.262	0.184	0.991	0.751	0.582	0.878	0.392	0.256	0.929	0.492	0.229	0.751
CYP11B2	**rs3097**	0.339	0.068	0.681	0.310	**0.039**	0.595	0.148	0.078	0.868	0.948	0.516	0.667	0.849	0.190	0.426	0.976	0.137	0.271
	rs4539	0.658	0.736	0.459	0.448	0.517	0.776	0.645	0.871	0.533	0.952	0.919	0.987	0.787	0.854	0.670	0.782	0.994	0.767
	I2C	0.744	0.779	0.579	0.707	0.677	0.477	0.863	0.978	0.842	0.166	0.603	0.308	0.373	0.709	0.534	0.295	0.780	0.393
	rs1799998	0.235	0.151	0.755	0.965	0.811	0.839	0.978	0.553	0.690	0.890	0.688	0.697	0.691	0.270	0.778	0.754	0.425	0.857

Abbreviations: **CWT score:** cumulative wall thickness score; ***CYP11B1:*** 11 beta-hydroxylase gene; ***CYP11B2*:** aldosterone synthase gene; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Two haplotypes were found to associate significantly with hypertrophy traits (Figure 3.31). Table 3.26 depicts the haplotype distribution observed across the CYP11B1/B2 locus, as well as the respective p-values for association with the investigated hypertrophy traits. Haplotype 8 was significantly associated with a 3.23 mm increase in mLVWT (p = 0.015), as well as a 1.73 mm increase in CWT score (p = 0.023) and a 0.11 increase in PC1 (p = 0.020). This haplotype was however only observed in 2.7 % of the cohort. In addition, haplotype 10, that was observed in only 1.9% of the cohort, associated significantly with a 2.34 mm increase in mPWT (p = 0.041).

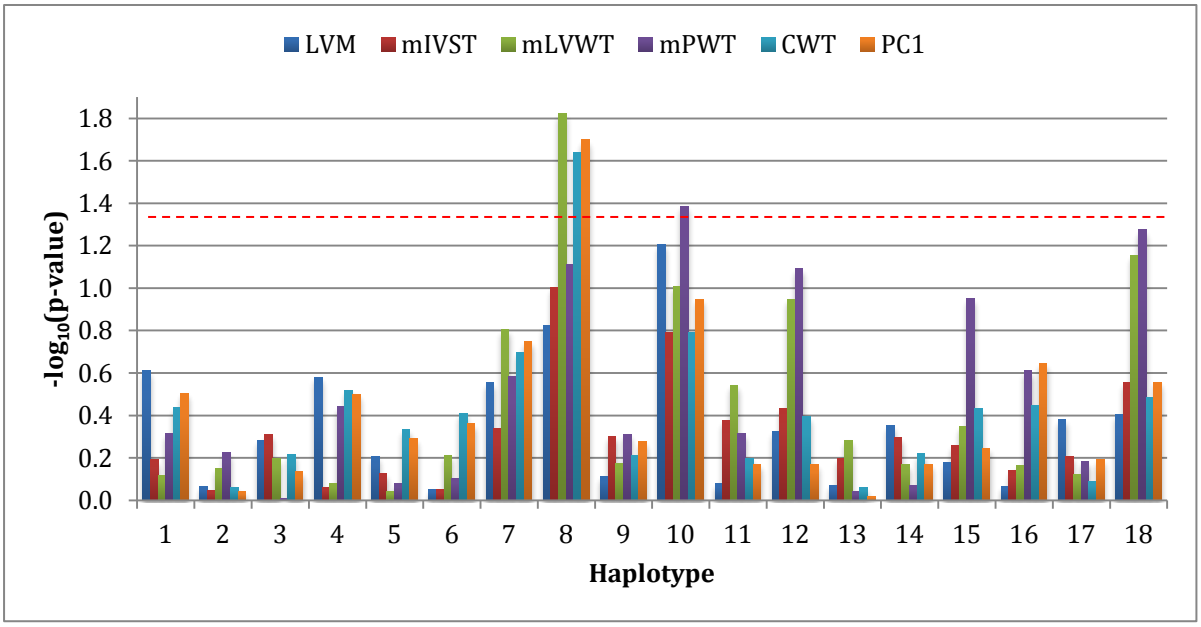


Figure 3.31 Summary of haplotype association results across CYP11B1/B2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.26 Haplotype distribution across the CYP11B1/B2 locus, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs1799998	I2C	rs4539	rs3097	rs6410	rs6387	rs4310186	freq.	p-value for test of association					
									LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	T	NC	G	C	C	A	C	0.108	0.244	0.644	0.763	0.481	0.364	0.312
2	C	NC	A	C	C	A	C	0.099	0.859	0.899	0.706	0.594	0.871	0.910
3	C	CV	A	T	T	G	G	0.098	0.521	0.488	0.637	0.975	0.606	0.731
4	C	CV	A	C	T	G	G	0.094	0.263	0.867	0.836	0.362	0.303	0.316
5	C	NC	A	C	T	G	C	0.056	0.618	0.743	0.908	0.833	0.462	0.509
6	T	NC	A	C	C	A	C	0.046	0.887	0.890	0.614	0.784	0.388	0.436
7	C	NC	G	C	C	A	C	0.030	0.279	0.460	0.157	0.261	0.200	0.178
8	C	NC	A	C	T	G	G	0.027	0.150	0.099	**0.015**	0.077	**0.023**	**0.020**
9	C	NC	G	C	T	G	G	0.019	0.774	0.499	0.669	0.487	0.614	0.526
10	C	CV	A	C	C	A	C	0.019	0.062	0.161	0.098	**0.041**	0.161	0.113
11	C	NC	A	C	C	G	G	0.015	0.832	0.420	0.286	0.482	0.633	0.676
12	T	NC	G	C	T	G	G	0.013	0.472	0.368	0.113	0.081	0.402	0.676
13	T	CV	G	C	C	A	C	0.010	0.848	0.632	0.522	0.912	0.873	0.960
14	C	CV	A	C	T	G	C	0.009	0.445	0.504	0.679	0.851	0.604	0.677
15	C	NC	A	C	C	G	C	0.009	0.659	0.552	0.448	0.112	0.370	0.566
16	C	CV	A	C	C	A	G	0.008	0.861	0.726	0.684	0.244	0.357	0.225
17	C	CV	G	T	T	G	G	0.008	0.414	0.618	0.751	0.654	0.813	0.638
18	C	NC	A	T	T	G	G	0.008	0.393	0.277	0.070	0.053	0.327	0.278

Abbreviations: **A:** adenine; **C:** cytosine; **CV:** converted; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **NC:** not converted; **PC1:** first principal component; **T:** thymine

Table 3.27 contains the p-values for interaction between the different HCM mutation groups and CYP11B1/B2 haplotypes to illustrate the differences in allelic effect of the different haplotypes between these groups.

Table 3.27 The p-values for interaction between HCM mutation groups and haplotypes of the CYP11B1/B2 locus, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.568	0.582	0.322	0.429	0.454	0.681	0.751	0.917	0.676	0.266	0.862	0.260	0.698	0.779	0.545	0.493	0.895	0.503
2	0.375	0.439	0.910	0.587	0.265	0.520	0.308	0.170	0.682	0.912	0.505	0.389	0.446	0.179	0.518	0.501	0.216	0.532
3	0.473	0.090	0.530	0.206	**0.035**	0.695	0.148	0.116	0.760	0.289	0.535	0.503	0.738	0.315	0.632	0.977	0.334	0.434
4	0.308	0.361	0.700	0.347	0.551	0.585	0.388	0.562	0.634	0.056	0.525	0.120	0.264	0.528	0.462	0.205	0.458	0.416
5	0.525	0.754	0.532	0.618	0.814	0.601	0.688	0.802	0.780	0.789	0.529	0.406	0.702	0.774	0.875	0.772	0.855	0.845
7	0.783	0.504	0.850	0.985	0.872	0.928	0.808	0.677	0.964	0.696	0.589	0.988	0.925	0.666	0.699	0.957	0.682	0.739
8	0.353	**0.004**	**0.001**	0.199	0.053	**0.005**	0.066	0.080	**0.002**	0.291	**0.041**	**0.006**	0.095	**0.039**	**0.001**	0.179	**0.016**	**0.001**

*Haplotypes 6, 9-18 not tested

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Haplotype 3 was found to exert a 3.56 mm higher effect on mIVST in the R92W group, compared to the A797T$_{MYH7}$ group (p = 0.035) (Table 3.27). Interestingly, haplotype 8 caused significantly different effects between the HCM mutation groups. This haplotype was significantly associated with significantly different effects in LVM (p = 0.001), mIVST (p = 0.005), mLVWT (p = 0.002), mPWT (p = 0.006), CWT score (p = 0.001), as well as PC1 (p = 0.001) in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group. Similarly, haplotype 8 was associated with significantly different effects on LVM (p = 0.004), mPWT (p = 0.041), CWT score (p = 0.039), as well as PC1 (p = 0.016) in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group. These results should, however, be interpreted with caution due to the low frequency of haplotype 8 in the cohort as small sample sizes increases the risk for spurious significance of results.

3.7.6 Mineralocorticoid receptor and 11 β-hydroxysteroid-dehydrogenase type 2

Figures 3.32 and 3.33 depict the chromosomal location and structure of the *HSD11B2* and *NR3C2* genes, as well as intragenic location of target polymorphisms within these genes, while Table 3.28 shows the observed LD structure within *NR3C2* in the present cohort.

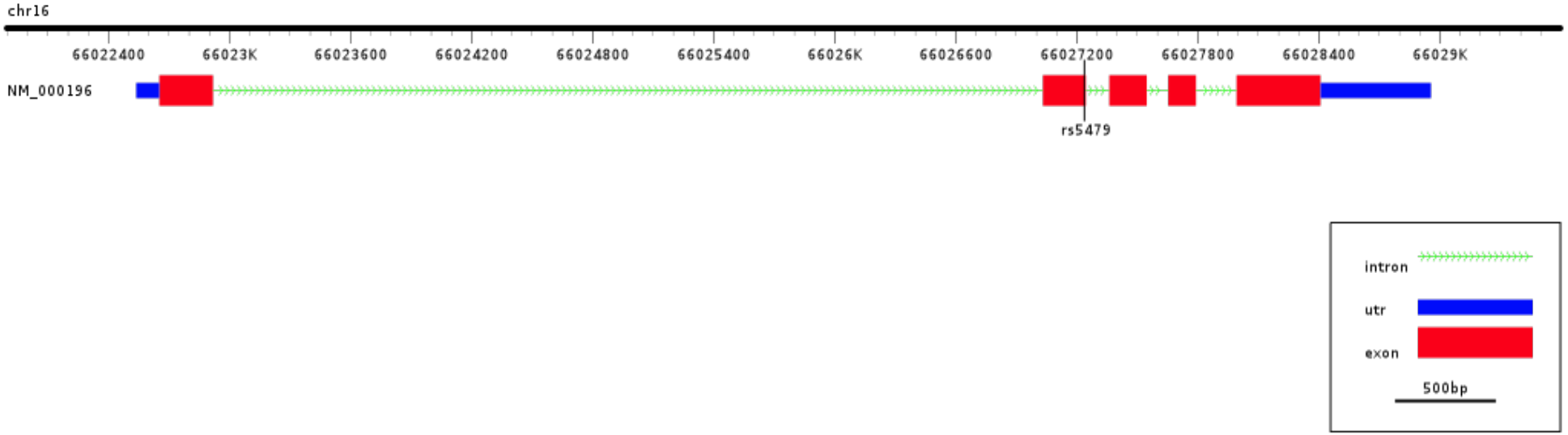


Figure 3.32 Scale diagram depicting chromosomal location and structure of the HSD11B2 gene, as well as intragenic location of the target polymorphism. *Arrows indicate direction of transcription.*

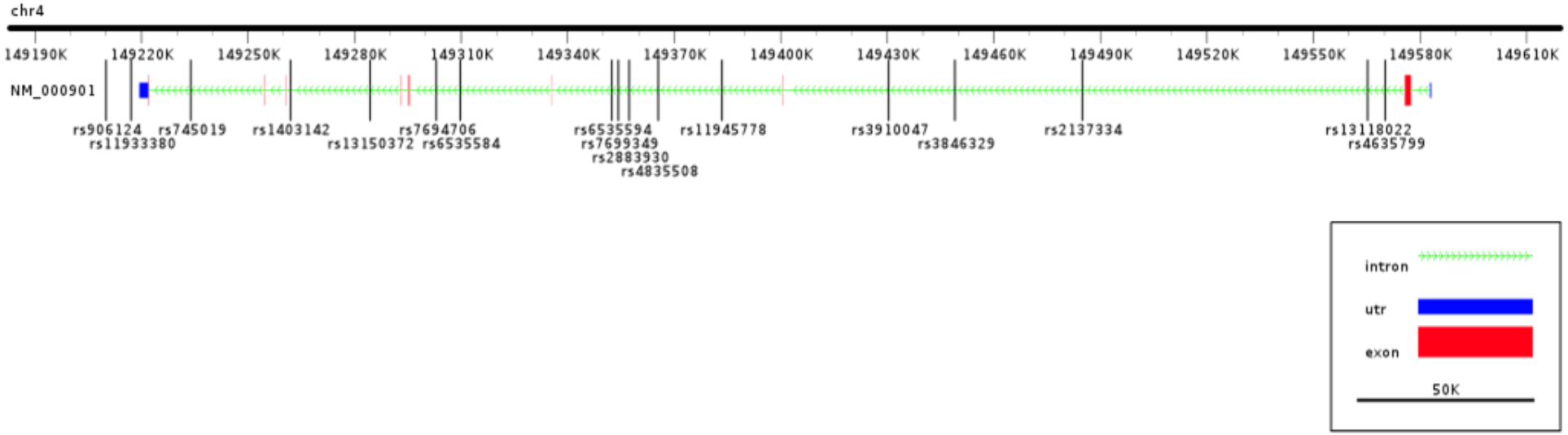


Figure 3.33 Scale diagram depicting chromosomal location and structure of the NR3C2 gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.28 Pairwise D' values as a representation of the observed LD structure within NR3C2 in the present cohort.

	rs906124	rs11933380	rs745019	rs1403142	rs13150372	rs7694706	rs6535584	rs6535594	rs7699349	rs2883930	rs4835508	rs11945778	rs3910047	rs3846329	rs2137334	rs13118022	rs4635799
rs906124		0.92	0.58	0.32	0.68	0.08	0.01	0.24	0.42	0.24	0.26	0.18	0.16	0.20	0.16	0.18	0.38
rs11933380			0.52	0.28	0.52	0.16	0.02	0.16	0.22	0.26	0.3	0.11	0.04	0.02	0.26	0.36	0.38
rs745019				0.66	0.38	0.18	0.22	0.78	0.42	0.37	0.16	0.14	0.17	0.18	0.29	0.04	0.59
rs1403142					1	1	0.66	0.39	0.06	0.04	0.32	0.02	0.18	0.03	0.01	0.11	0.56
rs13150372						1	0.84	0.16	0.16	0.54	0.98	0.06	0.04	0.04	0.05	0.04	0.52
rs7694706							0.88	0.45	0.17	0.21	0.14	0.05	0.04	0.02	0.07	0.16	0.23
rs6535584								0.63	0.17	0.08	0.31	0.01	0.04	0.04	0.06	0.36	0.31
rs6535594									0.93	0.38	0.35	0.24	0.09	0.12	0.22	0.05	0.25
rs7699349										0.37	0.21	0.2	0.07	0.05	0.11	0.01	0.28
rs2883930											0.38	0.09	0.03	0.01	0.03	0.14	0.37
rs4835508												0.03	0.68	0.62	0.04	0.07	0.39
rs11945778													0.76	0.79	0.02	0.45	0.41
rs3910047														1	0.38	0.36	0
rs3846329															0.35	0.04	0
rs2137334																0.37	0.04
rs13118022																	0.26
rs4635799																	

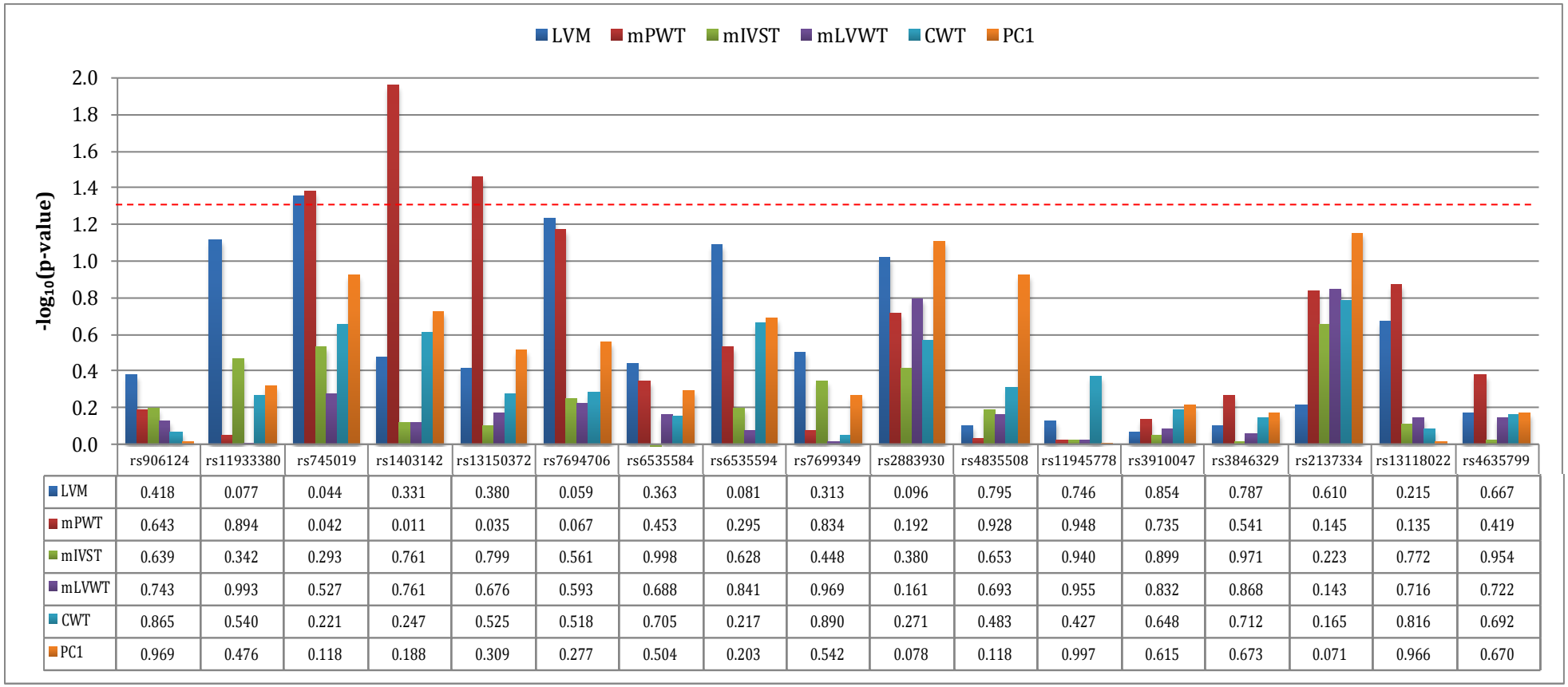
3.7.6.1 Mineralocorticoid receptor (*NR3C2*)

Rs3910047 and rs3846328 was found to be in complete LD (D' = 1), whereas almost complete LD was observed between rs906124 and rs11933380 (D' = 0.92), rs13150372 and rs4835508 (D' = 0.98), as well as between rs6535594 and rs7699349 (D' = 0.93) (Table 3.28). In addition, a very high degree of LD extends from rs1403142 to rs6535584 in *NR3C2*. D' values for the rest of the gene is, however, relatively weak, which is perhaps not surprising considering the size of the *NR3C2* gene.

Figure 3.34 depicts results of the single SNP association analysis for *NR3C2*, as well as the exact p-values for the tests of allelic association. The G-allele of rs745019 was significantly associated with en increase in LVM of 10.19 g (p =0.044), as well as an increase of 0.44 mm in mPWT (p =0.042). The rs1403142 G-allele, as well as the rs13150372 A-allele was significantly associated with respective decreases in mPWT of 0.54 mm (p = 0.011) and 0.63 mm (p = 0.035).

Table 3.29 depicts the p-values for interaction between HCM mutation group and *HSD11B2* or *NR3C2* genotypes, illustrating the differences in allelic effect of the particular variants between these groups.

There was a significant difference in effect size of 1.10 mm on mPWT in the R403W$_{MYH7}$ group, when compared to the A797T$_{MYH7}$ group for the *NR3C2* rs1403142 G-allele (p = 0.032) (Table 3.29). The rs7699349 T-allele was associated with a 20.15 g higher LVM (p = 0.041), 0.94 mm higher CWT score (p = 0.050), as well as a 0.06 higher PC1 score (p = 0.049) in the R92W$_{TNNT2}$ group compared to the R403W$_{MYH7}$ group. On the other hand the rs2883930 G-allele was associated with a 36.53 g lower effect on LVM in the R92W$_{TNNT2}$ group when compared to the R403W$_{MYH7}$ group (p = 0.039). Similarly, the rs2137334 T-allele was associated with a 20.29 g lower effect on LVM in the R92W$_{TNNT2}$ group compared to the A797T$_{MYH7}$ group (p = 0.049).



	rs906124	rs11933380	rs745019	rs1403142	rs13150372	rs7694706	rs6535584	rs6535594	rs7699349	rs2883930	rs4835508	rs11945778	rs3910047	rs3846329	rs2137334	rs13118022	rs4635799
LVM	0.418	0.077	0.044	0.331	0.380	0.059	0.363	0.081	0.313	0.096	0.795	0.746	0.854	0.787	0.610	0.215	0.667
mPWT	0.643	0.894	0.042	0.011	0.035	0.067	0.453	0.295	0.834	0.192	0.928	0.948	0.735	0.541	0.145	0.135	0.419
mIVST	0.639	0.342	0.293	0.761	0.799	0.561	0.998	0.628	0.448	0.380	0.653	0.940	0.899	0.971	0.223	0.772	0.954
mLVWT	0.743	0.993	0.527	0.761	0.676	0.593	0.688	0.841	0.969	0.161	0.693	0.955	0.832	0.868	0.143	0.716	0.722
CWT	0.865	0.540	0.221	0.247	0.525	0.518	0.705	0.217	0.890	0.271	0.483	0.427	0.648	0.712	0.165	0.816	0.692
PC1	0.969	0.476	0.118	0.188	0.309	0.277	0.504	0.203	0.542	0.078	0.118	0.997	0.615	0.673	0.071	0.966	0.670

Figure 3.34 Single polymorphism association results for NR3C2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between NR3C2 variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Table 3.29 The p-values for interaction between HCM mutation groups and NR3C2 or HSD11B2 genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values are indicated in bold red font and the corresponding effect sizes are discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
NR3C2	**rs906124**	0.600	0.574	0.962	0.623	0.740	0.430	0.518	0.629	0.804	0.673	0.176	0.475	0.962	0.513	0.549	0.755	0.408	0.318
	rs11933380	0.147	0.295	0.551	0.117	0.744	0.172	0.092	0.163	0.595	0.670	0.131	0.373	0.297	0.153	0.839	0.517	0.096	0.424
	rs745019	0.127	0.381	0.480	0.538	0.561	0.939	0.891	0.870	0.766	0.708	0.362	0.628	0.275	0.969	0.276	0.295	0.816	0.225
	rs1403142	0.663	0.645	0.366	0.822	0.099	0.075	0.809	0.149	0.107	0.085	0.783	**0.032**	0.823	0.115	0.104	0.852	0.166	0.156
	rs13150372	0.354	0.311	0.827	0.686	0.106	0.415	0.674	0.187	0.564	0.597	0.320	0.847	0.809	0.090	0.358	0.861	0.118	0.370
	rs7694706	0.534	0.185	0.539	0.724	0.213	0.430	0.981	0.390	0.442	0.936	0.807	0.886	0.859	0.453	0.403	0.922	0.316	0.429
	rs6535584	0.505	0.970	0.472	0.288	0.910	0.311	0.359	0.896	0.285	0.368	0.774	0.240	0.966	0.262	0.331	0.820	0.249	0.472
	rs6535594	0.143	0.410	0.372	0.200	0.196	0.766	0.275	0.249	0.851	0.499	0.670	0.706	0.105	0.310	0.368	0.203	0.494	0.424
	rs7699349	**0.041**	0.516	0.107	0.182	0.125	0.893	0.493	0.528	0.847	0.418	0.737	0.562	**0.050**	0.315	0.224	**0.049**	0.489	0.142
	rs2883930	**0.039**	0.116	0.471	0.487	0.467	0.950	0.753	0.138	0.304	0.900	0.847	0.759	0.389	0.105	0.652	0.434	0.088	0.549
	rs4835508	0.782	0.986	0.759	0.887	0.912	0.961	0.929	0.831	0.770	0.882	0.912	0.794	0.637	0.204	0.521	0.459	0.164	0.630
	rs11945778	0.225	0.685	0.112	0.469	0.924	0.410	0.652	0.552	0.309	0.115	0.817	0.166	0.186	0.958	0.192	0.209	0.999	0.199
	rs3910047	0.555	0.190	0.116	0.661	0.348	0.800	0.786	0.572	0.879	0.807	0.623	0.541	0.749	0.465	0.398	0.564	0.484	0.283
	rs3846329	0.511	0.188	0.094	0.649	0.312	0.761	0.802	0.607	0.888	0.551	0.815	0.431	0.739	0.476	0.391	0.543	0.504	0.271
	rs2137334	0.141	**0.049**	0.735	0.123	0.199	0.695	0.218	0.237	0.871	0.829	0.743	0.592	0.210	0.269	0.801	0.295	0.296	0.926
	rs13118022	0.497	0.319	0.858	0.280	0.160	0.910	0.512	0.399	0.947	0.518	0.113	0.459	0.909	0.574	0.707	0.920	0.476	0.601
	rs4635799	0.985	0.183	0.297	0.783	0.105	0.316	0.653	0.122	0.455	0.584	0.077	0.051	0.527	0.237	0.113	0.490	0.246	0.104
HSD11B2	**rs5479**	0.731	0.879	0.672	0.692	0.795	0.579	0.864	0.542	0.737	0.357	0.615	0.679	0.559	0.724	0.837	0.384	0.808	0.591

Abbreviations: **CWT score:** cumulative wall thickness score; ***HSD11B2*****:** 11 β-hydroxysteroid-dehydrogenase type 2 gene**; LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; ***NR3C2*****:** nuclear receptor subfamily 3, group C, member 2 gene**; PC1:** first principal component.

Haplotype analysis of the *NR3C2* gene yielded a large number of haplotypes with relatively small frequencies. Figure 3.35 depicts a summary of the association results for haplotypes with a frequency of more than 0.8 %. Five haplotypes associated significantly with one or more of the hypertrophy traits.

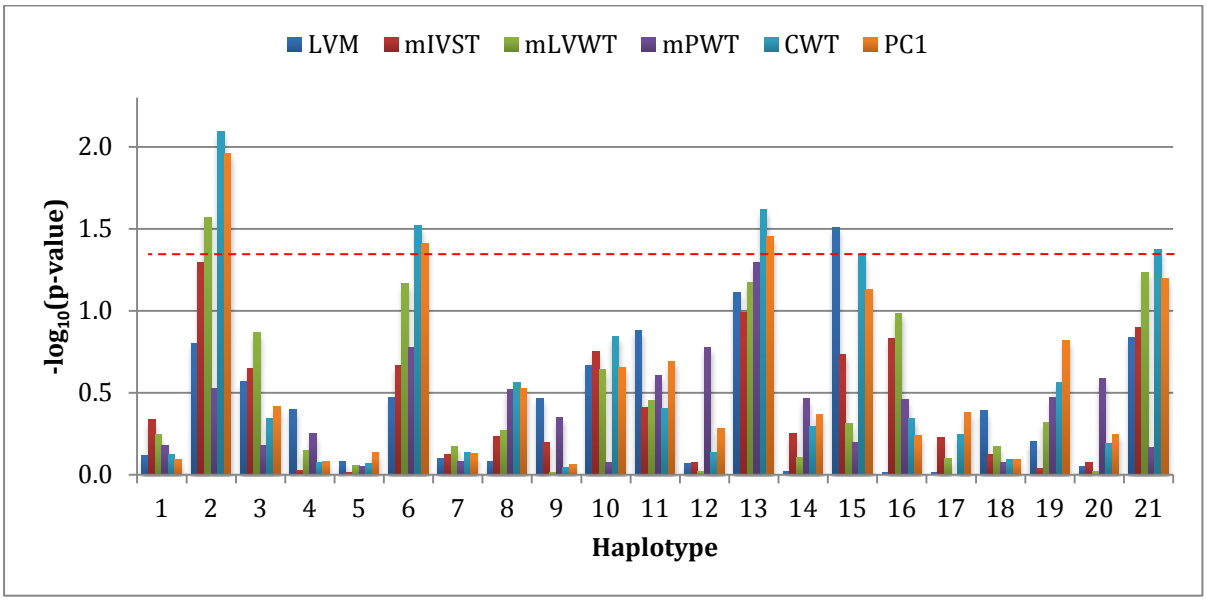


Figure 3.35 Summary of haplotype association results for NR3C2. *Bar graph indicates –log 10 transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.30 depicts the haplotypes with a frequency of more than 0.8 %, as well as the exact p-values for tests of association between the respective haplotypes and the investigated hypertrophy traits.

Table 3.30 Haplotype distribution within NR3C2, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes are indicated in the text.*

	rs906124	rs11933380	rs745019	rs1403142	rs13150372	rs7694706	rs6535584	rs6535594	rs7699349	rs2883930	rs4835508	rs11945778	rs3910047	rs3846329	rs2137334	rs13118022	rs4635799	freq.	p-value for test of association					
																			LVM	**mIVST**	**mLVWT**	**mPWT**	**CWT**	**PC1**
1	T	T	A	G	A	A	C	A	C	C	C	C	T	G	C	T	C	0.024	0.765	0.461	0.570	0.663	0.755	0.803
2	T	T	A	A	G	G	T	G	C	C	C	T	C	T	T	G	C	0.019	0.158	0.051	**0.027**	0.299	**0.008**	**0.011**
3	T	T	A	G	A	A	C	A	C	C	C	C	T	G	T	T	C	0.018	0.271	0.225	0.136	0.665	0.452	0.384
4	T	T	A	A	G	G	T	G	C	C	C	C	T	G	C	T	T	0.017	0.400	0.939	0.706	0.563	0.844	0.827
5	C	C	G	G	G	A	C	G	T	C	C	T	T	G	C	G	T	0.017	0.829	0.964	0.871	0.894	0.850	0.729
6	T	T	A	G	A	A	C	A	C	C	C	C	C	T	C	G	T	0.015	0.335	0.215	0.068	0.167	**0.030**	**0.039**
7	C	C	G	A	G	G	C	A	C	C	C	C	T	G	T	T	C	0.014	0.799	0.753	0.670	0.831	0.733	0.745
8	T	T	A	A	G	G	T	G	T	C	C	C	C	T	C	G	C	0.012	0.828	0.585	0.536	0.301	0.274	0.298
9	T	T	A	A	G	G	T	G	C	C	T	C	T	G	C	T	T	0.011	0.341	0.636	0.972	0.448	0.899	0.868
10	C	C	A	G	G	A	C	A	C	C	C	C	T	G	C	G	C	0.011	0.214	0.177	0.227	0.839	0.144	0.222
11	T	T	A	A	G	G	T	G	C	C	T	T	T	G	C	G	T	0.010	0.131	0.389	0.352	0.247	0.393	0.202
12	T	C	A	A	G	A	C	A	C	C	C	C	C	T	T	G	T	0.010	0.851	0.843	0.950	0.167	0.734	0.524
13	C	C	A	G	G	A	C	A	C	C	T	C	T	G	C	G	T	0.010	0.077	0.102	0.067	0.051	**0.024**	**0.035**
14	T	T	A	A	G	A	C	A	C	C	C	C	T	G	C	G	T	0.010	0.952	0.560	0.785	0.344	0.508	0.428
15	C	C	A	G	A	A	C	A	C	C	T	T	T	G	C	G	C	0.010	**0.031**	0.185	0.484	0.630	**0.045**	0.074
16	T	T	A	A	G	A	C	A	C	C	C	T	C	T	C	G	T	0.010	0.971	0.147	0.104	0.348	0.452	0.575
17	C	C	A	A	G	A	C	A	C	C	C	T	T	G	C	G	T	0.009	0.969	0.593	0.795	0.989	0.565	0.416
18	T	T	A	A	G	A	C	A	C	G	T	C	T	G	T	T	T	0.008	0.405	0.751	0.672	0.836	0.810	0.810
19	C	T	G	A	G	A	C	G	T	C	C	T	T	G	C	G	T	0.008	0.629	0.915	0.477	0.337	0.275	0.151
20	C	C	G	A	G	A	T	G	C	C	C	C	T	G	T	T	T	0.008	0.885	0.845	0.953	0.257	0.644	0.564
21	C	T	A	A	G	G	C	A	C	G	C	T	T	G	T	G	T	0.008	0.145	0.126	0.058	0.680	**0.042**	0.063

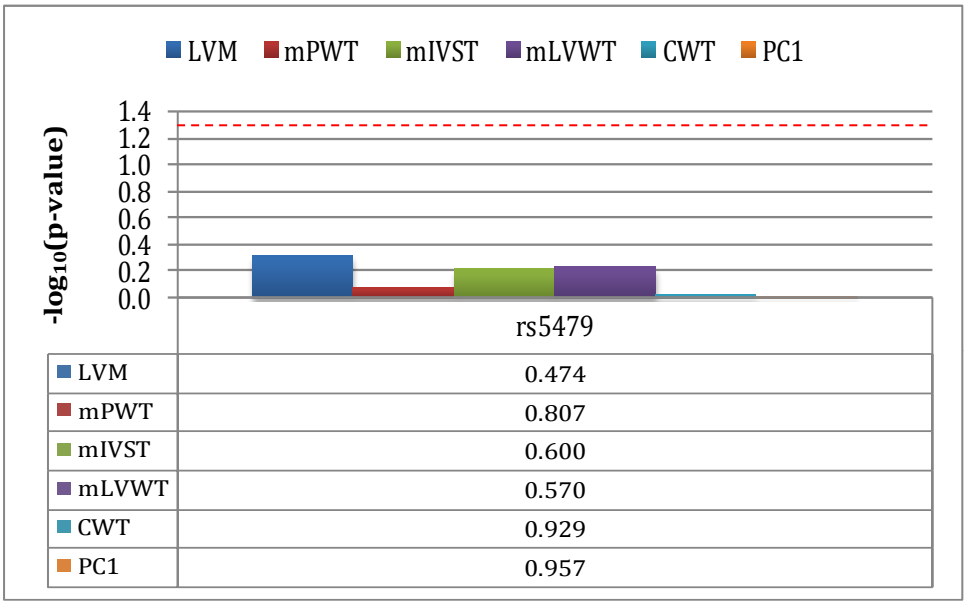
Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Haplotype 2 was significantly associated with a 2.55 mm increase in mLVWT (p = 0.027), a 1.96 mm increase in CWT score (p = 0.008), as well as a 1.22 increase in PC1 (p = 0.011). Haplotype 6 was associated with a 2.24 mm decrease in CWT score (p = 0.030) and a 0.14 decrease in PC1 (p = 0.039). On the other hand, haplotype 13 was significantly associated with a 4.03 mm increase in CWT score (p = 0.024) and a 0.25 increase in PC1 (p = 0.035). Significant evidence for association was found between haplotype 15 and a 60.10 g decrease in LVM (p = 0.031), as well as a decrease in CWT score of 2.94 mm (p = 0.045). Lastly, haplotype 21 was found to significantly increase CWT score by 5.11 mm (p = 0.042).

The tests for interaction between HCM mutation group and the *NR3C2* haplotypes, to determine the differences in allelic effect of these haplotypes between the mutation groups could unfortunately not be done due the low frequency of these haplotypes in the present HCM cohort.

3.7.6.2 11 β-hydroxysteroid-dehydrogenase type 2 (*HSD11B2*)

No statistically significant association was found between the investigated *HSD11B2* variant and any of the investigated hypertrophy traits (Figure 3.36).



	rs5479
LVM	0.474
mPWT	0.807
mIVST	0.600
mLVWT	0.570
CWT	0.929
PC1	0.957

Figure 3.36 Single polymorphism association results for HSD11B2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

3.7.7 Epithelial sodium channel subunits

Figures 3.37, 3.38 and 3.39 depict the chromosomal location and structure of the *SCNN1A*, *SCNN1B* and *SCNN1G* genes, as well as intragenic location of target polymorphisms within these genes. Tables 3.31 and 3.32 depict the D' values as a proxy for LD structure in the *SCNN1A* and *SCNN1B* genes, respectively.

Complete LD was observed between rs2286600 and rs10849446 (D' = 1), while a high degree of LD exists between rs11614164 and rs3782726 (D' = 0.86) in *SCNN1A* (Table 3.31). Intermediate D' values were further found for the region between rs7973914 and rs2286600, with the exception of rs7973914 and rs10849446, which showed no evidence for LD (D' = 0).

Complete LD was observed between several SNP pairs, whereas a relatively high degree of LD extends between rs11074555 and rs238547 in *SCNN1B* (Table 3.32). There was however no evidence for pairwise LD between rs11074555 and rs2303153, as well as between rs239345 and rs2303153 (D' = 0).

Little evidence for LD, i.e. a D' value of 0.1, was observed between rs5735 and rs4247210 in *SCNN1G*.

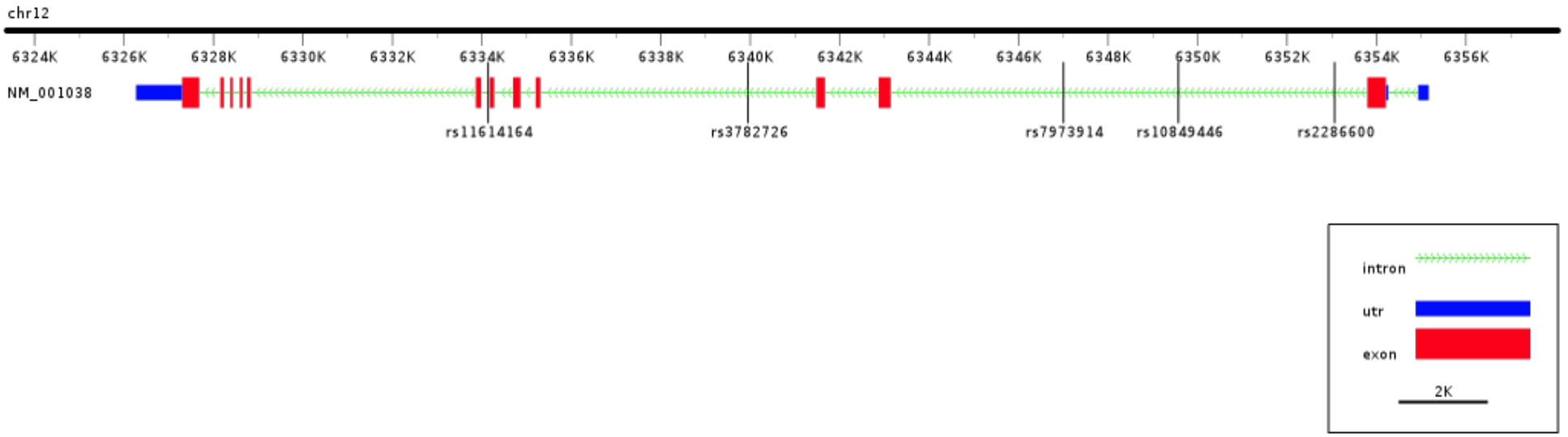


Figure 3.37 Scale diagram depicting chromosomal location and structure of the SCNN1A gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.31 Pairwise D' values as a representation of the observed LD structure within SCNN1A in the present cohort.

	rs11614164	rs3782726	rs7973914	rs10849446	rs2286600
rs11614164		0.86	0.72	0.41	0.38
rs3782726			0.64	0.22	0.16
rs7973914				0	0.02
rs10849446					1
rs2286600					

Figure 3.38 Scale diagram depicting chromosomal location and structure of the SCNN1B gene, as well as intragenic location of target polymorphisms. *Arrows indicate direction of transcription.*

Table 3.32 Pairwise D' values as a representation of the observed LD structure within SCNN1B in the present cohort.

	rs11074555	**rs9930640**	**rs239345**	**rs238547**	**rs8044970**	**rs152740**	**rs250563**	**rs2303153**
rs11074555		1.00	0.78	0.74	0.56	0.41	1.00	0
rs9930640			0.76	1.00	0.22	1.00	0.08	1.00
rs239345				0.66	0.40	0.36	1.00	0
rs238547					1.00	0.80	1.00	0.10
rs8044970						0.56	0.18	0.16
rs152740							1.00	0.38
rs250563								1.00
rs2303153								

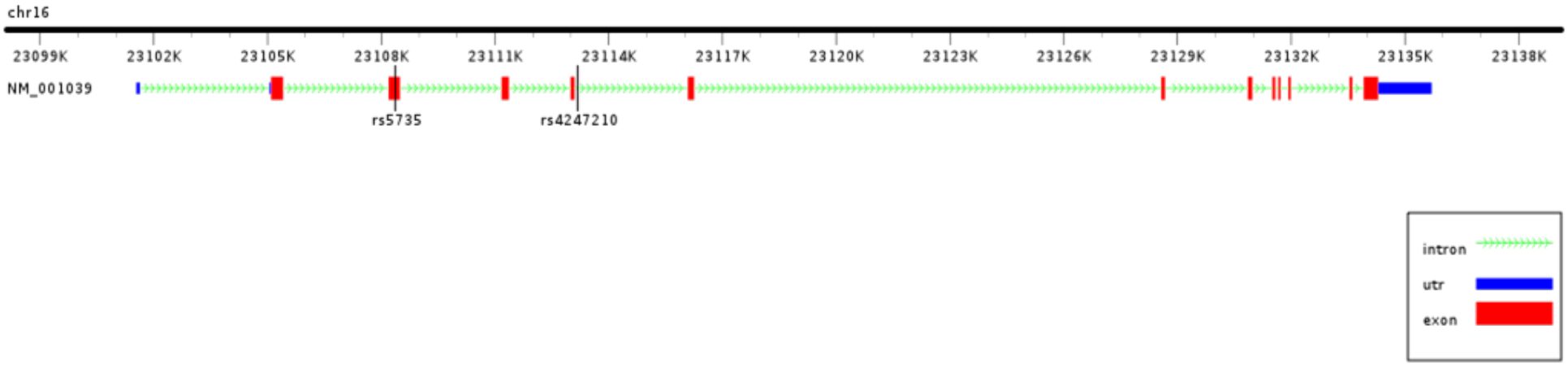
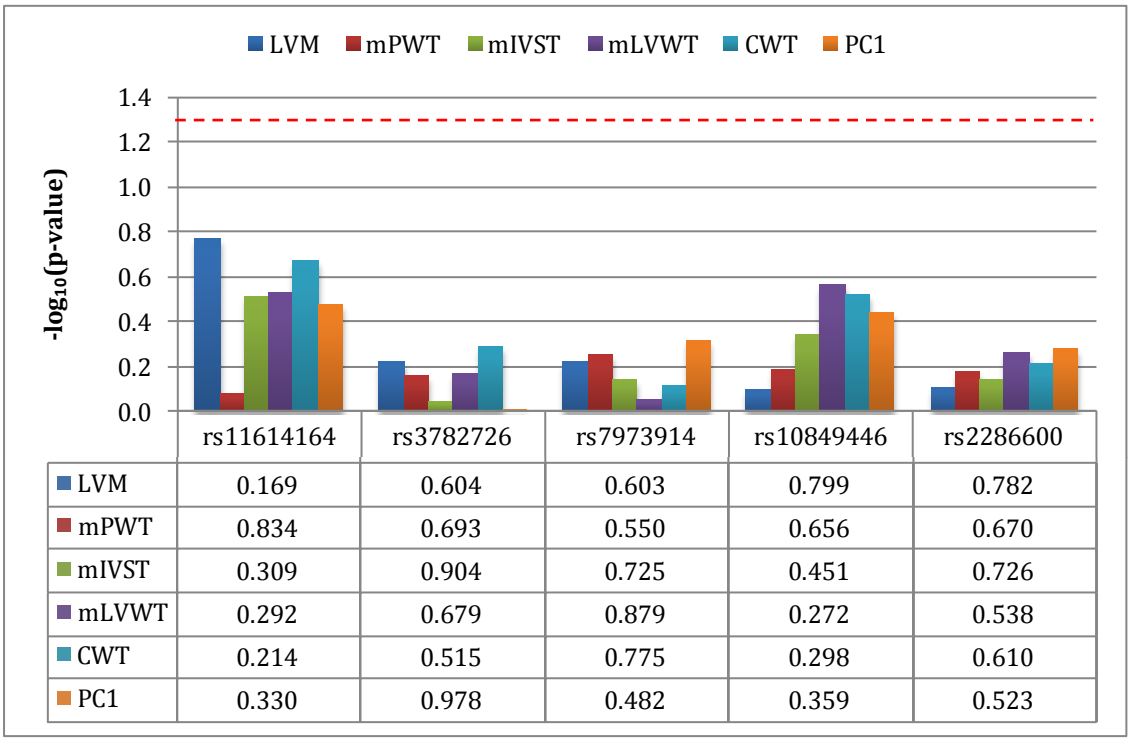


Figure 3.39 Scale diagram depicting chromosomal location and structure of the SCNN1G gene, as well as intragenic location of target polymorphisms. *D' = 0.1 between rs5735 and rs4247210. Arrows indicate direction of transcription.*

3.7.7.1 *SCNN1A*

There was no statistically significant evidence for association between any of the investigated *SCNN1A* SNPs and any of the hypertrophy traits (Figure 3.40).



	rs11614164	rs3782726	rs7973914	rs10849446	rs2286600
LVM	0.169	0.604	0.603	0.799	0.782
mPWT	0.834	0.693	0.550	0.656	0.670
mIVST	0.309	0.904	0.725	0.451	0.726
mLVWT	0.292	0.679	0.879	0.272	0.538
CWT	0.214	0.515	0.775	0.298	0.610
PC1	0.330	0.978	0.482	0.359	0.523

Figure 3.40 Single polymorphism association results for SCNN1A. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Table 3.33 depicts the p-values for interaction between HCM mutation group and *SCNN1A*, *SCNN1B* or *SCNN1G* genotypes, illustrating the differences in allelic effect of the particular variants between these groups. We did not find any significant differences in allelic effect of the investigated *SCNN1A*, *SCNN1B* and *SCNN1G* variants between the three mutation groups (Table 3.33).

Table 3.33 The p-values for interaction between HCM mutation groups and SCNN1A, SCNN1B or SCNN1G genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
SCNN1A	**rs11614164**	0.281	0.382	0.643	0.711	0.768	0.527	0.828	0.919	0.881	0.205	0.807	0.242	0.340	0.657	0.474	0.535	0.785	0.635
	rs3782726	0.783	0.383	0.717	0.341	0.455	0.636	0.558	0.767	0.679	0.331	0.837	0.361	0.541	0.553	0.815	0.763	0.709	0.955
	rs7973914	0.523	0.581	0.858	0.483	0.288	0.819	0.586	0.095	0.339	0.267	0.242	0.893	0.486	0.064	0.375	0.638	0.074	0.284
	rs10849446	0.655	0.392	0.880	0.785	0.271	0.609	0.854	0.279	0.545	0.342	0.417	0.659	0.669	0.203	0.654	0.643	0.173	0.632
	rs2286600	0.540	0.754	0.709	0.928	0.591	0.552	0.808	0.696	0.918	0.986	0.664	0.700	0.813	0.597	0.467	0.964	0.500	0.510
SCNN1B	**rs11074555**	0.976	0.379	0.392	0.940	0.600	0.681	0.993	0.888	0.887	0.936	0.435	0.519	0.875	0.814	0.705	0.816	0.730	0.577
	rs9930640	0.190	0.688	0.227	0.201	0.856	0.326	0.183	0.934	0.442	0.746	0.206	0.160	0.529	0.983	0.702	0.594	0.789	0.578
	rs239345	0.855	0.857	0.998	0.828	0.976	0.794	0.918	0.568	0.621	0.515	0.453	0.140	0.902	0.719	0.617	0.726	0.632	0.396
	rs238547	0.539	0.833	0.379	0.630	0.244	0.591	0.924	0.566	0.671	0.453	0.732	0.245	0.976	0.629	0.689	0.984	0.698	0.743
	rs8044970	0.582	0.961	0.531	0.522	0.780	0.697	0.346	0.805	0.465	0.497	0.424	0.920	0.299	0.870	0.378	0.307	0.894	0.371
	rs152740	0.625	0.449	0.852	0.748	0.478	0.767	0.520	0.260	0.727	0.303	0.832	0.395	0.545	0.293	0.754	0.590	0.232	0.614
	rs250563	0.852	0.290	0.394	0.764	0.797	0.558	0.632	0.744	0.856	0.898	0.327	0.420	0.850	0.693	0.864	0.794	0.903	0.875
	rs2303153	0.179	0.739	0.271	0.246	0.958	0.204	0.644	0.979	0.604	0.536	0.667	0.290	0.350	0.836	0.271	0.375	0.797	0.275
SCNN1G	**rs5735**	0.277	0.996	0.248	0.194	0.285	0.624	0.219	0.258	0.740	0.197	0.345	0.560	0.489	0.548	0.832	0.485	0.562	0.815
	rs4247210	0.452	0.382	0.956	0.249	0.198	0.985	0.433	0.625	0.727	0.139	0.055	0.790	0.475	0.895	0.540	0.451	0.869	0.534

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; ***SCNN1A*****:** sodium channel, nonvoltage-gated 1 alpha gene; ***SCNN1B*****:** sodium channel, nonvoltage-gated 1, beta gene; ***SCNN1G*****:** sodium channel, nonvoltage-gated 1, gamma gene

Two haplotypes were found to associate significantly with LVM (Figure 3.41). Table 3.34 contains the haplotype distribution and the respective p-values for the tests of association between these haplotypes and the investigated hypertrophy traits. Haplotype 1 was present in 17.4% of the entire HCM cohort and associated significantly with a 15.90 g decrease in LVM (p = 0.037). Conversely, haplotype 10 was present in only 1.8 % of the cohort and associated with a 36.90 g increase in LVM (p = 0.030).

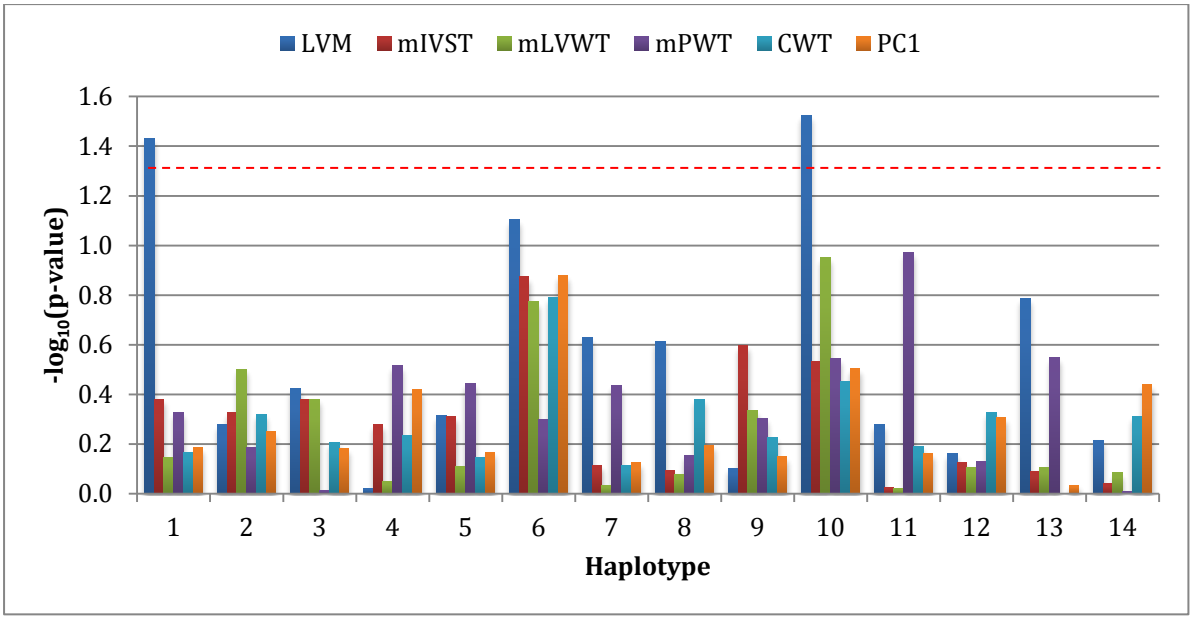


Figure 3.41 Summary of haplotype association results for SCNN1A. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.34 Haplotype distribution within SCNN1A, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes are indicated in the text.*

	rs11614164	rs3782726	rs7973914	rs10849446	rs2286600	freq.	p-value for test of association					
							LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	T	T	A	G	0.174	**0.037**	0.417	0.717	0.472	0.686	0.654
2	A	T	C	A	G	0.167	0.528	0.472	0.317	0.655	0.481	0.564
3	G	G	C	A	G	0.085	0.377	0.416	0.417	0.968	0.623	0.657
4	A	T	T	C	A	0.065	0.951	0.526	0.895	0.306	0.585	0.382
5	G	G	C	C	A	0.060	0.486	0.489	0.780	0.359	0.717	0.684
6	A	T	C	C	A	0.055	0.079	0.134	0.169	0.503	0.162	0.132
7	A	T	T	A	A	0.029	0.236	0.770	0.929	0.368	0.772	0.752
8	G	G	T	A	G	0.025	0.244	0.804	0.840	0.700	0.417	0.64
9	A	G	T	A	G	0.018	0.790	0.254	0.461	0.497	0.594	0.711
10	G	G	T	C	A	0.018	**0.030**	0.294	0.112	0.286	0.354	0.312
11	G	T	C	A	G	0.018	0.526	0.943	0.952	0.107	0.647	0.69
12	G	G	C	C	G	0.017	0.692	0.749	0.786	0.745	0.472	0.494
13	A	G	C	A	G	0.010	0.164	0.818	0.782	0.284	0.999	0.924
14	G	T	C	C	A	0.010	0.609	0.913	0.825	0.982	0.487	0.364

*Haplotypes < 0.01 not indicated

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.35 contains the p-values for interaction between the different HCM mutation groups and *SCNN1A* haplotypes to illustrate the differences in allelic effect of the different haplotypes between these groups. Haplotype 3 was found to have a significantly different effect on LVM between the R92W$_{TNNT2}$ and A797T$_{MYH7}$ groups (Table 3.35). This haplotype was associated with a 44.61g lower effect on LVM in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group. Similarly, haplotype 6 caused a 4.02 mm lower effect on mIVST (p = 0.013), a 3.69 mm lower effect on mLVWT (p = 0.010), a 1.88 mm lower effect on CWT score (p = 0.013), as well as a 0.12 lower effect on PC1 (p = 0.024). Haplotypes 7, 9 and 12-14 were not tested due to too small sample sizes for the three HCM mutation groups as a consequence of low haplotype frequency.

Table 3.35 The p-values for interaction between HCM mutation groups and SCNN1A haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

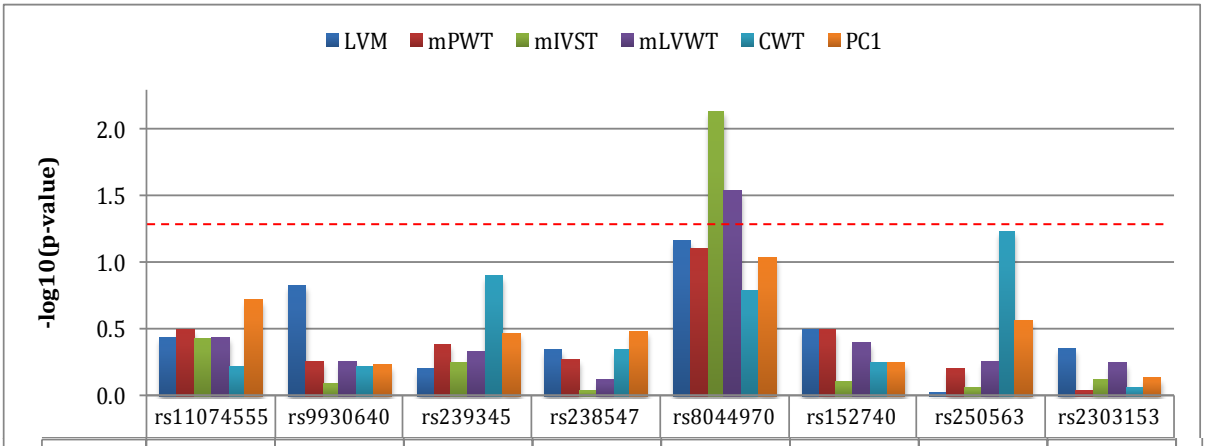
Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.659	0.416	0.725	0.662	0.251	0.506	0.256	0.182	0.854	0.151	0.111	0.901	0.507	0.055	0.263	0.545	0.063	0.265
2	0.462	0.769	0.646	0.366	0.366	0.911	0.928	0.894	0.836	0.691	0.364	0.691	0.646	0.740	0.898	0.703	0.871	0.836
3	0.149	**0.017**	0.649	0.260	0.074	0.797	0.355	0.061	0.563	0.227	0.183	0.839	0.372	0.056	0.695	0.421	0.095	0.751
4	0.960	0.345	0.070	0.643	0.221	0.196	0.479	0.232	0.497	0.336	0.512	0.473	0.692	0.469	0.641	0.552	0.503	0.994
5	0.584	0.491	0.830	0.528	0.322	0.880	0.429	0.439	0.667	0.945	0.770	0.820	0.273	0.357	0.507	0.282	0.510	0.433
6	0.209	0.355	0.594	0.106	**0.013**	0.579	0.166	**0.010**	0.364	0.781	0.152	0.298	0.095	**0.013**	0.687	0.209	**0.024**	0.515
8	0.598	0.726	0.365	0.344	0.437	0.080	0.140	0.248	0.007	0.393	0.664	0.197	0.208	0.473	0.042	0.482	0.379	0.113
10	0.641	0.663	0.824	0.964	0.961	0.985	0.914	0.670	0.865	0.319	0.279	0.706	0.919	0.969	0.933	0.709	0.805	0.810
11	0.635	0.578	0.980	0.841	0.958	0.710	0.481	0.619	0.689	0.645	0.255	0.417	0.558	0.917	0.523	0.548	0.981	0.432

*Haplotypes 7, 9 and 12-14 not tested

Abbreviations: **CWT:** cumulative wall thickness; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.7.2 *SCNN1B*

The G-allele of rs8044970 was associated with a significant decrease of 1.66 mm in mIVST (p = 0.007), as well a significant decrease of 1.28 mm in mLVWT (p = 0.029) as seen in Figure 3.42.



	rs11074555	rs9930640	rs239345	rs238547	rs8044970	rs152740	rs250563	rs2303153
LVM	0.363	0.149	0.619	0.460	0.069	0.319	0.960	0.441
mPWT	0.325	0.557	0.410	0.536	0.077	0.319	0.623	0.927
mIVST	0.378	0.798	0.571	0.910	0.007	0.786	0.861	0.758
mLVWT	0.359	0.552	0.464	0.749	0.029	0.401	0.552	0.572
CWT	0.615	0.615	0.125	0.455	0.163	0.563	0.059	0.867
PC1	0.194	0.597	0.337	0.334	0.092	0.571	0.269	0.735

Figure 3.42 Single polymorphism association results for SCNN1B. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Table 3.36 contains the p-values for interaction between HCM mutation group and *SCNN1B* haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. Haplotypes 6, 7, 9-14 and 16-26 were not tested due to unequal distribution of these haplotypes among the HCM mutation groups, which resulted in insufficient sample sizes for the analysis.

Haplotype 5 was found to have a significantly different effect on the respective mutation groups (Table 3.36). This haplotype was associated with a 56.78 g increased effect on LVM (p = 0.030), a 2.62 mm increased effect on mIVST (p = 0.047) and a 1.39 mm increased effect on mPWT (p = 0.049) in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group. Similarly, haplotype 5 was associated with an 8.79 mm increased effect on mLVWT (p = 0.028), a 4.40 mm increased effect on the CWT score (p = 0.010), as well as a 0.28 increased effect on PC1 (p = 0.034).

Table 3.36 The p-values for interaction between HCM mutation groups and SCNN1B haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.695	0.607	0.884	0.540	0.189	0.450	0.504	0.151	0.404	0.374	0.431	0.941	0.476	0.206	0.561	0.400	0.155	0.538
2	0.260	0.760	0.492	0.179	0.397	0.089	0.162	0.844	0.236	0.669	0.348	0.730	0.137	0.627	0.134	0.151	0.826	0.204
3	0.252	0.379	0.073	0.737	0.097	0.528	0.897	0.155	0.469	0.865	0.100	0.235	0.627	0.357	0.279	0.716	0.270	0.290
4	0.842	0.513	0.635	0.963	0.445	0.425	0.708	0.295	0.486	0.596	0.719	0.280	0.727	0.144	0.050	0.539	0.191	**0.036**
5	0.389	**0.030**	0.528	0.745	**0.047**	0.072	0.200	0.222	**0.028**	0.739	**0.049**	0.102	0.158	0.096	**0.010**	0.292	0.127	**0.034**
8	0.323	0.551	0.159	0.281	0.942	0.257	0.339	0.458	0.642	0.627	0.534	0.943	0.463	0.889	0.503	0.456	0.891	0.495
15	0.958	0.750	0.771	0.582	0.964	0.600	0.653	0.950	0.683	0.366	0.192	0.959	0.401	0.659	0.606	0.355	0.593	0.598

*Haplotypes 6, 7, 9-14 and 16-26 not tested

Abbreviations: **CWT:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Figure 3.43 provides an overview of the results obtained from the haplotype association analyses of *SCNN1B*, while Table 3.37 depicts the haplotype distribution observed for *SCNN1B,* as well as the respective p-values for association between these haplotypes and the heritable hypertrophy traits. Four haplotypes were found to associate significantly with hypertrophy traits. Haplotype 5 was observed in 5.9% of the cohort and was associated with a 0.083 mm increase in mPWT (p = 0.046). Haplotype 9 was significantly associated with a 0.94 mm decrease in mPWT (p = 0.046), as well as a 1.09mm decrease in CWT score (p = 0.020) as a 0.068 decrease in PC1 (p = 0.022).

Interestingly, haplotype 19 was found to associate significantly with almost all the investigated hypertrophy traits (Figure 3.43). This haplotype was significantly associated with a decrease in mIVST of 2.80 mm (p = 0.023), a 2.58 mm decrease in mLVWT (p = 0.016), a 1.22 mm decrease in mPWT (p = 0.041), a 1.48mm decrease in CWT score (p = 0.015), as well as a 0.093 decrease in PC1 (p = 0.025) (Table 3.37). In addition, haplotype 20 was associated with a 3.88 mm increase in mIVST (p = 0.012) and a 3.63 mm increase in mLVWT (p = 0.015).

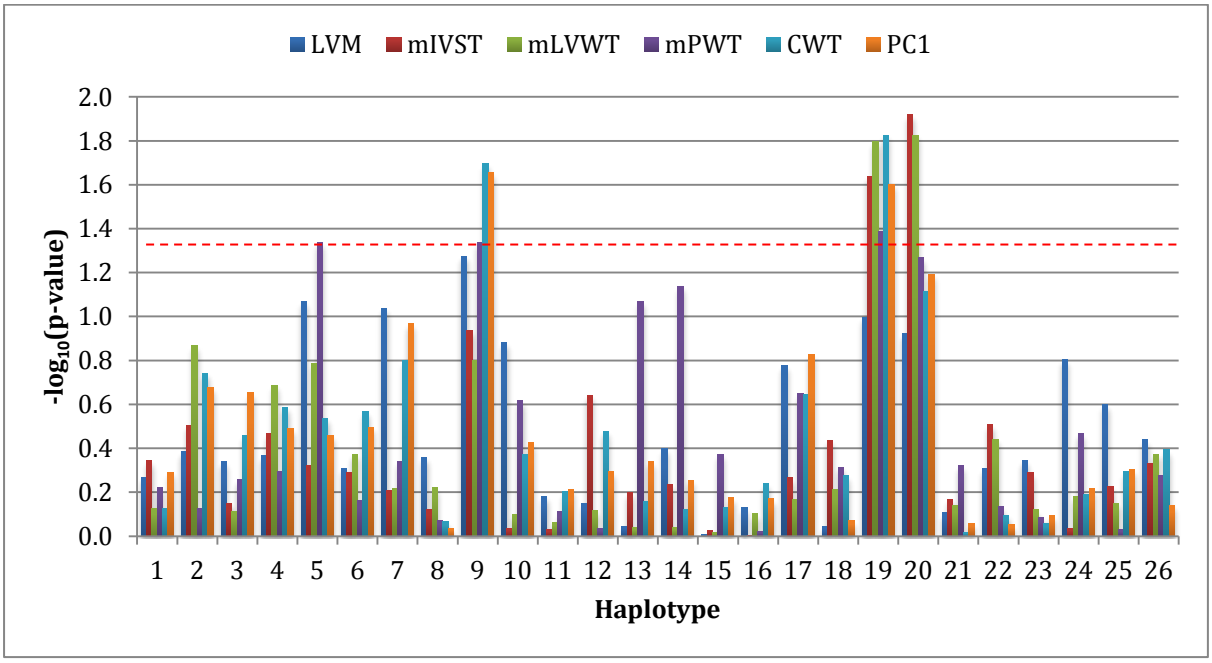


Figure 3.43 Summary of haplotype association results for SCNN1B. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

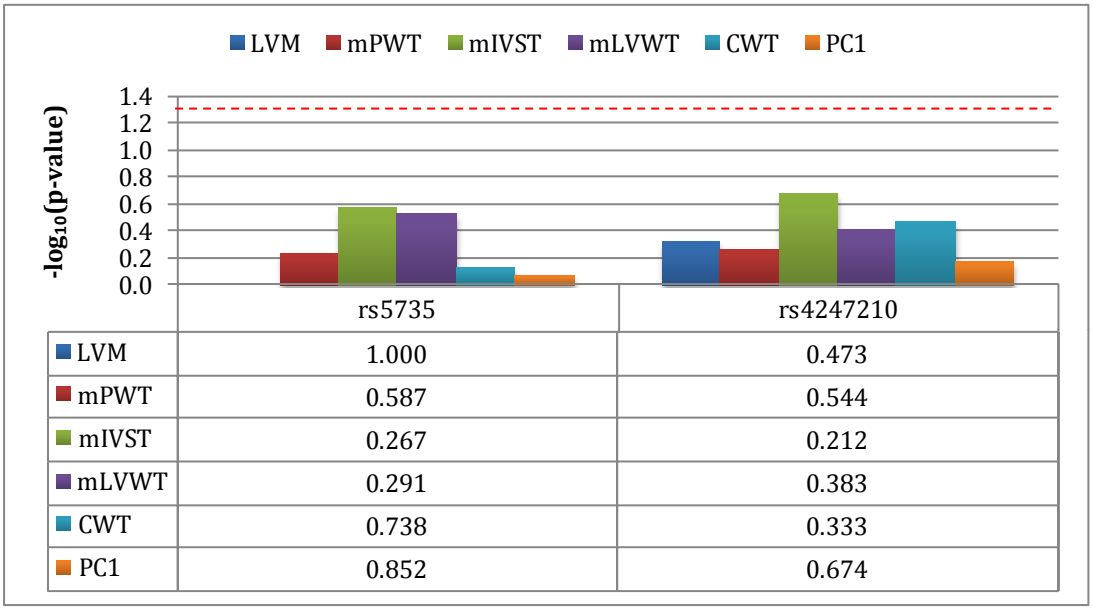
Table 3.37 Haplotype distribution within SCNN1B, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes are indicated in the text.*

	rs11074555	rs9930640	rs239345	rs238547	rs8044970	rs152740	rs250563	rs2303153	freq.	p-value for test of association					
										LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	T	G	T	C	G	T	C	G	0.100	0.540	0.454	0.744	0.598	0.744	0.512
2	C	G	A	C	T	A	C	G	0.074	0.413	0.312	0.135	0.750	0.182	0.211
3	T	G	T	T	T	A	C	G	0.072	0.456	0.708	0.769	0.553	0.348	0.222
4	T	G	T	T	T	A	C	C	0.062	0.430	0.339	0.205	0.505	0.258	0.322
5	T	G	T	C	T	T	C	G	0.059	0.085	0.476	0.164	**0.046**	0.292	0.348
6	T	G	T	C	T	T	C	C	0.040	0.490	0.511	0.424	0.684	0.271	0.319
7	C	G	A	T	T	A	C	G	0.028	0.092	0.621	0.605	0.455	0.159	0.107
8	C	G	A	C	T	A	C	C	0.028	0.437	0.756	0.602	0.848	0.858	0.920
9	C	G	A	C	T	T	C	G	0.024	0.053	0.116	0.158	**0.046**	**0.020**	**0.022**
10	C	A	A	C	T	T	C	G	0.023	0.131	0.920	0.795	0.242	0.423	0.374
11	T	G	T	C	G	T	C	C	0.022	0.660	0.933	0.861	0.773	0.623	0.611
12	T	G	T	T	T	A	T	G	0.022	0.707	0.228	0.761	0.925	0.332	0.508
13	T	G	A	C	T	A	C	G	0.018	0.900	0.635	0.913	0.085	0.697	0.456
14	C	G	T	C	T	T	C	G	0.017	0.399	0.579	0.910	0.073	0.755	0.555
15	C	G	T	C	T	A	C	G	0.017	0.980	0.941	0.961	0.424	0.741	0.668
16	T	G	A	C	T	T	C	G	0.017	0.741	0.987	0.790	0.946	0.575	0.674
17	C	G	T	C	G	A	C	G	0.014	0.167	0.542	0.680	0.224	0.227	0.148
18	C	G	T	C	G	T	C	G	0.012	0.903	0.367	0.614	0.484	0.528	0.849
19	T	G	A	C	G	A	C	G	0.012	0.101	**0.023**	**0.016**	**0.041**	**0.015**	**0.025**
20	T	G	T	T	T	T	C	G	0.012	0.119	**0.012**	**0.015**	0.054	0.077	0.064
21	C	A	T	C	G	T	C	G	0.011	0.782	0.680	0.727	0.475	0.957	0.873
22	C	A	A	C	T	A	C	G	0.010	0.489	0.309	0.361	0.733	0.806	0.888
23	C	G	A	T	T	A	C	C	0.010	0.451	0.510	0.758	0.821	0.876	0.804
24	T	G	T	C	T	A	C	G	0.010	0.156	0.920	0.662	0.340	0.645	0.608
25	T	G	A	C	T	A	C	C	0.009	0.250	0.595	0.707	0.934	0.508	0.496
26	C	G	A	C	G	T	C	G	0.008	0.364	0.467	0.423	0.528	0.404	0.724

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

3.7.7.3 *SCNN1G*

We did not find any statistically significant evidence for association between any of the *SCNN1G* variants and the investigated hypertrophy traits (Figure 3.44).



	rs5735	rs4247210
LVM	1.000	0.473
mPWT	0.587	0.544
mIVST	0.267	0.212
mLVWT	0.291	0.383
CWT	0.738	0.333
PC1	0.852	0.674

Figure 3.44 Single polymorphism association results for SCNN1G. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

No statistically significant evidence for association was found between the identified *SNNN1G* haplotypes and the investigated hypertrophy traits (Figure 3.45). Table 3.38 depicts the haplotype distribution within *SCNN1G*, as well as the respective p-values for tests of allelic association.

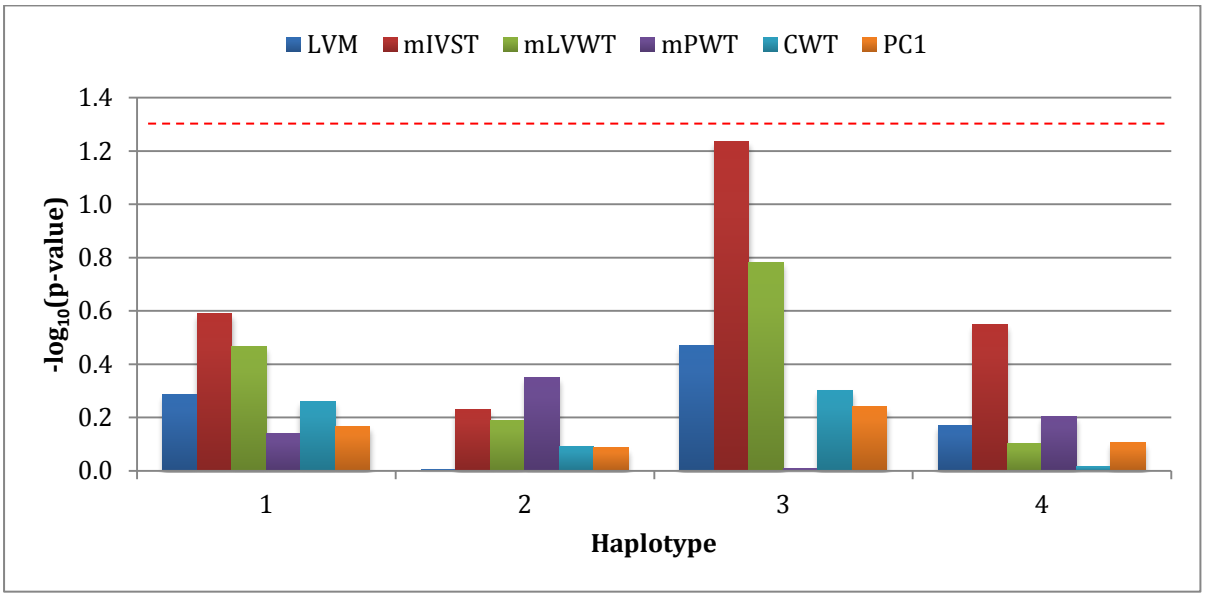


Figure 3.45 Summary of haplotype association results for SCNN1G. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.38 Haplotype distribution within SCNN1G, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates.*

	rs5735	rs4247210	freq.	p-value for test of association					
				LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	C	C	0.329	0.518	0.257	0.340	0.726	0.550	0.680
2	C	G	0.285	0.989	0.586	0.645	0.445	0.810	0.820
3	T	G	0.071	0.339	0.058	0.165	0.980	0.499	0.575
4	T	C	0.059	0.673	0.282	0.789	0.623	0.960	0.782

Abbreviations: **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.39 contains the p-values for interaction between HCM mutation group and *SCNN1G* haplotypes, illustrating the differences in allelic effect of the identified haplotypes between these groups.

Table 3.39 The p-values for interaction between HCM mutation group sand SCNN1G haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.503	**0.027**	0.183	0.516	**0.006**	0.057	0.397	**0.004**	0.067	0.878	0.118	0.200	0.758	**0.001**	**0.006**	0.902	**0.004**	**0.011**
2	0.175	0.496	**0.045**	0.572	0.289	0.127	0.807	0.095	0.081	0.132	0.977	0.107	0.216	0.148	**0.013**	0.157	0.290	**0.020**
3	0.521	0.059	0.576	0.491	**0.002**	0.218	0.724	**0.025**	0.281	0.552	**0.009**	0.307	0.355	**0.019**	0.484	0.314	**0.011**	0.440
4	0.667	0.705	0.260	0.758	0.703	0.342	0.692	0.838	0.393	0.666	0.587	0.181	0.666	0.688	0.903	0.804	0.904	0.834

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

We identified three haplotypes that had differential effects on the three HCM mutation groups (Table 3.39). Haplotype 1 was associated with a 21.05 g lower effect on LVM (p = 0.027), a 3.27 mm lower effect on mIVST (p = 0.006), a 3.44 mm lower effect on mLVWT (p = 0.004) and a 1.99 mm lower effect on the CWT score in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group. In addition this haplotype was associated with a 2.04 mm lower effect on the CWT score (p = 0.006) and a 0.13 lower effect on PC1 in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group.

Haplotype 2 was found to have a 32.79 g higher effect on LVM (p = 0.045), as well as a 1.93 mm higher effect on the CWT score (p = 0.013) in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group (Table 3.39). Haplotype 3 resulted in a 5.59 mm increased effect on mIVST (p = 0.002), as well as an increased effect of 4.56 mm mLVWT, 2.09 mm on mPWT (p = 0.009) and 2.47 mm on the CWT score in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (Table 3.39).

3.7.8 Na^+/K^+-ATPase subunits

Figures 3.46, 3.47, 3.48 and 3.49 depict the chromosomal location and structure of the *ATP1A1*, *ATP1A2*, *ATP1B1* and *ATP1B3* genes, as well as intragenic location of target polymorphisms within these genes.

The two SNPs investigated in *ATP1A1*, viz. rs10924074 and rs850609, was found to be in complete LD (D' = 1). Intermediate D' values were observed between rs7548116 and rs6695366 (D' = 0.48), as well as between rs6695366 and rs11585375 (D' = 0.42), while scant evidence exists for LD between rs7548116 and rs11585375 (D' = 0.1) in *ATP1A2* (Figure 3.47). In addition, very little evidence exists for LD between the variants investigated in *ATP1B1*; LD values were D' = 0.16 between rs1200130 and rs1358714, D' = 0.36 between rs1200130 and rs1040503 and D' = 0.06 between rs1358714 and rs1040503 (Figure 3.48).

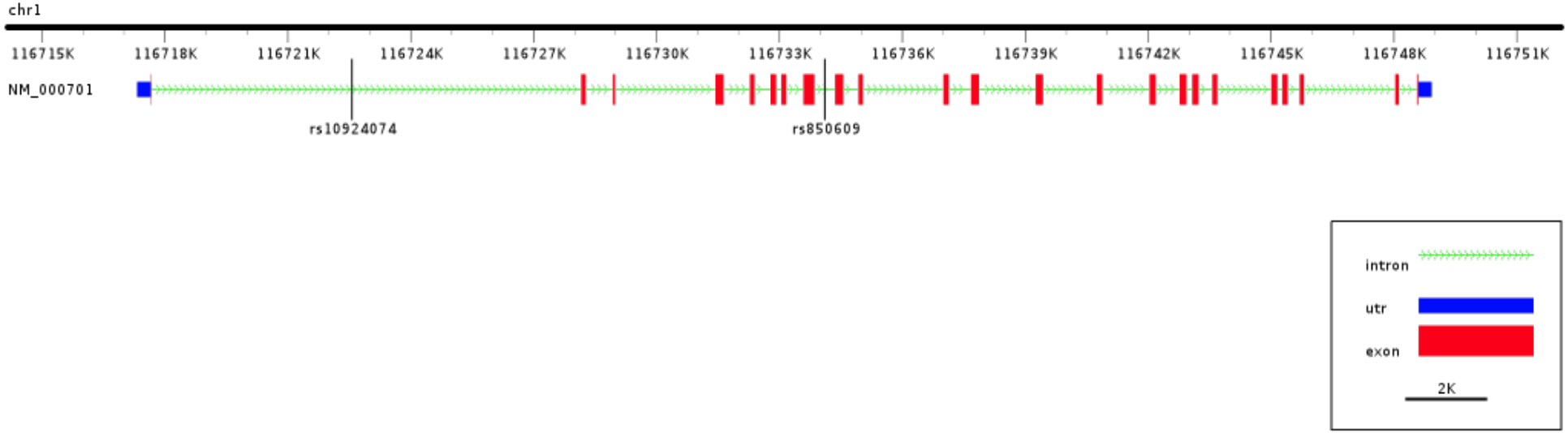


Figure 3.46 Scale diagram depicting chromosomal location and structure of the ATP1A1 gene, as well as intragenic location of target polymorphisms. *The two SNPs were found to be in complete LD (D' = 1). Arrows indicate direction of transcription.*

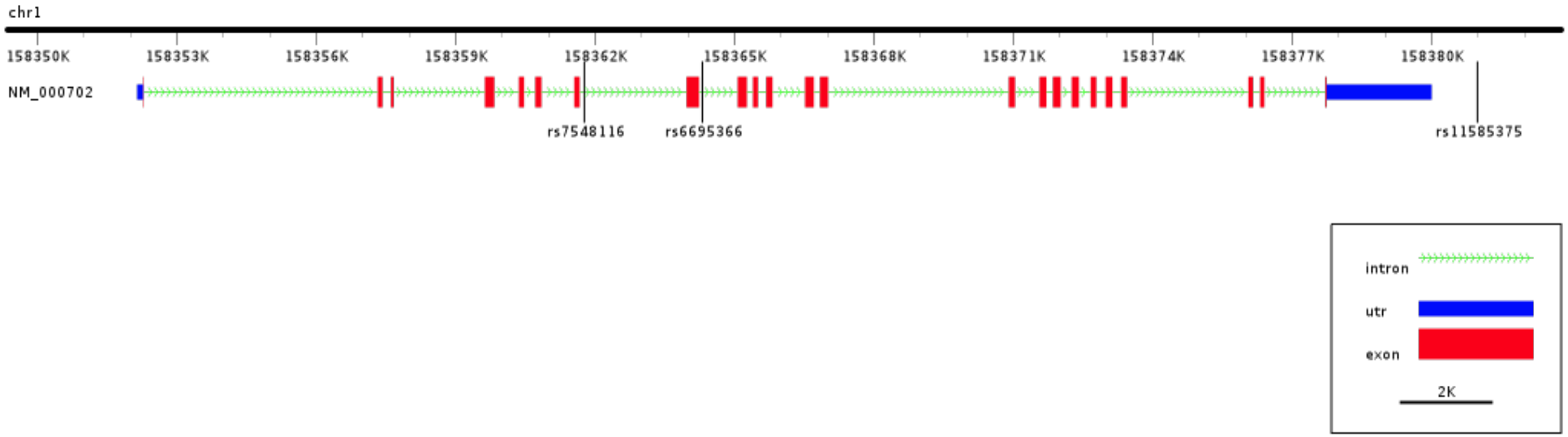


Figure 3.47 Scale diagram depicting chromosomal location and structure of the ATP1A2 gene, as well as intragenic location of target polymorphisms. *LD values were D' = 0.48 between rs7548116 and rs6695366, D' = 0.10 between rs7548116 and rs11585375 and D' = 0.42 between rs6695366 and rs11585375. Arrows indicate direction of transcription.*

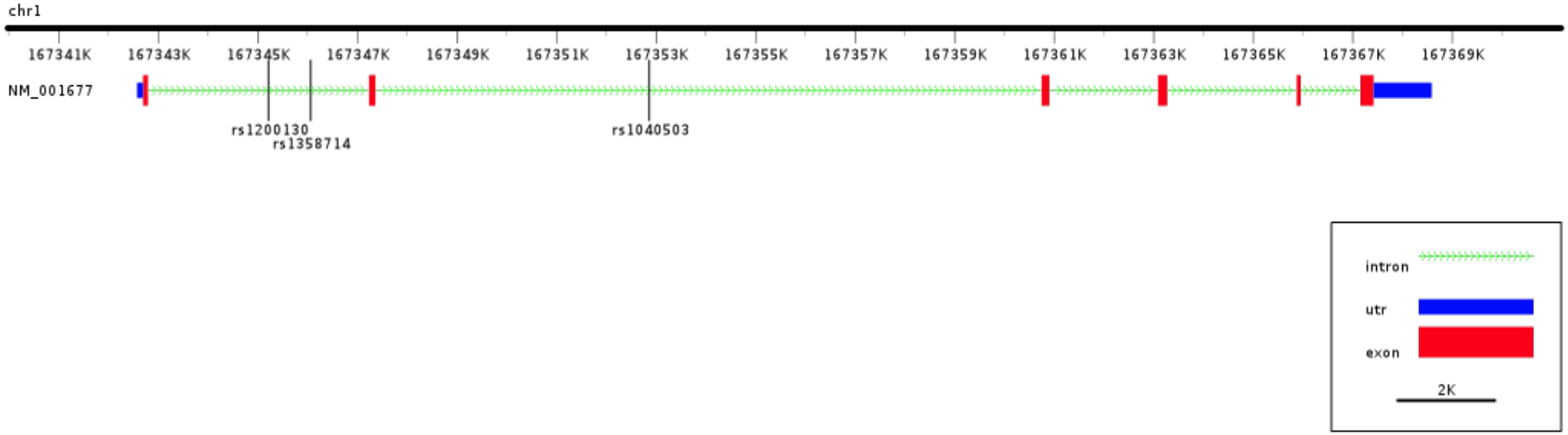


Figure 3.48 Scale diagram depicting chromosomal location and structure of the ATP1B1 gene, as well as intragenic location of target polymorphisms. *LD values were D' = 0.16 between rs1200130 and rs1358714, D' = 0.36 between rs1200130 and rs1040503 and D' = 0.06 between rs1358714 and rs1040503. Arrows indicate direction of transcription.*

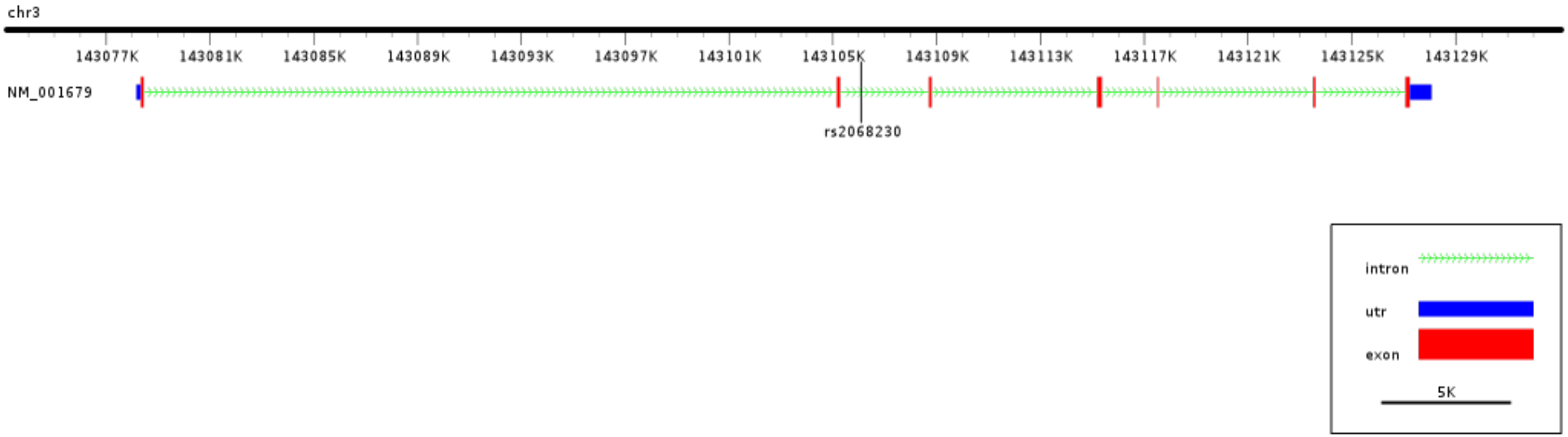
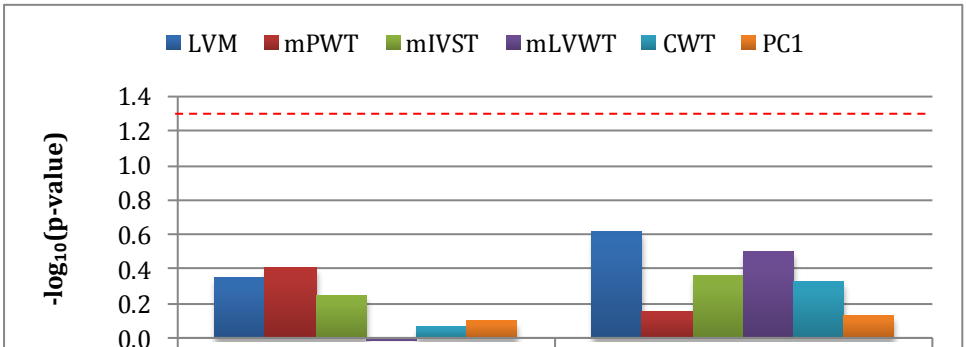


Figure 3.49 Scale diagram depicting chromosomal location and structure of the ATP1B3 gene, as well as intragenic location of target polymorphism. *Arrows indicate direction of transcription.*

3.7.8.1 *ATP1A1*

There was, however, no statistically significance for association between the investigated *ATP1A1* SNPs and the hypertrophy traits (Figure 3.50).



	rs10924074	rs850609
LVM	0.445	0.239
mPWT	0.387	0.689
mIVST	0.558	0.430
mLVWT	0.989	0.314
CWT	0.860	0.466
PC1	0.790	0.731

Figure 3.50 Single polymorphism association results for ATP1A1. *Bar graph indicates $-\log_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of $p < 0.05$, and effect sizes for significant associations are indicated in the text.*

Table 3.40 depicts the p-values for interaction between HCM mutation group and *ATP1A1*, *ATP1A2*, *ATP1B1* or *ATP1B3* genotypes, illustrating the differences in allelic effect of the particular variants between these groups.

The rs850609 SNP in *ATP1A1* was found to have significantly different effects on mPWT (p = 0.040), CWT score (p = 0.040) and PC1 (p = 0.043) in the $R92W_{TNNT2}$ group versus the $A797T_{MYH7}$ group (Table 3.40). The T-allele of this polymorphism was found to have an increased effect of 1.40 mm on mPWT, 1.94 mm on CWT and 0.12 on PC1 (Table 3.40).

Table 3.40 The p-values for interaction between HCM mutation groups and ATP1A1, ATP1A2, ATP1B1 or ATP1B3 genotype, illustrating the differences in allelic effect of the particular variants between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

		LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
		R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
ATP1A1	**rs10924074**	0.637	0.765	0.902	0.717	0.609	0.411	0.694	0.328	0.193	0.997	0.218	0.227	0.922	0.390	0.460	0.890	0.370	0.460
	rs850609	0.169	0.157	0.626	0.336	0.069	0.806	0.420	0.136	0.849	0.121	**0.040**	0.820	0.273	**0.040**	0.700	0.272	**0.043**	0.719
ATP1A2	**rs7548116**	0.588	0.360	0.222	0.591	0.912	0.527	0.933	0.210	0.313	0.524	0.450	0.227	0.830	0.686	0.613	0.815	0.740	0.636
	rs6695366	0.378	0.987	0.341	0.347	0.709	0.432	0.339	0.472	0.557	0.196	0.132	0.620	0.176	0.394	0.309	0.255	0.356	0.447
	rs11585375	0.425	0.793	0.590	0.950	0.560	0.547	0.963	0.441	0.503	0.917	0.430	0.405	0.822	0.459	0.381	0.875	0.488	0.442
ATP1B1	**rs1200130**	0.094	0.881	0.084	0.458	0.958	0.504	0.308	0.942	0.378	0.897	0.114	0.202	0.436	0.684	0.283	0.421	0.682	0.271
	rs1358714	0.761	0.516	0.826	0.992	0.933	0.936	0.893	0.960	0.920	0.796	0.693	0.555	0.416	0.717	0.576	0.373	0.806	0.466
	rs1040503	0.122	0.314	0.497	0.212	0.414	0.566	0.134	0.195	0.733	0.389	0.329	0.998	0.137	0.158	0.787	0.204	0.237	0.803
ATP1B3	**rs2068230**	0.791	**0.030**	0.097	0.563	**0.002**	**0.033**	0.589	**0.002**	**0.029**	0.851	0.051	0.055	0.894	**0.004**	**0.021**	0.916	**0.009**	**0.019**

Abbreviations: ***ATP1A1*****:** ATPase, Na^+/K^+ transporting, alpha 1 polypeptide gene; ***ATP1A2*****:** ATPase, Na^+/K^+ transporting, alpha 2 polypeptide gene; ***ATP1B1*****:** ATPase, Na^+/K^+ transporting, beta 1 polypeptide gene; ***ATP1B3*****:** ATPase, Na^+/K^+ transporting, beta 3 polypeptide gene; **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

Figure 3.51 shows a graphical summary of the results for the haplotype association analysis for *ATP1A1*. Table 3.41 shows the observed haplotype distribution for *ATP1A1,* as well as the exact p-values for the respective tests of association between these haplotypes and the investigated hypertrophy traits. Haplotype 4 was observed in 2.2% of the cohort and associated significantly with 1.48 mm increase in mPWT (p = 0.021).

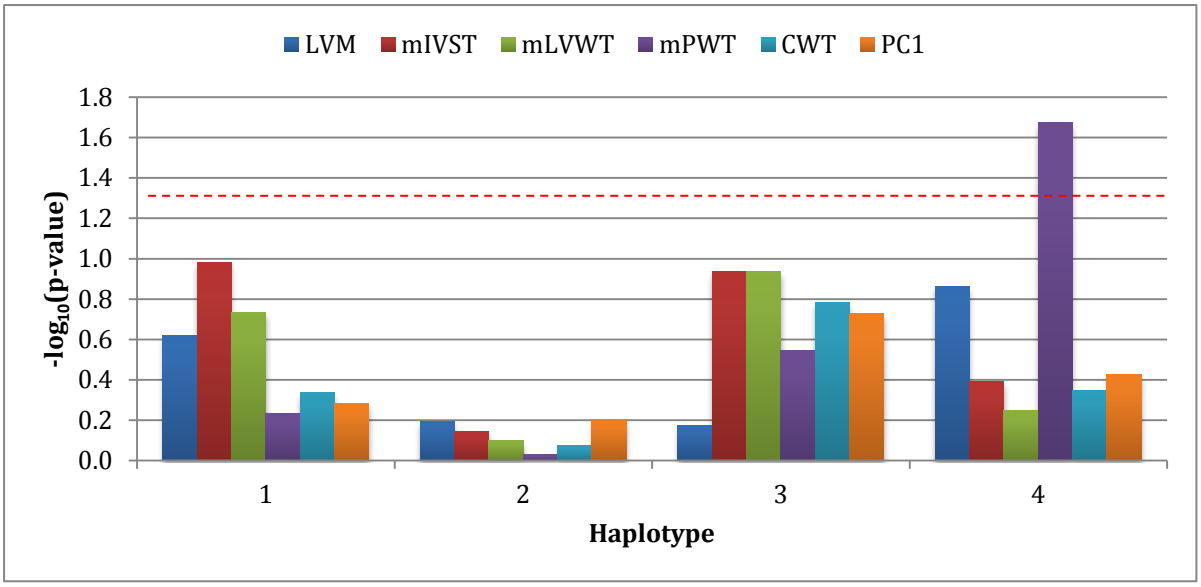


Figure 3.51 Summary of haplotype association results for ATP1A1. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.41 Haplotype distribution within ATP1A1, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes indicated in the text.*

	rs10924074	rs850609	freq.	p-value for test of association					
				LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	A	0.765	0.241	0.104	0.184	0.587	0.462	0.523
2	G	A	0.112	0.641	0.716	0.799	0.934	0.843	0.627
3	A	T	0.101	0.670	0.115	0.115	0.284	0.165	0.186
4	G	T	0.022	0.137	0.406	0.567	**0.021**	0.449	0.376

Abbreviations: **A:** adenine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.42 contains the p-values for interaction between HCM mutation group and *ATP1A1* haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. Haplotype 2 was found to have 1.78 mm increased effect on mPWT in the R92W$_{TNNT2}$ (p = 0.038), as well as a 1.81 mm increased effect on mPWT in the R403W$_{MYH7}$ group (p = 0.031), compared to the A797T$_{MYH7}$ group. Haplotype 3 had a 44.70 g higher effect on LVM in the R92W$_{TNNT2}$ group, compared to the R403W$_{MYH7}$ group (p = 0.040).

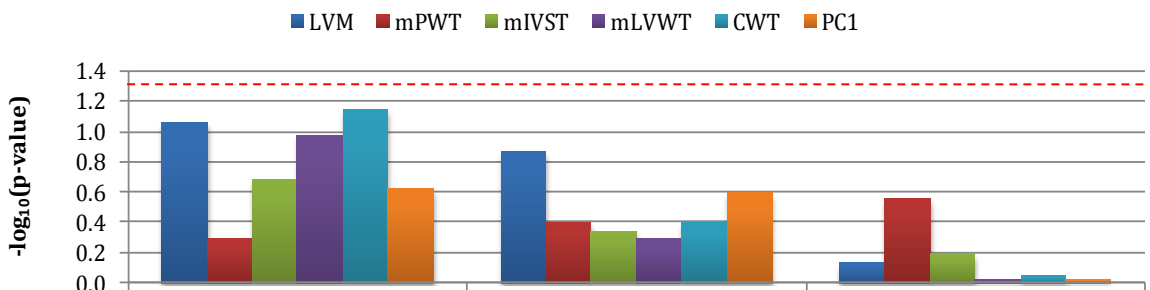
Table 3.42 The p-values for interaction between HCM mutation groups and ATP1A1 haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.676	0.991	0.418	0.368	0.329	0.232	0.347	0.933	0.774	0.863	0.299	0.305	0.714	0.479	0.801	0.683	0.407	0.641
2	0.534	0.766	0.379	0.792	0.106	0.259	0.926	0.119	0.146	0.712	**0.038**	**0.031**	0.687	0.120	0.332	0.721	0.111	0.294
3	**0.040**	0.334	0.210	0.147	0.063	0.854	0.161	0.200	0.787	0.132	0.395	0.424	0.397	0.172	0.741	0.321	0.146	0.793
4	0.523	0.632	0.857	0.188	0.305	0.736	0.375	0.998	0.335	0.725	0.797	0.916	0.878	0.913	0.986	0.852	0.725	0.845

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.8.2 *ATP1A2*

We did not find any statistically significant evidence for association between the *ATP1A2* SNPs and hypertrophy traits (Figure 3.52). In addition, the investigated *ATP1A2* SNPs were not found to have differential effects on the three HCM mutation groups (Table 3.40).



	rs7548116	rs6695366	rs11585375
LVM	0.086	0.135	0.731
mPWT	0.506	0.394	0.273
mIVST	0.206	0.456	0.644
mLVWT	0.107	0.504	0.953
CWT	0.071	0.401	0.896
PC1	0.239	0.252	0.941

Figure 3.52 Single polymorphism association results for ATP1A2. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Two haplotypes within *ATP1A2* associated significantly with hypertrophy traits (Figure 3.53). Haplotype 1 within *ATP1A2* was present in 29% of the cohort and associated significantly with an 11.1 g decrease in LVM (p = 0.027), as well as a 0.97 mm decrease in mIVST (p = 0.016) (Table 3.43). However, the exact opposite of this haplotype, i.e. the rarer haplotype 7, was associated with a 44.10 g increase in LVM (p = 0.025).

Table 3.44 contains the p-values for interaction between HCM mutation group and *ATP1A2* haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. Haplotype 4 was found to be associated with a 46.65 g increased effect on LVM in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (p = 0.035).

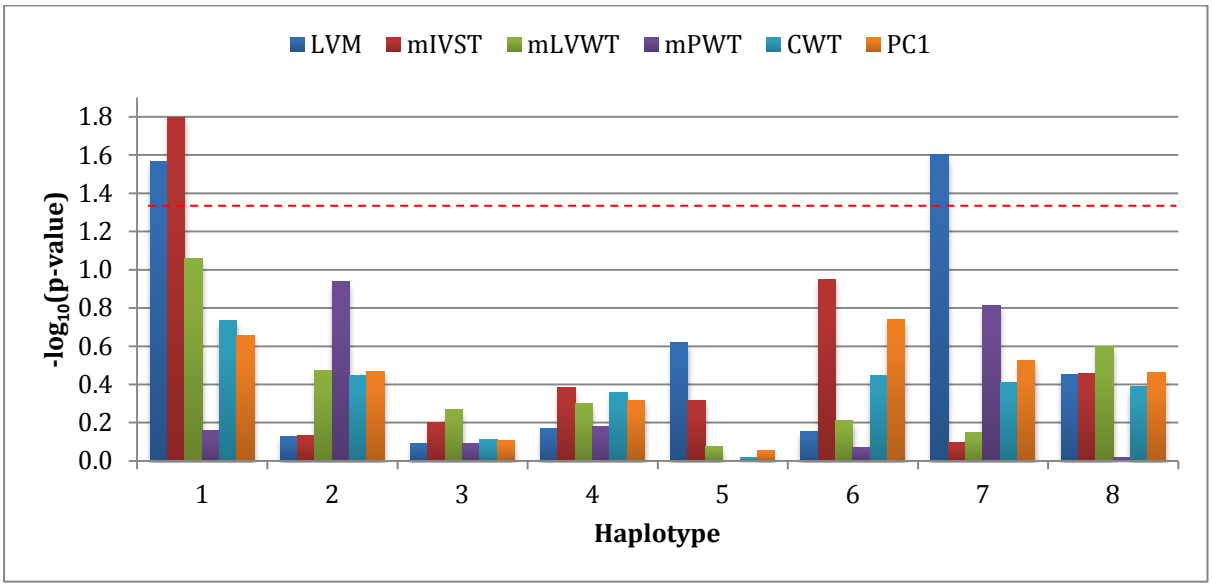


Figure 3.53 Summary of haplotype association results for ATP1A2. *Bar graph indicates –log 10 transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.43 Haplotype distribution within ATP1A2, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes are indicated in the text.*

	rs7548116	rs6695366	rs11585375	freq.	p-value for test of association					
					LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	A	A	A	0.290	**0.027**	**0.016**	0.087	0.695	0.184	0.221
2	A	A	G	0.174	0.748	0.737	0.337	0.115	0.358	0.339
3	T	A	A	0.139	0.813	0.626	0.538	0.809	0.777	0.781
4	T	A	G	0.077	0.676	0.414	0.499	0.661	0.437	0.484
5	T	G	A	0.051	0.241	0.484	0.837	0.990	0.955	0.884
6	A	G	A	0.021	0.700	0.112	0.614	0.854	0.359	0.181
7	T	G	G	0.019	**0.025**	0.805	0.711	0.153	0.391	0.299
8	A	G	G	0.010	0.352	0.351	0.252	0.962	0.406	0.346

Abbreviations: **A:** adenine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

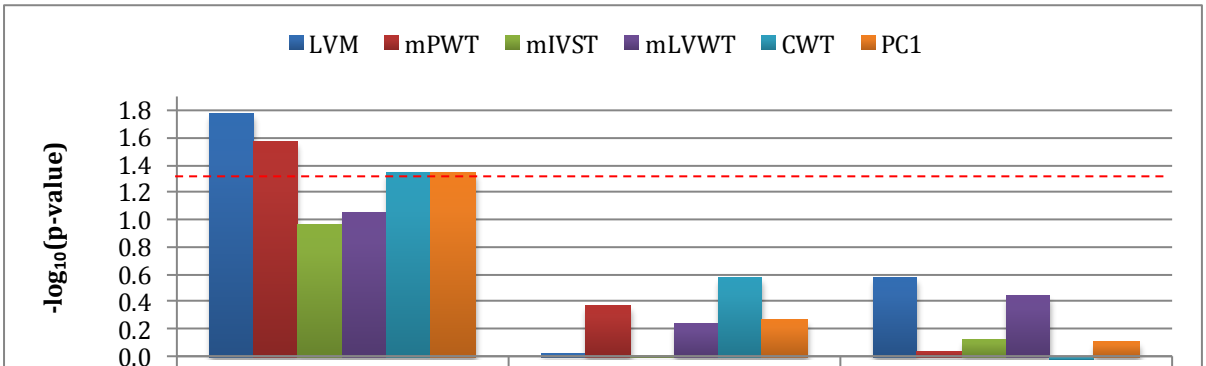
Table 3.44 The p-values for interaction between HCM mutation group and ATP1A2 haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.357	0.774	0.471	0.544	0.928	0.568	0.416	0.432	0.890	0.900	0.945	0.944	0.367	0.563	0.668	0.425	0.622	0.693
2	0.970	0.089	0.116	0.883	0.260	0.358	0.797	0.179	0.309	0.898	0.274	0.367	0.980	0.435	0.483	0.929	0.475	0.564
3	0.101	0.527	0.232	0.128	0.461	0.319	0.242	0.690	0.146	0.200	0.488	0.077	0.217	0.763	0.303	0.354	0.850	0.425
4	0.696	**0.035**	0.114	0.869	0.872	0.795	0.871	0.618	0.655	0.904	0.610	0.869	0.997	0.770	0.883	0.872	0.820	0.785
5	0.873	0.260	0.355	0.348	0.497	0.143	0.203	0.804	0.226	0.688	0.928	0.708	0.304	0.851	0.327	0.500	0.922	0.510

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.8.3 *ATP1B1*

The rs1200130 SNP within *ATP1B1* was significantly associated four hypertrophy traits, viz. LVM (p = 0.017), mPWT (p = 0.027), CWT score (p = 0.045), as well as PC1 (p = 0.046) (Figure 3.54). The T-allele of this polymorphism was associated with a 12.50g increase in LVM, 0.393 mm increase in mPWT, a 0.38 mm increase in CWT score, as well as a 0.02 increase in PC1. The investigated *ATP1B1* SNPs were not found to have differential effects on the three HCM mutation groups (Table 3.40).



	rs1200130	rs1358714	rs1040503
LVM	0.017	0.950	0.270
mPWT	0.027	0.418	0.895
mIVST	0.110	0.965	0.742
mLVWT	0.089	0.569	0.362
CWT	0.045	0.270	0.993
PC1	0.046	0.525	0.757

Figure 3.54 Single polymorphism association results for ATP1B1. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

Two *ATP1B1* associated significantly with hypertrophy traits (Figure 3.55). Table 3.45 shows the observed haplotype distribution for *ATP1B1,* as well as the p-values for the respective tests of association between these haplotypes and the investigated hypertrophy traits. Haplotype 2 was present in 14.5% of the cohort and associated significantly with a 20.20 g decrease in LVM (p = 0.006). In contrast, haplotype 5 was associated with a 0.83 mm increase in mPWT (p = 0.043).

Table 3.46 contains the p-values for interaction between HCM mutation group and *ATP1B1* haplotypes, illustrating the differences in allelic effect of the particular haplotypes between

these groups. Haplotype 6 was associated with a 1.94 mm lower effect on the CWT score in the R92W$_{TNNT2}$ group, compared to the R403W$_{MYH7}$ group (p = 0.040). Additionally, haplotype 7 was associated with a 2.27 mm higher effect on mPWT in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (p = 0.019). Haplotype 8 was not included in this analysis due to a low MAF.

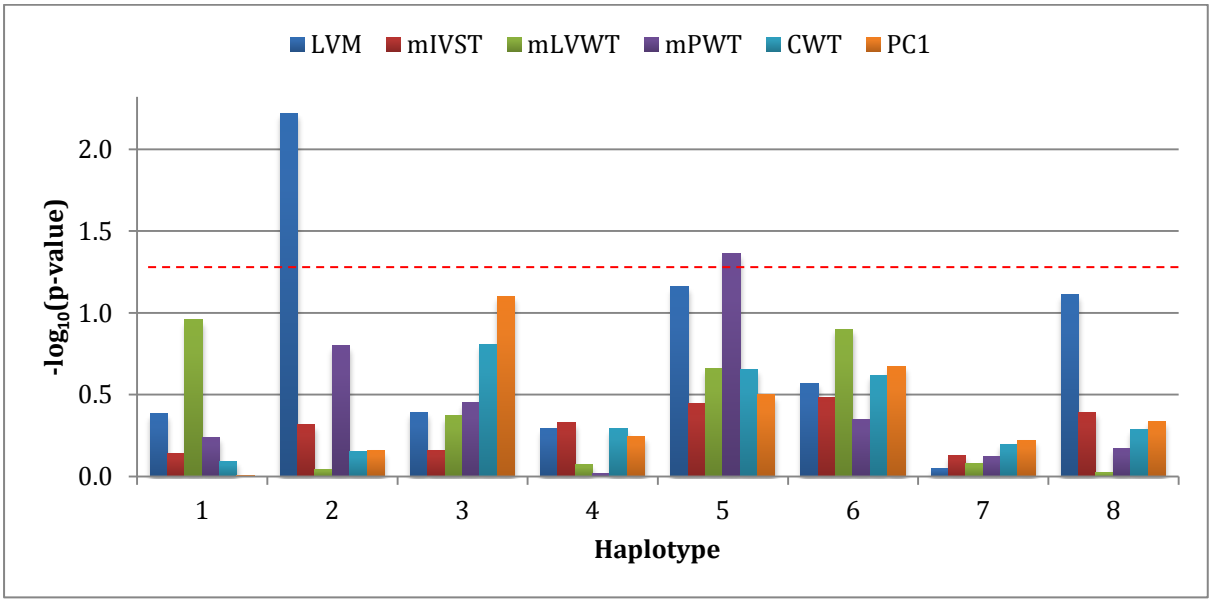


Figure 3.55 Summary of haplotype association results for ATP1B1. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between the observed haplotypes and investigated hypertrophy traits. Dashed red line indicates a significance level of p < 0.05 and effect sizes for significant associations are indicated in the text.*

Table 3.45 Haplotype distribution within ATP1B1, as well as the respective p-values for tests of allelic association. *All analyses were adjusted for the primary HCM-causal mutation, as well as other known hypertrophy covariates. Effect sizes are indicated in the text.*

	rs1200130	rs1358714	rs1040503	freq.	p-value for test of association					
					LVM	mIVST	mLVWT	mPWT	CWT	PC1
1	C	G	A	0.186	0.414	0.724	0.110	0.574	0.808	0.990
2	C	G	G	0.145	**0.006**	0.482	0.899	0.159	0.707	0.691
3	C	A	G	0.142	0.408	0.691	0.426	0.350	0.156	0.079
4	C	A	A	0.130	0.507	0.465	0.844	0.955	0.511	0.572
5	T	A	G	0.091	0.069	0.355	0.219	**0.043**	0.221	0.317
6	T	G	G	0.064	0.268	0.329	0.126	0.446	0.241	0.212
7	T	A	A	0.052	0.891	0.744	0.836	0.751	0.635	0.602
8	T	G	A	0.017	0.077	0.405	0.944	0.674	0.517	0.463

Abbreviations: **A:** adenine; **C:** cytosine; **CWT:** cumulative wall thickness score; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component; **T:** thymine

Table 3.46 The p-values for interaction between HCM mutation groups and ATP1B1 haplotypes, illustrating the differences in allelic effect of the particular haplotypes between these groups. *Significant p-values indicated in bold red font and effect sizes discussed in the text.*

Haplotype	LVM			mIVST			mLVWT			mPWT			CWT score			PC1		
	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T	R92W vs R403W	R92W vs A797T	R403W vs A797T
1	0.796	0.472	0.243	0.910	0.422	0.275	0.782	0.820	0.538	0.911	0.598	0.632	0.949	0.588	0.493	0.906	0.652	0.718
2	0.106	0.548	0.231	0.502	0.933	0.545	0.749	0.888	0.690	0.855	0.630	0.919	0.924	0.824	0.963	0.952	0.697	0.853
3	0.615	0.457	0.272	0.938	0.403	0.443	0.856	0.460	0.429	0.780	0.146	0.140	0.297	0.218	0.052	0.192	0.400	0.060
4	0.068	0.257	0.213	0.486	0.229	0.983	0.492	0.408	0.830	0.861	0.494	0.820	0.553	0.496	0.795	0.606	0.936	0.623
5	0.288	0.800	0.408	0.129	0.174	0.926	0.315	0.438	0.851	0.905	0.943	0.838	0.192	0.645	0.403	0.197	0.832	0.275
6	0.051	0.384	0.225	0.335	0.492	0.105	0.103	0.726	0.184	0.318	0.555	0.614	**0.040**	0.436	0.166	0.064	0.225	0.421
7	0.758	0.224	0.264	0.291	0.590	0.556	0.339	0.226	0.974	0.262	**0.019**	0.545	0.191	0.092	0.849	0.231	0.086	0.945

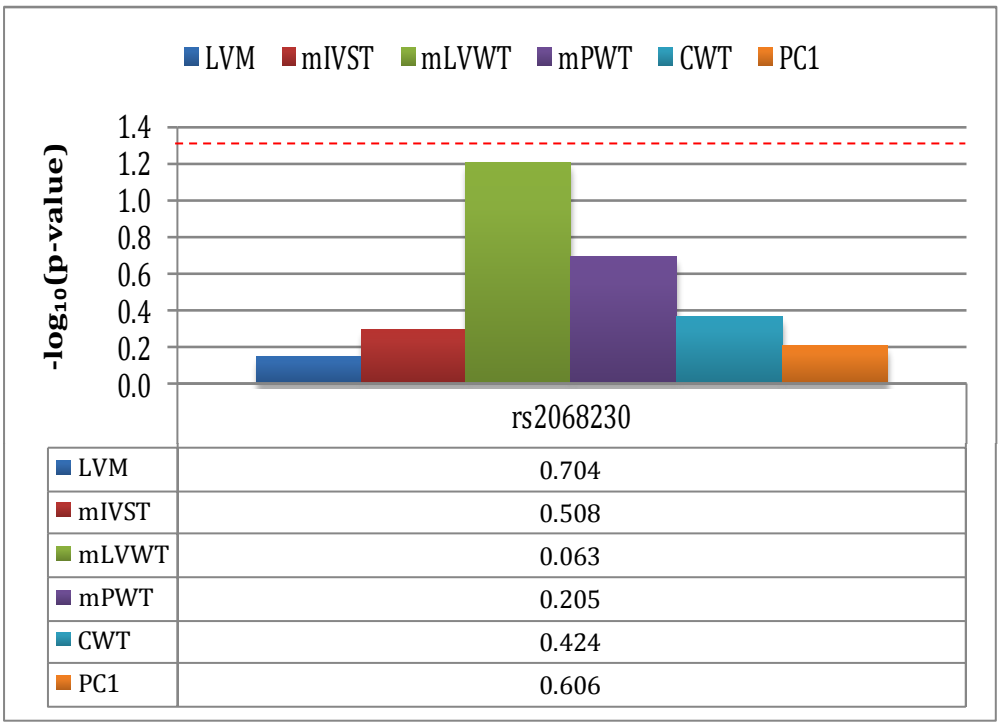
* Haplotype 8 not tested

Abbreviations: **CWT score:** cumulative wall thickness score; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **mPWT:** maximum posterior wall thickness; **PC1:** first principal component

3.7.8.4 *ATP1B3*

The rs2068230 SNP in *ATP1B3* showed the most pronounced difference in effect between the respective HCM mutation groups (Table 3.40). The T-allele of this polymorphism caused a 25.70 g higher LVM in the R92W$_{TNNT2}$ group, when compared to the A797T$_{MYH7}$ group (p = 0.030). In addition, the rs2068230 T-allele was associated with a 3.56 mm higher mIVST in the R92W$_{TNNT2}$ versus the A797T$_{MYH7}$ group, as well as a 2.68 mm higher mIVST in the R403W$_{MYH7}$ versus the A797T$_{MYH7}$ group. A similar effect was also observed for mLVWT, CWT score and PC1. The rs2068230 T-allele caused a 3.34 mm higher on effect mLVWT, a 1.88 mm higher CWT score, as well as a 0.12 higher PC1 in the R92W$_{TNNT2}$ group compared to the A797T$_{MYH7}$ group. Also, this allele resulted in a 2.68 mm higher effect on mLVWT, a 1.84 mm higher CWT score, as well as a 0.12 higher PC1 in the R403W$_{MYH7}$ versus the A797T$_{MYH7}$ group.

However, there was no statistically significant evidence for association between the rs2068230 SNP in *ATP1B3* and any of the hypertrophy traits (Figure 3.56).



	rs2068230
LVM	0.704
mIVST	0.508
mLVWT	0.063
mPWT	0.205
CWT	0.424
PC1	0.606

Figure 3.56 Single polymorphism association results for ATP1B3. *Bar graph indicates –log$_{10}$ transformed p-values for the tests of association between variants and investigated hypertrophy traits. The table below the graph indicates exact p-values for additive tests of allelic association. The red line in the graph indicates a significance level of p < 0.05, and effect sizes for significant associations are indicated in the text.*

3.8 Optimal selection

Previous studies analysed a set of five "pro-LVH" polymorphisms within the RAAS (Kaufman et al., 2007; Ortlepp et al., 2002; Perkins et al., 2005), constituting a set of SNPs that together confer an increased risk of hypertrophy development in HCM. The underlying hypothesis was that, as RAAS contribution to hypertrophy typically results in modest effect sizes, the compound effect of a number of variants might result in larger effects. However, as indicated previously, the full extent of the RAAS has not yet been considered in studies of hypertrophy modification, and thus the five pro-LVH polymorphisms may be considered a fairly limited approach to this matter.

Therefore, we used stepwise selection to identify markers among the greater RAAS study performed here, to identify a subset of markers that independently predicted an increase in at least one of the four selected hypertrophy indices. We identified nine such markers. Complete genotype data were not available for all the individuals for all traits and these analyses were consequently performed on the subset of individuals that had complete genotype and phenotype data for the respective traits.

The effect of each of the nine alleles that conferred an increased risk for hypertrophy development is listed in Table 3.47. These effects are given per allele as for all other results in this chapter. The combined effect of one of each of the nine alleles listed in Table 3.47 resulted in a 127.80 g increase in LVM, as well as a 13.97 mm increase in mIVST, a 14.67 mm increase in mLVWT, as well as a 7.85 mm increase in PC1.

As a comparison, we also modelled the previously described five "pro-LVH" polymorphisms to gauge their collective effect on hypertrophy in HCM in order to comment on the effectiveness of these markers as hypertrophy risk predictors in the present HCM cohort. As a caveat, we did not use the precise *AGTR1* polymorphism, rs5186, used in the previous studies, but we included the neighbouring rs5182 in our analyses, which is in LD with the rs5186 polymorphism in the 1000Genomes CEU low coverage panel as well as an English Caucasian and Chinese population (Abdollahi et al., 2007; Su et al., 2007).

However, we found that none of these polymorphisms were independently associated with any of the hypertrophy traits (Table 3.48). In addition, we observed markedly smaller compound effects for these alleles than for the nine SNPs that we had identified. The combined effect of all five 'pro-LVH" polymorphisms resulted in a 27.40 g increased LVM, a 2.15 mm increased mIVST, a 2.22 mm increased mLVWT, as well as a 1.49 mm increased PC1.

Table 3.47 Allelic effects of variants predicting a significant increase in hypertrophy in the present cohort.

			LVM	mIVST	mLVWT	PC1
Gene	**Polymorphism**	**Allele**	(n = 120)	(n = 120)	(n = 120)	(n = 105)
ACE	rs4356	C	15.0	1.47	1.49	0.60
AGT	rs4762	C	13.3	1.35	1.44	1.54
AGTR2	rs1403543	G	9.30	0.93	0.75	0.38
ATP1B1	rs1200130	T	12.3	0.64	0.74	0.83
M6PR	rs1805725	A	23.3	1.95	2.00	0.80
NR3C2	rs745019	G	19.0	1.98	1.97	0.88
NR3C2	rs1403142	G	0.20	0.86	1.11	0.58
NR3C2	rs13150372	G	21.5	2.60	2.95	1.58
SCNN1B	rs8044970	T	13.8	2.19	2.21	0.65
Combined effect on hypertrophy trait			127.8	13.97	14.67	7.85
Average effect per allele			14.2	1.55	1.63	0.87

Abbreviations: **A:** adenine; ***ACE*:** Angiotensin converting enzyme 1 gene; ***AGTR2*:** angiotensin II receptor, type 2 gene; ***ATP1B1*:** ATPase, Na^+/K^+ transporting, beta 1 polypeptide gene; **C:** cytosine; **G:** guanine; **LVM:** left ventricular mass; ***M6PR***: mannose-6-phosphate receptor gene; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; ***NR3C2*:** nuclear receptor subfamily 3, group C, member 2 gene; **PC1:** first principal component; ***SCNN1B*:** sodium channel, non-voltage-gated 1, beta gene; **T:** thymine

Table 3.48 Allelic effects of the five "pro-LVH" polymorphisms on hypertrophy traits in the present cohort.

			LVM	mIVST	mLVWT	PC1
Gene	**Polymorphism**	**Allele**	(n = 119)	(n = 119)	(n = 119)	(n = 105)
ACE	rs4340	I	5.20	0.54	0.41	0.33
AGT	rs699	T	1.70	0.82	0.72	0.03
AGTR1	rs5182	C	3.90	0.40	0.49	0.37
CYP11B2	rs1799998	T	6.30	0.05	0.04	0.00
CMA	rs1800875	G	10.3	0.35	0.56	0.77
Combined effect on hypertrophy trait			27.4	2.15	2.22	1.49
Average effect per allele			5.50	0.43	0.44	0.30

Abbreviations: ***ACE*:** Angiotensin converting enzyme 1 gene; ***AGTR1*:** angiotensin II receptor, type 1 gene; **C:** cytosine; ***CMA1:*** cardiac chymase gene; ***CYP11B2*:** aldosterone synthase gene; **G:** guanine; **LVM:** left ventricular mass; **mIVST:** maximum interventricular septal thickness; **mLVWT:** maximum left ventricular wall thickness; **PC1:** first principal component; **T:** thymine

This smaller effect is perhaps to be expected as we used four more polymorphisms than the previous studies. However, we also found that the average effect per variant was higher in our set of polymorphisms, which is indicative of a better predictive value per variant. The average effect per allele on LVM was 14.20 g for our set of polymorphisms, whereas the average "pro-LVH" allele predicted a 5.50 g increase on LVM. Moreover, the average allele in our set of polymorphisms predicted an increase of 1.55 mm, 1.63 mm and 0.87 mm in mIVST, mLVWT and PC1, respectively. However, the average "pro-LVH" allele only predicted increases of 0.43 mm, 0.44 mm and 0.30 in mIVST, mLVWT and PC1, respectively.

Chapter 4

Discussion

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4.1 HCM as a complex disorder

There is a large spectrum along which genetic variation can contribute to disease. Despite disease-causing mutations that would understandably have a seminal influence on a given disease, there exists the possibility of a number of factors, genetic or otherwise, that can impact on the eventual disease phenotype. This rings true particularly for complex diseases, where the eventual phenotype is characteristically influenced by a multitude of environmental and genetic factors of modest effect. However, it is also true for diseases that are not typically thought of as complex conditions.

It is now uncontested that the individual sarcomeric mutations that cause HCM are not sufficient, in and of themselves, to entirely explain the variability seen in the extent and distribution of hypertrophy in this disease. This is best illustrated in families with HCM, where we see a wide range of cardiac phenotypes in affected family members who share a common disease-causing mutation (Erdmann et al., 2001; Fananapazir and Epstein, 1994).

Despite its monogenic origin, previous findings point towards a number of factors that dynamically influence the eventual disease phenotype of a particular HCM patient. In particular, the LVH seen in HCM is partly attributable to environmental influences as well as to the complex interaction of a number of modifier genes (Marian, 2002). These genetic modifiers are neither necessary, nor sufficient to cause the disease, but they have the ability to significantly alter the presentation and progression of hypertrophy. In isolation, most modifier genes probably do not alter the HCM phenotype by much, but the composite effect of a number of modifier loci might have a significant effect on LVH development. However, exceptions may occur in HCM cases harbouring multiple sarcomeric mutations. For example, Biesiadecki et al. demonstrated that an R111C substitution in the cardiac troponin I gene countered the effects of a DCM-causing cardiac troponin T splice-variant in the hearts of wild turkeys (Biesiadecki et al., 2004), as turkeys with both mutations did not exhibit the DCM phenotype. Further functional analyses showed that this effect can be explained by the fact that the R111C substitution leads to a lowered binding affinity of troponin I for troponin T, which compensates for the increased binding affinity caused by the troponin T splice-variant (Biesiadecki et al., 2004).

Aside from environmental factors, there are a number of genetic modifiers that have been proposed for HCM, including tumour necrosis factor (TNF)-alpha (Patel et al., 2000), peroxisome proliferator-activated receptor gamma (PPARG) (Wang et al., 2007), as well as

genes involved in Ca^{2+}-homeostasis (Chiu et al., 2007; Friedrich et al., 2009) and myocardial energetics (Ho, 2010b).

In the present study we explored the contribution of variations in genes encoding RAAS components to hypertrophy development in HCM. This system was previously investigated for its potential impact on HCM, and a number of RAAS variants have been shown to significantly alter the HCM phenotype (Marian, 2002; Orenes-Pinero et al., 2011). However, the bulk of these studies focussed on single polymorphisms within a core set of genes, viz. *AGT*, *ACE*, *CMA*, *AGTR1* and *CYP11B2*. One drawback of these single variant approaches is that it neglects the effect of epistasis, while another is that these studies typically report relatively modest effect sizes, which is difficult to interpret clinically.

A few studies attempted to address these issues by investigating the compound effect of a set of five "pro-LVH" polymorphisms within these genes (Kaufman et al., 2007; Ortlepp et al., 2002; Perkins et al., 2005). However, this approach still appears to be overly simplistic when one takes into account that there is a complex biological interplay between all the components of the entire RAAS. Moreover, these variants might not offer a fair representation of RAAS variability in all population groups, while the contribution of the renin portion of the pathway, as well as the downstream RAAS effectors had remained largely neglected by this limited approach.

We, therefore, investigated the contribution of the entire RAAS to hypertrophy development by investigating not only the "pro-LVH" genes, but also including some genes that have not previously been investigated within the context of HCM. To this end we utilised a single SNP, as well as a phased haplotype association approach. Lastly, we provide a set of polymorphisms that best represented the overall contribution of the RAAS to hypertrophy in our cohort in an attempt to inform on hypertrophy risk stratification.

4.2 Genetic association studies to identify modifiers in HCM

The previous decade saw a 1000% increase in published genetic association studies (Donahue and Allen, 2005). In general, and specifically in cardiovascular research, this is partly attributable to the invaluable contribution on the HapMap project, which provided a catalogue of human genetic variation and LD structure for a core set of population groups (International HapMap Consortium., 2005; Manolio et al., 2008; Musunuru and Kathiresan, 2008), as well as rapid advances in the technology for detecting and genotyping SNPs (Morton and Collins, 2002; Palmer and Cardon, 2005; Ragoussis, 2009).

While association studies using single polymorphisms present certain challenges, as well as the likelihood of detecting only small individual effects (Little et al., 2009), that does not necessarily mean that genetic association studies are of little value in the study of complex diseases, and more specifically HCM. However, it is wise to be mindful of the inherent shortcomings and strengths of this approach.

At its core, genetic association studies investigate whether a correlation exists between a particular genetic variant and a disease trait. It does not, however, establish causality (Donahue and Allen, 2005). Statistical evidence for association between a particular allele and a phenotypic trait can arise from three situations (Cardon and Palmer, 2003). Firstly, the allele itself might be functional and exert a direct effect on the expression of the phenotype. Secondly, the allele might be in LD or correlated with a functional allele. However, a third possibility exists, viz. spurious allelic association, where the significant association is purely due to chance.

It is vital to ensure the reproducibility of a study due to the basic caveats of association studies. In HCM there are a number of factors that influence the eventual hypertrophic phenotype and failure to adjust for these variables impacts directly on the reproducibility and interpretability of a particular study. In addition, there are a few study design concerns that pertain specifically to HCM association studies. We will now discuss these basic concerns and caveats of association studies, which an emphasis on studies aimed at identifying hypertrophy modifiers in HCM.

4.2.1 Power

Statistical power in an association study refers to the probability that a test statistic reflects a true association (or lack thereof) between a genomic variant and a specific disease trait (Gordon and Finch, 2005). Power should therefore be the starting consideration for any association study. While it is not always possible to do specific power calculations in the advent of a study, it remains critical to take the relative power of a particular study into account when interpreting its results and one should consequently always bear the relative power in mind when designing a genetic association study.

Traditionally, the power of a modifier study relies on the marker allele frequency, the effect size within the cohort, as well as the true effect size, and the number of confounders for which adjustment is made (Colhoun et al., 2003; Donahue and Allen, 2005). In addition, errors in phenotypic classification and genotype errors contribute significantly to the power of an association study (Gordon and Finch, 2005). At present, no comprehensive method for

calculating the power of an association study in extended families (such as the present cohort) exists, while, in fact, very few case-control HCM studies have reported power calculations.

Although our cohort size can be considered modest by current population association study-standards, it is simply the reality that HCM cohorts will typically be relatively small, as explained in Table 1.1 in Chapter 1, seeing that HCM prevalence is estimated to be 1 in 500 in young adults (Maron et al., 1995). This makes the logistics of enrolling extremely large cohorts a challenge; in fact, the cohorts described here are some of the largest reported cohorts of individuals carrying the same founder mutation. The impact of cohort size on the statistical power of an association study can, however, not be ignored and results from smaller studies should consequently be interpreted with caution. In this study, we, nonetheless, made every effort to include every individual who gave informed consent within the 27 founder families.

4.2.2 Marker selection

Effective marker selection in conjunction with the currently available high-throughput genotyping technology, which provides better quality, specificity and time-efficiency (Kwok and Chen, 2003; Tsuchihashi and Dracopoli, 2002) can significantly improve the quality of an association study. It is not practical or in most cases financially feasible, to genotype all the known variants in a gene of interest in an association study. In fact, some markers might not be informative in the population in question. Moreover, disease susceptibility loci and marker allele frequencies might vary between different ethnic groups (Bamshad, 2005; Frazer et al., 2009; Ioannidis et al., 2004; Jorde and Wooding, 2004). Previous studies have found that a RAAS polymorphism that serves as a marker for hypertrophy in one population might have a very low MAF in a different population (Wang et al., 2008).

It is therefore necessary to prioritise certain genetic polymorphisms or makers. Markers chosen for genetic association studies basically fall into two main categories, namely polymorphisms chosen with the prior probability of functionality or markers chosen with the possibility of correlation (LD) with a true functional variant.

Functional variants have the advantage of an increased prior likelihood of involvement in association studies, but on the other hand, these mutations typically have low heterozygosity values, as they impact directly on disease phenotypes and are subject to greater evolutionary selection than common variants with modest or no impact on disease phenotypes (Shastry, 2007). Another possible disadvantage to this selection method is that there is no comprehensive list of all the possible functional variants in the human genome, or any genome for that matter,

at present. Researchers are therefore left to select variants that have a previously published functional effect or to select markers that confer marked changes on mRNA stability or the eventual amino acid sequence of the resulting protein, by, for example, influencing mRNA splicing or through non-synonymous exonic mutations. This approach is simply not possible for all genes and is, therefore, limited to well-characterised genes.

However, the power of a particular study might be hampered by the use of genetic markers that have a low MAF, especially when one considers the small effect sizes that seem to be the norm in HCM hypertrophy modifier studies (Lechin et al., 1995; Mayosi et al., 2003), and the profound impact that these two parameters have on the statistical power to avoid spurious results (Donahue and Allen, 2005). It is, therefore, preferable (and common practice) to select markers with relatively high MAFs for modifier studies in complex disorders, especially if the intended cohort is relatively small, rather than functional variants with low MAFs.

Using LD to guide marker selection allows for adequate, efficient and population-specific coverage of the gene under investigation, with the added benefit of the ability to prioritise markers with a fairly high MAF to ensure adequate statistical power to detect true significant effects. This approach to marker selection has been shown to reduce genotyping effort in association studies aimed at mapping quantitative trait loci (such as cardiac hypertrophy), without much loss of power (Weiss and Clark, 2002; Zhang et al., 2002; Zhang and Sun, 2005).

It is, in addition, not necessary to cover all the informative markers in a population with a high MAF. By selecting markers based on their ability to capture most of the haplotype structure in a particular genomic area, we can limit the number of polymorphisms needed for a particular genetic association study. The degree of LD in the particular area of interest will in this case dictate the number of variants required for adequate coverage of a gene (Zhang et al., 2004). LD patterns typically vary greatly across the human genome, with some regions of low LD interspersed with regions of high LD (Gabriel et al., 2002; Johnson et al., 2001; Zhang et al., 2004); LD also varies between different population groups and subgroups. Previous studies proposed that only a small number of tag SNPs are required to capture most of the haplotype structure in high LD regions of the human genome (Johnson et al., 2001; Patil et al., 2001; Stram, 2004). In addition, previous reports state that LDU maps have greater power when compared to the centi-Morgan/kb map and that LDU maps are a powerful tool for disease gene association mapping using LD (Collins et al., 2004; Maniatis et al., 2005).

In the present study we prioritised markers with a MAF higher than 0.05, using LD-guided marker selection. HapMap CEU and YRI LD maps were used to select markers to cover the *AGT*, *ACE*, *ACE2*, *CMA* and *AGTR1* genes by capturing the main haplotypes across these genes, while LDU mapping was used to select markers to cover the rest of the genes in the present study. There are currently no studies published in which the superiority of either method over the other has been assessed, to the best of our knowledge, and we, therefore, chose to use the LDU method over the tag SNP method, as the theoretical number of SNPs required to cover a chosen gene is generally fewer using the LDU method, resulting in better cost-effectiveness.

It must however still be mentioned that while the use of markers with high MAFs offer benefits over scarcer functional variants in the search for modifier variants in HCM, the probability exists that the resultant association studies will not directly indentify functional variants that impact on gene function, but rather point towards genetic loci that warrant further investigation.

4.2.3 Linkage disequilibrium (LD)

In other words, the possibility then exists that variants that associate significantly with a disease phenotype are not in themselves functional, but that they are in LD with genetic variants that confer a true biological impact on the RAAS and, consequently, hypertrophy. In these cases we cannot pinpoint the precise location of a functional variant based on the results of a genetic association study alone as that particular variant is not only in LD with other (possibly rare or even yet undiscovered) mutations and polymorphisms, but also structural variants, the majority of which have not yet been identified (Frazer et al., 2009; McCarroll et al., 2008).

In essence, genetic association points out a genetic region that correlates significantly with a particular phenotype. The size of this region is dependent on the measure of LD in that particular study population. For instance, Reich et al. found that LD typically extends 60 kb from common alleles in a United States population of north-European descent, while LD in a Nigerian population extends markedly less far (Reich et al., 2001).

Drawing from examples within the RAAS, haplotype studies showed that aldosterone synthesis is affected by genotypes at *CYP11B2,* as well as the neighbouring *CYP11B1,* as LD exists across the entire CYP11B1/B2 locus in Caucasians (Imrie et al., 2006; Alvarez-Madrazo et al., 2009). The pattern of LD is, however, not uniform across populations of different ethnic origin (Conrad et al., 2006; Gabriel et al., 2002; Hinds et al., 2005). For instance, we generally see a higher

degree of LD in Caucasian populations than in African populations (Henn et al., 2011; Reich et al., 2001; Shifman et al., 2003).

Patterns of LD are, therefore, variable across the genome and between different populations (Ardlie et al., 2002a; Conrad et al., 2006; Liu et al., 2004; McVean et al., 2004). Markers in close proximity to a particular functional genetic variant might, consequently, show less or more LD than markers further away, and it is presently not possible to predict which of theses markers will be in strongest LD with another neighbouring functional variant (Cardon and Bell, 2001). Consequently, significant associations with some markers might not be identified in a region that contains a disease-modifying variant, while significant associations might be detected at directly adjacent markers, which can lead to inconsistent results across studies (Cardon and Bell, 2001). Additionally, ignoring LD in association studies can inflate false positive rates (Schaid et al., 2002).

It is, therefore, important to be familiar with the LD structure of the particular population under investigation. Ideally, one would use an LD map constructed from LD information from the particular population group under investigation. This is, however, not always possible as comprehensive LD maps for all the possible ethnic populations do not exist at present. The recent release of information of HapMap3 populations (Altshuler et al., 2010), which adds seven more populations to the initial four HapMap populations, offer the possibility of better-matched LD maps for some populations, such as the Maasai in Kinyawa, Kenya, but detailed LD information is, however, still lacking for most populations.

In the absence of such a specific map, we used LD information from both the HapMap CEU and YRI populations as a proxy for our population for marker selection purposes as our cohort is comprised of individuals of mixed ancestry, as well as Caucasians, and due to the fact that the full datasets for these populations were available at the time of marker selection for this project.

4.2.4 Population stratification

However, false positive associations might also originate from undetected population substructure in any genetic association study (Cardon and Palmer, 2003; Clayton et al., 2005; Cooper et al., 2008; Hu and Ziv, 2008; Ioannidis et al., 2001; Koller et al., 2004). Association between a specific genotype and disease trait could, therefore, be confounded by population stratification in a study population consisting of a mixture of two or more subpopulations with different allele frequencies and disease risks, leading to a false positive association between a

certain variant and a particular disease trait (Colhoun et al., 2003), especially if the allele frequencies of the variant are vastly different in the subpopulations involved in the study.

However, previous studies that modelled the possible effects of population stratification reported that its effects, if left unadjusted for, are likely to be small in most cases (Ardlie et al., 2002b; Ioannidis et al., 2004; Millikan, 2001; Wacholder et al., 2000; Wang et al., 2004). In addition, a meta-analysis of 697 studies on 43 gene-disease associations provided evidence against large effects of population stratification, by showing consistent associations across different ethnic groups (Ioannidis et al., 2004).

It is, however, still advisable to protect against the possible confounding effects of underlying population stratification in studies where small or moderate effect sizes are expected, as is usually the case in complex associations. This is because these studies typically require larger sample sizes and the effect of population stratification increases markedly with an increase in sample size (Little et al., 2009; Marchini et al., 2004).

Family-based studies using the transmission disequilibrium test offer a unique solution as within family association is robust against population admixture and stratification as untransmitted and transmitted share the same genetic ancestry (Abecasis et al., 2000; Laird and Lange, 2006; Ott et al., 2011). However, to ensure maximum power, family-based studies need to incorporate the between-family information as well, which is still vulnerable to stratification issues (Price et al., 2010).

Mixed effects models offer a comprehensive and practical approach to simultaneously address confounding due to family structure, cryptic relatedness and population stratification (Peloso et al., 2011; Price et al., 2010). Basically, this approach models disease phenotypes using a mixture of random and fixed effects. Fixed effects include the investigated marker, as well as optional covariates, such as age or BSA, while random effects are based on a phenotypic covariance matrix, modelled as the sum of non-heritable and heritable random variation (Price et al., 2010). Modelling population structure or ethnicity as a fixed effect provides an adequate measure to address population stratification issues, but requires running principal component analysis to infer genetic ancestry in the cohort in order to remove the effects of population stratification entirely (Price et al., 2010).

In the present study we incorporate both the within- and the between family components of association in favour of increased statistical power, using mixed models. The use of these

models enabled us to (among others) adjust for family-relatedness and, consequently, admixture, to a certain degree. In addition we modelled self-reported ethnicity as a fixed effect to further adjust for population stratification.

4.2.5 Confounders

Confounding variables are factors that are related to both the disease and the particular disease trait under investigation. An apparent association between a disease trait and a given genetic polymorphism can sometimes, therefore, actually be attributed to confounding effects (Campbell and Rudan, 2002; Cordell and Clayton, 2005). The effect size of these confounding variables is, however, jointly related to its correlation with the factor under investigation, as well as to the eventual outcome; multiple confounders can contribute anywhere from modest effects to a substantial compound effect (Campbell and Rudan, 2002).

Cardiac hypertrophy, in general, and in the context of HCM has a multifactorial origin, arising from a complex interaction between causal mutations and a multitude of susceptibility genes and environmental factors (Balakumar and Jagadeesh, 2010; Xu et al., 2010b). If one then considers these environmental influences and genetic factors that could impact on disease presentation and progression, and could, therefore, possibly confound statistical analyses, it becomes essential to take the underlying etiology of the disease into account when designing an HCM association study.

Previous studies have reported correlations between body size (Chumlea et al., 2009; Garner et al., 2000), age (Fleg and Strait, 2011; Lasky-Su et al., 2008), gender (Maass et al., 2004; Okin et al., 2000), ethnicity (Okin et al., 2000), blood pressure (Roman et al., 2010), hypertension (Kraja et al., 2011; Levy et al., 1990b; Puntmann et al., 2010; Sipola et al., 2011), heart rate (Saba et al., 2001) and LVH. In HCM in particular, one must also consider the possible differential effect of the distinct disease-causing mutations, or mutations in distinct genes, on the extent and distribution of cardiac hypertrophy (Ackerman et al., 2002; Arad et al., 2005; Marian and Roberts, 2001; Moolman et al., 1997; Varnava et al., 2001; Watkins et al., 1995a).

In addition, previous studies reported context-dependent associations between RAAS variants and hypertrophy traits, where such associations were dependent on, among others, ethnicity (Jin et al., 2011b; Kuznetsova et al., 2005; Wang et al., 2008), blood pressure (Tang et al., 2002) and the primary HCM causal mutation (Perkins et al., 2005; Tesson et al., 1997).

Weak replication rates of genetic associations aimed at identifying modifiers for HCM might, therefore, reflect inconsistent adjustment for relevant hypertrophy confounders; failure to account for confounding variables directly influences the reproducibility of a particular study (Cardon and Palmer, 2003). In the present study, we adjusted all analyses for the identity of the primary HCM-causing mutation (R92W$_{TNNT2}$, R403W$_{MYH7}$, or A797T$_{MYH7}$), self-reported ethnicity, hypertension diagnosis, mean arterial pressure, sex, BSA, heart rate and age at clinical assessment. We did, however, not adjust for circulating Ang II levels or other measures of plasma RAAS activity, which has the potential to mask or exaggerate the contribution of individual RAAS component to hypertrophy development.

4.2.6 Phenotypic definition and distribution

Phenotypic heterogeneity and errors in phenotypic classification, as well as genotype errors contribute significantly to the power of an association study (Gordon and Finch, 2005), while weak replication rates in genetic association results may also reflect inconsistency in phenotypic definition across studies (Cardon and Palmer, 2003). The responsibility of the researcher is, therefore, to minimize the chance for spurious results through careful study design and relevant statistical methods.

In this study, we used a number of quantitative traits to describe hypertrophy. There are possible advantages to investigating quantitative phenotypes such as blood pressure or LVM, rather than a qualitatively described disease state, which can be difficult to ascertain precisely in diseases with diagnostic criteria that require human interpretation and discretion (Newton-Cheh and Hirschhorn, 2005). Dichotomous traits, such as disease state, are sometimes difficult to pinpoint with exact precision, as is the case in some psychiatric disorders and, for example, hypertension, where criteria for diagnosis can vary between research groups. But, more importantly, most dichotomous traits do not give an indication of the severity of the disease. The heritability, i.e. the proportion of trait variance that can be ascribed to genetic factors as explained in section 2.7.5, also relies strongly on the accuracy of this measurement. The heritability of the particular trait then, in turn, affects the strength of the association study. Quantitative traits that are easily measured in a large number of individuals, and that show high heritability, are therefore preferable in association studies (Newton-Cheh and Hirschhorn, 2005).

In an analysis of published randomized trials evaluating the effects of antihypertensive therapy on left ventricular morphology, as assessed by echocardiography, Cuspidi et al. showed that the phenotypic definition of LVH is extremely variable across studies (Cuspidi et al., 2008), where

some studies used LVM as a proxy for LVH, while others used maximal left ventricular wall thickness. In HCM, the definition of LVH poses a potential problem. As discussed in Chapter 1, the phenotypic expression of HCM varies greatly between individuals, particularly with regard to the extent and distribution of hypertrophy. Cardiac hypertrophy in HCM is mostly asymmetrical, and HCM patients rarely exhibit uniform concentric hypertrophy. It is, therefore, difficult to quantify the extent and distribution of hypertrophy in a whole cohort using a single measurement.

The most commonly studied phenotype in HCM is LVM, as determined by echocardiography, as this approach makes use of a readily available clinical tool (Myerson et al., 2002) and it provides a more comprehensive measure of the extent of hypertrophy than a single wall thickness measurement could do. The extent and distribution of LVH in HCM is, however, extremely variable and asymmetric, as discussed earlier, while echocardiographically determined LVM is derived from geometric assumptions; hence echocardiographically determined LVM may be an inaccurate measure of total LVH in the context of HCM. On the other hand, cardiac MRI is credited with being a more precise and reproducible measure of LVM than M-mode and 2D-echocardiography, although the substantial costs involved in the infrastructure prohibits many investigators from using this technique. Moreover, the heritability of echocardiographically-determined LVM is estimated to be between 0.17 and 0.69 as discussed earlier, while ECG and MRI measures are reported to be more heritable (Busjahn et al., 2009; Mayosi et al., 2002). These estimates are however heavily cohort-specific and sensitive to hypertrophy confounders. Thus, a great need exists for a clear consensus on the most appropriate measure of hypertrophy across HCM association studies.

On the other hand, single measurements are difficult to interpret clinically as no single measurement could give the most accurate representation of the degree of hypertrophy in all affected individuals, given the asymmetry and the variability of the distribution of LVH in HCM. One way to address this issue is to use composite scores that encompass a number of hypertrophy measurements to describe the extent of hypertrophy. A number of such scores, such as the Wigle score (Wigle et al., 1985) and the Maron-Spirito score, exist (Spirito and Maron, 1990); however, it is still uncertain which composite score offers the best estimate of the extent of hypertrophy. It is also debatable whether these two scores offer the best description of ventricle-wide LVH. The Maron-Spirito score is a quantitative appraisal of the extent of hypertrophy and is the sum of the maximum wall thickness obtained from four left ventricular segments at the mitral and papillary muscle levels of the heart. This score might, however, not perform equally well across all HCM patient groups in accurately describing the extent and

distribution of LVH, especially where the hypertrophy in localised to a certain region, like, for instance, in the case of a less frequent form of HCM where LVH is restricted to the apex. A weighted sum of the cardiac wall thickness measurements might offer a better appraisal of overall LVH in such a scenario. The Wigle score, on the other hand, is potentially vulnerable to differences in interpretation between clinicians as it is a semi-quantitative score consisting of a number of subjective scores added together to form an overall impression of the extent of hypertrophy.

In the absence of a consensus measurement that accurately reflects the degree and distribution of hypertrophy in HCM; we investigated six hypertrophy measurements. In total, 16 wall thickness measurements were taken at three levels of the heart to estimate the distribution of hypertrophy, using 2D and M-mode echocardiography, by a single cardiologist. We then used mLVWT, mPWT and mIVST to indicate the distribution of hypertrophy. Additionally, we determined LVM by echocardiography and two composite hypertrophy scores as an indication of the extent of hypertrophy. The CWT score is the sum of all 16 cardiac wall thickness measurements, while the composite hypertrophy score derived by principal component analysis, PC1, best described the variability in these 16 echocardiographically determined wall thickness measurements in our cohort.

It must also be mentioned that most statistical packages for association analysis of quantitative traits assume a normal distribution of trait values (Diao and Lin, 2006; Lange et al., 2002). Outliers with extreme values can, however, influence the analysis outcome, leading to inappropriately low p-values if the trait distribution deviates from the assumed normal distribution (Newton-Cheh and Hirschhorn, 2005). This can be addressed using appropriate statistical transformation to achieve normality (Newton-Cheh and Hirschhorn, 2005). In the present study, we transformed all hypertrophy traits to approximate normality using quantile normalization, prior to the association analysis (Pilia et al., 2006).

4.2.7 Multiple testing

Multiple testing in any cohort can result in an increased probability of obtaining a false positive result (type I error). A popular solution for this is to use a Bonferroni adjustment. This method assumes that all the performed tests are independent and each p-value is subsequently multiplied with the number of tests performed. However, Bonferroni corrections can be overly stringent and possibly over-correct for false-positives by assuming complete independence; this is obviously not the case in family-based association studies (Cardon and Bell, 2001; Perneger, 1998). Furthermore, Bayesian methods for multiple testing correction require prior knowledge

of the probability of involvement, which is presently unknown for most genetic variants (Campbell and Rudan, 2002; Thomas and Clayton, 2004).

The hypertrophy traits investigated in the present study are correlated as they are all at least in part derived from the same 16 wall thickness measurements, while a degree of LD, although sometimes not complete, exists between our chosen markers. The tests that we perform are therefore not entirely independent and we consequently did not use corrections for multiple testing.

That said, one must also consider that overly conservative adjustment for multiple testing, especially when there is currently no consensus on the appropriate statistical method for multiple testing corrections in extended families and complex phenotypes, might leave true biological effects undetected if initial exploratory studies are discarded or discouraged in favour of controlling the false-positive rate (Thomas and Clayton, 2004). However, the necessity for valid replication cannot be overstated in genetic association studies, given the statistical possibility of false positive and negative results (Ioannidis et al., 2001) and we agree that initial studies, such as the present investigation, that do not employ multiple testing corrections should be viewed as "hypothesis generating" but can, upon subsequent replication, be of value in elucidating the molecular underpinning of cardiovascular disease (Crossman and Watkins, 2004).

4.2.8 Compound genetic effects

Finally, the possibility exists that a given association study identifies a real correlation between a variant and a disease phenotype, but nevertheless would not reproducible if the underlying genetic effect of the variant is weak (Hirschhorn et al., 2002). This is particularly applicable in association studies on complex cardiovascular phenotypes, such as cardiac hypertrophy in HCM, where relatively small effect sizes are typically reported (Donahue and Allen, 2005).

In the following discussion on the association results, we provide estimates of effect size in an attempt to quantify the contribution of RAAS variants to hypertrophy. The effect sizes reported here are modest, although comparable to those reported by previous studies (Lechin et al., 1995; Mayosi et al., 2003; Sookoian et al., 2008; Wang et al., 2006). The clinical relevance might, however, improve if one considers the compound effect of a number of RAAS polymorphisms, where each contributes a modest amount to the eventual hypertrophic phenotype.

However, the compound effect of these RAAS variants on hypertrophy might not necessarily be additive in nature. The influence of epistasis where the effects of one locus is amplified, altered or even masked by another locus (Cordell, 2002), has not yet been considered extensively in HCM. It is conceivable that such an interaction should exist between RAAS variants due to their complex biological functions; moreover, such epistatic effects have been reported between RAAS variants in atrial fibrillation (Tsai et al., 2004), coronary atherosclerosis (Ye et al., 2003), hypertension (Williams et al., 2004) and coronary artery disease (Tsai et al., 2007). There is, therefore, a need for a comprehensive analysis that focuses on the compound effects of multiple hypertrophy modifier loci within the RAAS to gain the most accurate understanding of its role in hypertrophy development.

While we did not perform formal statistical analyses to detect epistasis in the present study, we investigated RAAS gene haplotypes as a proxy for within gene epistatic effects. In addition, we aimed to identify a subset of variants from multiple RAAS genes that, together, confer a measurable risk of LVH development in the present cohort as a proxy for epistatic effects between genes.

4.3 Use of haplotypes in association studies

The use of haplotypes offers an advantage over single SNP association approaches by providing additional power for mapping disease modifier genes while factoring in the interdependency among genetic markers studies, as it considers the compound effect of a number of SNP loci (Clark, 2004; Liu et al., 2008)

Haplotypes are constituted of the alleles present at multiple genetic markers inherited from the same parent (Ott and Lucek, 1998). Haplotypes are vital to elucidating and understanding the LD pattern across the human genome as LD measures derived from marker pairs, such as D' and r^2, cannot precisely capture higher-order interdependency among markers (Ardlie et al., 2002a; Liu et al., 2008; Weiss and Clark, 2002). The best way to understand genomic LD patterns is in fact to know the actual inherited haplotypes (Daly et al., 2001; Liu et al., 2008).

Virtually all genetic association methods that are based on single markers can be applied to the analysis of haplotypes, as previously identified haplotypes can be considered as alleles for a single multi-allelic marker (Liu et al., 2008). It has been suggested that haplotype-based association tests offer improved power, compared to single SNP association tests in both population-based and family-based approaches (Akey et al., 2001; Bader, 2001; Botstein and Risch, 2003; Clark, 2004; De La Vega et al., 2005; Li and Jiang, 2005; Martin et al., 2000; Morris

and Kaplan, 2002; Zaykin et al., 2002; Zhang et al., 2002). This is hypothesised to be due to the ancestral genetic structure that is captured by these haplotypes (Akey et al., 2001).

The power of single variant-based association methods rely on the measure of LD between the disease-susceptibility locus and the investigated marker locus. The difficulty is that these methods do not necessarily incorporate LD information from flanking markers, which can potentially lead to a loss of power (Liu et al., 2008).

Haplotype-based methods are regarded as being more powerful than single SNP studies, as these consider LD information from multiple markers simultaneously. For instance, the possibility exists that several markers within a small genomic region might be in LD with both the disease locus and with each other. In such a scenario, single marker-based LD methods might not capture all the available LD information which is contained in multi-locus haplotypes (Akey et al., 2001; Morris and Kaplan, 2002). Akey et al. found that when a distance of 1 cM is assumed between adjacent markers and the disease susceptibility locus is in the middle of these markers, the sample sizes required by a two- or four marker locus haplotype association test is approximately one-half and one-quarter, respectively, of the sample size required for the equivalent single marker association test (Akey et al., 2001).

Moreover, haplotypes offer improved power to detect disease QTLs when using haplotypes compiled of markers selected to capture the LD structure in a gene (Li et al., 2006). In addition, haplotypes of two or more SNPs generally have a higher probability than individual SNPs of showing useful LD with a disease mutation, although exceptions are described (Garner and Slatkin, 2003). Therefore, the use of haplotypes offers an attractive approach to complex disease mapping as haplotypes can be tested for association as a proxy for untyped causal variants (Newton-Cheh and Hirschhorn, 2005). However, this depends on whether haplotypes can capture other markers more efficiently than multiple markers considered independently. It has been suggested that multi-marker combinations of tag SNPs may capture more untyped variants and therefore allow these SNPs to be used more efficiently (Newton-Cheh and Hirschhorn, 2005). However, this might not be completely true in all situations. In a population such as that investigated in the present study, where a detailed LD map of the specific population is not available, one has to use other, better characterised populations as a proxy for tag-SNP selection with the understanding that some information might still be lost through this approach due to the difference in LD structure between the proxy- and the true study population.

The use of haplotypes in association studies, furthermore, allows for the simultaneous investigation of multiple potentially disease-modifying variants. This would allow association studies to pick up effects of markers that have undetectable effects when considered individually, but act along with others though epistatic effects to markedly influence the phenotype. In addition, it allows one to test haplotypic combinations of markers with weak individual effects for yet stronger combined effects (Newton-Cheh and Hirschhorn, 2005). For instance, previous studies have shown that combined effects of multiple sequence variants on promoter activity or protein structure may actually precipitate or exacerbate the disease phenotype, even when the individual SNPs had poor predictive power (Drysdale et al., 2000; Joosten et al., 2001; Kankova et al., 2010).

In addition, it is known that the functionality of a particular protein is influenced and sometimes actually determined by how it is folded. Protein folding is determined by the amino acid sequence of the protein, which is in turn determined by DNA sequence variation. Several nucleotide changes within the coding regions of the same gene, constituting a particular haplotype, can consequently interact to have a marked effect on the eventual protein and consequently its function (Clark, 2004; Schaid, 2004). A number of such examples in humans exists in the literature (Clark et al., 1998; Hollox et al., 2001; Tavtigian et al., 2001).

Notwithstanding the increase in statistical power, the use of haplotypes in preference to SNPs adds complexity to association studies. Haplotypes can unfortunately be technologically demanding and expensive to measure with direct molecular techniques (Douglas et al., 2001; Michalatos-Beloin et al., 1996; Tost et al., 2002; Yan et al., 2000). Most studies consequently preferentially used haplotypes that were inferred or estimated statistically from multilocus genotyping data for QTL mapping (Bagos, 2011; Cardon and Abecasis, 2003; Schaid, 2004).

However, the number of possible haplotypes increases dramatically with the addition of each new marker and many haplotypes will consequently have low frequencies, while the possibility for ambiguity of the actual haplotype increases due to incomplete datasets that is frequent with larger extended families. Constructing an individual's haplotypes using genotype data from close relatives can decrease ambiguity in the haplotype (haplotype inference), while phased haplotypes can offer substantial improvements to association studies (Becker and Knapp, 2002; Browning and Browning, 2011; Clark, 2004; Liu et al., 2008; Rohde and Fuerst, 2001; Tewhey et al., 2011). Thus, the incorporation of pedigree information has been shown to improve the precision for haplotype frequency estimation and the accuracy for haplotype reconstruction (Zhang and Zhao, 2006). Haplotype ambiguity might, however, still be an issue of concern when

a large number of markers is used, as the likelihood of incomplete datasets increases (Hodge et al., 1999; Van Steen et al., 2007).

There are numerous computational and statistical methods for determining or inferring haplotypes using pedigrees, each with its own strengths and weaknesses, as reviewed previously (Liu et al., 2008; Schaid, 2004). Basically, exact likelihood-based methods can only handle small pedigrees with fewer markers, due to the extensive computations required, while approximate likelihood-based methods are more suited to larger pedigrees and many marker loci (Liu et al., 2008; Schaid, 2004)

For the present study we used SimWalk2 (Sobel and Lange, 1996) as this method can infer a most likely pair of haplotypes for each individual in large, extended pedigrees, while dealing with a certain proportion of missing data. However, one disadvantage to haplotype association analysis is the fact that no method for haplotype estimation or determination exists for X-linked markers at present. Software for phased haplotype estimation is currently limited to autosomal genes, while it would be nearly impossible to correctly assign and phase haplotypes using manual haplotype assignment in large, extended pedigrees, such as those in the present study; especially where there are some genotypes missing, due to differing marker genotyping efficiencies.

4.4 Heritability

The heritability statistic, h^2, refers to the proportion of variability in the disease phenotype under investigation that can be attributed to genetic factors. We expect that most diseases with familial clustering should have at least some genetic component, but it is essential in genetic association studies to know what proportion of variability in the quantitative phenotype chosen to represent disease variability can be explained by environmental influences and what proportion is attributable to genetic factors, to ensure that we identify true genetic modifiers. If no significant heritable component can be found for a particular disease trait, there is simply no grounds for continuing with a genetic association study, as there is no genetic component influencing variability of the disease phenotype.

However, a particular modifier variant will not necessarily explain the entire heritable component of a disease; in fact, we find that most complex disease heritabilities rely on the effects of a number of modifier loci, each explaining only a modest proportion of the heritability of a particular trait (Manolio et al., 2009).

The heritability value reported here for the CWT score and mLVWT is in accordance with earlier values reported for cardiac wall thickness (Mayosi et al., 2002; Swan et al., 2003). However, previously published heritability estimates for echocardiographically determined LVM ranged from 17% to 69% in different populations, with the lowest values reported in healthy adults and the highest in monozygotic twins (Bella et al., 2004; De Simone et al., 2007; Jin et al., 2011a; Juo et al., 2005; Kotchen et al., 2000; Mayosi et al., 2002; Post et al., 1997; Swan et al., 2003). Despite the range of LVM heritability values reported in these studies, all of them agreed that adjustment for known hypertrophy confounders such as age, sex and blood pressure significantly affected these values. In the Northern Manhattan Family Study, for instance, the heritability of LVM was estimated at 65% in a model adjusted for age and gender, while this value dropped to 49% in a model adjusted for age, sex, weight, height, systolic blood pressure, diabetes, and antihypertensive medication (Juo et al., 2005). Furthermore, the heritability of LVM can also be influenced by the method used to determine the trait: LVM, as determined by cardiac MRI, was estimated at 84% after adjustment for age and sex in a recent study on twins (Busjahn et al., 2009).

The heritability estimates reported in this multigenerational HCM founder family cohort then offers a relevant appraisal of the heritability of the six investigated traits, as it is independent of the known confounding effects of the primary HCM causal mutation, as well as other known hypertrophy covariates (Table 3.4). After adjustment for these covariates, we report a strong heritable component for all six hypertrophy traits used in this study.

4.5 Results from the association analyses

4.5.1 Angiotensinogen

Kang et al. crossed angiotensinogen-deficient mice with transgenic mice expressing the rat angiotensinogen gene exclusively in the brain and liver; the resulting crossbred animals did not express angiotensinogen in the kidney or the heart (Kang et al., 2002). Interestingly, these crossbred animals showed markedly reduced cardiac hypertrophy and fibrosis when compared to mice expressing angiotensinogen in the liver, brain and heart, even though blood pressure was similar (Kang et al., 2002). Moreover, transgenic mice overexpressing the rat angiotensinogen gene developed chronic hypertension coupled with extensive cardiac hypertrophy and impaired cardiac function (Xu et al., 2009). These studies provide evidence for the hypertrophic effects of angiotensinogen.

Studies aimed at investigating the effect of *AGT* polymorphisms on LVH, both within the context of hypertension and HCM, have, however, yielded inconsistent results, despite the clear

biological link between *AGT* and cardiac hypertrophy. The M235T (rs699) SNP, in particular, has been associated with LVM in some hypertensive cohorts (Jeng, 1999; Karjalainen et al., 1999), but not all (Iwai et al., 1995; Kauma et al., 1998). Two meta-analyses revealed that this polymorphism serves as a marker for hypertension in Caucasians, although no correlation could be found between this polymorphism and cardiovascular complications (Sethi et al., 2003; Staessen et al., 1999).

There are of course a number of factors that could account for these weak replication rates in association studies as discussed in the previous sections, but inconsistent association between *AGT* polymorphisms and LVH most likely stems from inconsistent adjustment for common hypertrophy confounders, coupled with a difference in allele frequency of *AGT* variants across populations of different ethnic origin (Corvol et al., 1999; Sethi et al., 2003; Staessen et al., 1999), as well as the varying *AGT* haplotype structure across populations (Nakajima et al., 2002).

In the EPOGH study on 824 individuals 221 nuclear families from three Caucasian populations, respectively from Poland, Russia and Italy, Kuznetsova et al. reported a strong context dependence for association between *AGT* -532 C/T and -6 G/A polymorphisms and LVM_{index}, as well as mean cardiac wall thickness (Kuznetsova et al., 2005). For instance, they found a significant association between both LVM_{index} and mean cardiac wall thickness and the -532 C/T polymorphism, alone and combined with the -6 G/A polymorphism in a haplotype, in Slavic, but not Italian males in population-based, as well as family-based analyses. However, in women, LVM_{index} was neither associated with single *AGT* variants nor with the haplotypes. They also reported that the significant association that they observed between these polymorphisms and both hypertrophy indices were dependent on age, gender, ecogenetic context and appeared to be modulated by the trophic effects of salt intake on LVM.

A similar effect was observed in 605 predominantly Caucasian patients obtained from the HyperGEN cohort (Tang et al., 2002). LVM, as well as LVM_{index}, and the M235-T allele was negatively associated in hypertensive patients from this cohort, but positively associated in normotensive patients, in a model adjusted for the potential confounding effect of weight, height, age, sex, systolic blood pressure, diastolic blood pressure, presence of diabetes, and antihypertensive medication use. The authors attributed these findings to the differential effects of the respective antihypertensive medications or other unknown hypertrophy confounders (Tang et al., 2002). In addition, studies on animals expressing human -6 G/A | M235T haplotype

combinations report that the cardiovascular effects of these haplotypes were largely dependent on environmental influences (Grobe et al., 2010).

Similarly, we find inconsistent study designs and adjustment for relevant hypertrophy confounders in HCM. Previous studies reported a higher M235-T allele frequency in Japanese HCM patients, when compared to their unaffected siblings and offspring (Ishanov et al., 1997; Kawaguchi, 2003). A similar study on 150 South Indian HCM (90 sporadic HCM and 60 familial HCM) patients and 165 age- and sex-matched healthy controls, without known hypertension or LVH, also reported significant differences in M235T allele frequencies between patients with sporadic HCM and controls, although these findings were not replicated in patients with familial HCM (Manohar Rao et al., 2010). In contrast, Yamada et al. found no significant association between this variant and non-familial HCM in a Japanese cohort (Yamada et al., 1997). Such studies are, however, difficult to interpret clinically, due to small sample sizes, inadequate adjustment for known hypertrophy confounders and more importantly, due to the fact that these studies did not investigate a clearly defined HCM phenotype, especially when the underlying heterogeneity in HCM presentation is well documented.

Brugada et al. reported that none of the commonly studied *AGT* SNPs, viz. M235T, T174M, and -6 G/A, had a significant influence on a composite LVH score or LVM in a cohort of 108 unrelated HCM patients (Brugada et al., 1997). Similarly, the M235T polymorphism was not found to associate significantly with left ventricular wall thickness in a cohort of 389 unrelated patients with HCM (Perkins et al., 2005).

In the present study, we found significant evidence for association between rs4762 (T174M) and the CWT score. The T-allele of this polymorphism was found to significantly decrease the CWT score by 1.03 mm. We did however not find association between the well-studied *AGT* M235T (rs699) or -6 G/A (rs5051) polymorphisms and any of the investigated hypertrophy indices. In contrast to previous investigations (Brugada et al., 1997; Ishanov et al., 1997; Kawaguchi, 2003; Manohar Rao et al., 2010; Perkins et al., 2005; Yamada et al., 1997), we adjusted all analyses for the primary HCM-causal mutation, as well as for other known hypertrophy covariates. This might be an explanation for the inconsistent association between *AGT* variants and hypertrophy. In fact, our results show that the M235T and -6 G/A polymorphisms had significantly different allelic effects on LVM between the R403W$_{MYH7}$ and A797T$_{MYH7}$ groups.

Curiously, we did not find a statistically significant association between any of the identified *AGT* haplotypes and the investigated hypertrophy traits. This is in agreement with a previous study on 777 individuals from the HyperGEN study, which also found no significant association between *AGT* haplotypes and LVH, after adjustment for age, sex, hypertension status, and heart rate (Rasmussen-Torvik et al., 2005).

One possible explanation is that the effects of the rs4762 could have been diluted or masked in the 5-SNP haplotype. However, in terms of causality, it remains difficult to pinpoint the variant responsible for association significant associations with *AGT* variants due to the tight LD structure of the *AGT* gene observed in the present and other investigations (Rasmussen-Torvik et al., 2005).

Previous studies have reported that the significant associations seen with the M235T variant are actually attributable to the fact that this variant is in tight LD with the -6G/A variant in the promoter of the *AGT* gene (Inoue et al., 1997; Tang et al., 2002). However, studies with transgenic mice expressing either the -6G/235M or the -6A/235T human *AGT* haplotype, found that both transgenes exhibited the same transcriptional activity and produced similar plasma levels of human Angiotensinogen (Cvetkovic et al., 2002). Due to fact that LD extends over a few kilobases in the UTR region of this gene, there might be more, yet unidentified, *cis* acting genetic factors influencing the effect of *AGT* on cardiac hypertrophy and more studies are, therefore, needed to fully elucidate the contribution of this locus to hypertrophy in general and within the context of HCM.

4.5.2 Renin and renin-associated genes

The RAAS can be inhibited efficiently at several levels of the cascade, but interruption of Ang I generation by renin is considered most efficacious as direct renin inhibition uniquely decreases plasma renin activity, which is not achieved through ACE and Ang receptor blockers (Balakumar and Jagadeesh, 2010a; Westermann et al., 2008b). The recent development of a direct renin inhibitor, aliskiren, therefore renewed interest in renin as a potential therapeutic target in cardiac hypertrophy management (Sever et al., 2009; Verdecchia et al., 2008).

Direct renin inhibitors were also proven to be at least as effective as ACE inhibition and Ang receptor blockade in LVH reduction in double-transgenic rats and mice expressing human *REN* and *AGT* (Major et al., 2008; Pilz et al., 2005), as well as in spontaneously hypertensive rats (Van Esch et al., 2010). Aliskiren was also shown to reduce cardiac remodelling and hypertrophy after myocardial infarction in mice, independent of its effect on blood pressure (Westermann et

al., 2008a). The recent ALLAY study on 465 patients with hypertension tested the effectiveness of aliskiren on LVH reduction, while comparing its effects to the Ang-receptor blocker losartan (Solomon et al., 2009). LVM_{index} was significantly reduced in both treatment groups, independent of blood pressure lowering effects. There were, however, no statistically significant differences in effect between the two treatment groups, leading the authors to conclude that aliskiren was as effective as losartan in reducing LVH (Solomon et al., 2009).

While there is ample evidence reporting an association between *REN* polymorphisms and hypertension (Ahmad et al., 2005b; Frossard et al., 2001), to the best of our knowledge, there are no studies that investigate the effects of *REN* gene variants on hypertrophy development.

We therefore complement the findings of these animal studies and clinical trials by reporting a significant association between *REN* haplotype 4 (GGTGGC) and a decrease of 29.1 g in LVM. Two other haplotypes were also found to be associated with different effects between the different HCM mutation groups. Haplotype 1 (GTCTAC) was associated with a 50.33 g lower effect on LVM in the $R92W_{TNNT2}$ group compared to the $R403W_{MYH7}$ group, while haplotype 7 (GTCTGC) associated with a significantly higher effect of 4.05 mm on the CWT score and a 0.25 higher PC1 in the $R92W_{TNNT2}$ group than in the $R403W_{MYH7}$ group. Interestingly, these haplotypes only differ in one respect, namely the allele at rs10900555. This points towards a context dependent effect of this SNP, but further research is needed to confirm these findings as haplotype 7 was only observed in 2.6 % of the cohort.

We did, however, not find association between single *REN* variants and any of the investigated hypertrophy traits (Figure 3.10). This might be due to the fact that these polymorphisms act together to markedly affect hypertrophy development, while the individual effects are too weak to detect.

In the present study we also investigated the effect of three proteins that associate with renin function *in vivo* on hypertrophy development in HCM, viz. RnBP, M6PR and the PRR. Research on these proteins is sparse at present, but preliminary studies show that the effects of these three proteins might be crucial in understanding renin's cellular hypertrophic functions that are independent of blood pressure regulation (Nguyen et al., 2003).

Previous studies have shown that the heart can generate renin locally from circulating prorenin by proteolytic cleavage and non-proteolytic activation through the PRRs in myocardial tissues (Nguyen et al., 2002; Nguyen and Danser, 2008; Nguyen and Muller, 2010; Reudelhuber et al.,

1994). Prorenin might also offer significant physiological relevance in hypertrophy development as plasma concentrations of prorenin is ten times greater than that of renin (Danser et al., 1998), while circulating prorenin levels may reach as high as 100 times the level of renin under conditions of renal damage and cardiac hypertrophy (Susic et al., 2008).

Transgenic rats expressing prorenin exclusively in the liver demonstrated a 400-fold increase in plasma prorenin. These animals exhibited, but developed severe liver fibrosis, as well as cardiac hypertrophy, despite normal plasma renin levels and blood pressure (Veniant et al., 1996). Later, Saris et al. found that prorenin bound to the PRR activated the p38 MAPK/HSP27 pathway in neonatal rat cardiomyocytes (Saris et al., 2006), which they postulated to explain the severe hypertrophy observed by Veniant et al.

Earlier studies have reported that renin and prorenin *per se* exerts hypertrophic cellular effects, independent of Ang II generation, possibly through involvement of the PRR (Methot et al., 1999; Nguyen et al., 1996; Prescott et al., 2002). Later, studies showed that renin and prorenin are able to induce DNA synthesis and activate profibrotic, inflammatory and hypertrophic signalling pathways that function independent of Ang II generation through binding with the PRR (Huang et al., 2006; Huang et al., 2007b; Ichihara et al., 2006; Nguyen and Muller, 2010). This includes activation of the p42/p44 MAPK intracellular pathways, release of PAI-1, as well as TGF-β1 (Cousin et al., 2010). Interestingly, these pro-hypertrophic signalling cascades are not inhibited by ACE inhibitors, aliskiren or AT_1R blockers (Balakumar and Jagadeesh, 2010a).

Recently, Cruciat et al. proposed a renin-independent mechanism for the PRR, as this protein acted as an adaptor between Wnt receptors and the v-H^+-ATPase complex in human cultured cells (Cruciat et al., 2010). This link between Wnt signalling and the PRR is especially intriguing as aberrant Wnt signalling has been linked to cardiac hypertrophy (Balakumar and Jagadeesh, 2010b). Connelly et al. later confirmed the co-localization of PRR with v-H^+-ATPase in the heart and reported an increased expression of PRR in the hearts of transgenic animals with diabetic cardiomyopathy (Connelly et al., 2011). These animals developed diastolic dysfunction, interstitial fibrosis and cardiomyocyte hypertrophy, while PRR expression was reduced with renin inhibition, which resulted in improved cardiac structure and function (Connelly et al., 2011).

The PRR therefore offers an Ang II-independent link between renin, prorenin and cardiac hypertrophy. However, in the present study, we found no statistically significant evidence for association between the investigated *ATP6AP2* variants and any of the hypertrophy traits as

evident from Figure 3.13. On the other hand, we observed a difference in effect size caused by the rs2968917 polymorphism in *ATP6AP2* in the R403W$_{MYH7}$ group compared to the A797T$_{MYH7}$ group, which might point toward context-dependent effects. We were, unfortunately, not able to estimate haplotypes for *ATP6AP2* as there are currently no documented methods available for estimating haplotypes for X-linked genes.

Furthermore, human renin and prorenin contains the M6P signal that is necessary to bind to M6P/IGFII receptors and these receptor have been shown to bind prorenin and renin on cardiomyocytes (Van den Eijnden et al., 2001; Van Kesteren et al., 1997a), and to generate renin from prorenin through proteolytic cleavage (Saris et al., 2001a). However, binding of prorenin to M6P/IGFII receptors on the cell membrane of neonatal rat cardiomyocytes resulted in enhanced DNA and protein synthesis in the presence of angiotensinogen, but not in its absence, while intracellular prorenin activation through these receptors did not result in intracellular or extracellular Ang II generation, leading the authors to conclude that these effects were mediated through the catalytic activity of prorenin *per se*, rather than intracellular activation (Saris et al., 2002). The M6P/IGFII receptor has consequently been suggested rather to act as a clearance receptor, as it exclusively binds glycosylated forms of prorenin and renin, which is followed by rapid internalization of the M6PR/(pro)renin complex, intracellular proteolytic activation to mature renin and subsequent degradation (Nguyen, 2006).

However, another avenue of research suggests that this receptor is essential during early cardiac development (McCormick et al., 1996), while complete M6P/IGFII receptor knockout results in foetal overgrowth and neonatal lethality (Wylie et al., 2003). In addition, a lone study associating M6P/IGFII receptor down-regulation with decreased sensitivity of cardiomyocytes to TNF- and hypoxia-induced apoptosis, suggests that this receptor might be involved in cell growth and apoptotic signalling pathways in the heart (Chen et al., 2004).

In the present study we find strong evidence for association between *M6PR* variants and LVH as evident from Figures 3.14 and 3.15. The C-allele of rs1805725 in *M6PR* was significantly associated with a 15.1 g decrease in LVM, a 1.28 mm decrease in mIVST, a 0.62 mm decrease in CWT score, as well as a 0.04 decrease in PC1. Interestingly, both haplotypes containing the C-allele for the rs987917 SNP, viz. AC and CC, were found to associate significantly with hypertrophy traits (Table 3.12). When this C-allele is accompanied by an A-allele at rs1805725, the resulting haplotype is associated with a 0.67 mm increase in mLVWT. However, when the rs987917 C-allele is accompanied by a C-allele at rs1805725, the resulting haplotype is associated with much larger effects, namely a 44.9 g decrease in LVM, a 3.73 mm decrease in

mIVST, a 3.39 mm decrease in mLVWT, a 1.59 mm decrease in CWT score, as well as a 0.10 decrease in PC1. The rs1805725 polymorphism, therefore, appears to have a strong independent effect on hypertrophy development, which is strengthened by the rs987917 polymorphism.

A preliminary HapMap and NCBI gene search showed no flanking genes with obvious cardiovascular functions, so it is unlikely that the association signal is originating from a neighbouring gene. Further research is therefore warranted to fully elucidate the cardiac-specific functions of this gene in order to clarify the link between *M6PR* variants and cardiac hypertrophy observed in the present study. One might speculate that improper function of the *M6PR* might lead to prorenin accumulation, which has been shown to exert hypertrophic effects (Veniant et al., 1996), but there is currently no evidence to back this hypothesis.

In vitro studies have shown that the RnBP is able to inhibit renin's activity upon binding to it (Takahashi et al., 1994). In renal homogenates, RnBP forms a tight heteromeric complex with renin, designated as "high molecular weight renin" (Takahashi et al., 1983), the formation of which is dependent on a leucine zipper motif in RnBP (Inoue et al., 1991). Binding of RnBP to renin has been shown to inhibit angiotensinogen to Ang I conversion in mouse pituitary AtT-20 cells transfected with human renin and RnBP cDNAs, while the expression of RnBP inhibits active renin secretion in a dose-dependent manner (Inoue et al., 1992). The T61C polymorphism in intron 6 of *RENBP* was later shown to associate significantly with increased plasma prorenin, as well as with the renin/prorenin ratio, but not with circulating renin, blood pressure, heart rate or LVM (Knoll et al., 1997).

However, knockout mice lacking RnBP were normotensive, with normal renin levels, and did not exhibit any obvious adverse phenotypes (Schmitz et al., 2000). On the other hand, Bohlmeyer et al. showed that RnBP was selectively activated in failing human hearts (Bohlmeyer et al., 2003). Using ribonuclease protection assays, this group found increased *RENBP* gene expression in failing hearts from patients with ischaemic and idiopathic dilated cardiomyopathies, when compared to healthy, non-failing hearts (Bohlmeyer et al., 2003). In addition, reverse transcriptase-PCR demonstrated *RENBP* expression in endothelial cells, but not in the cardiomyocytes of the patients with non-failing hearts. However, *RENBP* expression was not detectable in endothelial cells, but was selectively activated in cardiomyocytes of failing hearts (Bohlmeyer et al., 2003). One can then speculate that RnBP only has cardiovascular functions under certain pathological conditions, which might possibly relate to the cardioprotective effects proposed for RnBP though its inhibitory effect on renin. RnBP could

possibly be recruited to decrease renin activity, which would lead to decreased Ang I and ultimately Ang II, which has known hypertrophic effects on the heart.

In the present study, the A-allele of rs762656 in *RENBP* was found to significantly decrease a single hypertrophy measurement, mPWT, by 0.51 mm. However, as with *ATP6AP2*, we were unable to estimate haplotypes for the X-linked *RENBP*. The evidence for involvement of RnBP in hypertrophy development is in addition comparatively weak when the present results are viewed in conjunction with the discussed results from animal studies. More research is, therefore, needed to elucidate the cardiac effects of RnBP *per se* and consequently the hypertrophic effects of *RENBP* gene variants.

4.5.3 Angiotensin converting enzymes

This *ACE* gene remains the most commonly studied candidate modifier gene for HCM as it is responsible for conversion of Ang I to the active Ang II, as well as the inactivation of bradykinin, which exerts strong cardiovascular effects in opposing directions. While Ang II promotes hypertension and cardiac hypertrophy, bradykinin exerts cardioprotective effects.

A strong correlation exists between ACE and blood pressure, which is evident from the fact that ACE inhibitors are currently the gold standard in hypertension treatment (Paulis and Unger, 2010). However, earlier studies have demonstrated that plasma ACE activity is significantly related to LVM, independent of systemic blood pressure (Schunkert et al., 1997). Moreover, meta-analyses of clinical trials demonstrate that ACE inhibitors are able to effectively reduce LVH, even after adjustment for treatment duration and change in blood pressure (Dahlof et al., 1992; Klingbeil et al., 2003; Schmieder et al., 1996).

Most association studies on *ACE* focus on the I/D polymorphism (rs4340) in intron 16 of this gene, as it is thought to be functional, exerting an incremental effect on plasma and tissue ACE levels, where II-homozygotes exhibit the lowest levels of plasma and tissue ACE, heterozygotes the intermediate and DD-homozygotes the highest (Marian, 2002; Tiret et al., 1992). However, the I/D variant is not located in a coding region and does not exert any clear effect on gene transcription, which points to a very real possibility that it might only be a marker for a functional variant in close proximity (Cox et al., 2002; Keavney et al., 1998).

A recent meta-analysis on echocardiographic phenotypes from 8979 individuals from 38 association studies (Jin et al., 2011b) reported that both DD homozygotes and ID heterozygotes had a higher LVM or LVM_{index} than II homozygotes. However, these data were compiled from

studies with inconsistent adjustment for lifestyle and environmental confounders of hypertrophy. In addition, the authors reported a significant publication bias towards studies reporting an association between LVM and the I/D polymorphism, which may have led to an overestimation of association in the combined cohort (Jin et al., 2011b).

On the other hand, the Framingham Heart study, which had a smaller, but still fairly large cohort of 2439 individuals, did not find significant evidence for linkage or association between the I/D polymorphism and LVM (Lindpaintner et al., 1996). This study clearly adjusted all association and linkage analyses for sex, age, height, weight, systolic blood pressure, and the presence of diabetes, ischemic heart disease or congestive heart failure.

A number of studies have investigated the effects of this polymorphism in HCM, but when we examine these studies more closely, it becomes apparent that the underlying designs of these studies are too diverse to draw definitive conclusions regarding the effect of this polymorphism on the HCM phenotype. For instance, three earlier studies (Pfeufer et al., 1996; Rai et al., 2008; Yoneya et al., 1995) looked at allele frequency differences between HCM patients and controls, which does not offer any information on the link between this polymorphism and LVH *per se*.

In the present study we did not find significant evidence for association between the I/D polymorphism and any of the investigated hypertrophy traits (Figure 3.19), which is consistent with findings from previous HCM cohorts (Buck et al., 2009; Coto et al., 2010; Osterop et al., 1998; Yamada et al., 1997), but in contrast to others (Doolan et al., 2004; Lechin et al., 1995; Tesson et al., 1997). All these studies have comparatively modest sample sizes ranging from 62 to 545, but they differ largely on the adjustment for relevant confounders, which might account for the discrepant results as association between this polymorphism and hypertrophy indices is context-dependent and sensitive to hypertrophy confounders (Sayed-Tabatabaei et al., 2006). For instance, none of these studies adjusted their analyses for the primary HCM-causal mutation, which might specifically have an impact on the outcome of *ACE* I/D association studies in HCM.

In families harbouring HCM causal mutations in either the *MYBPC* or *MYH7* genes, Tesson et al. reported a mutation-specific association between the I/D polymorphism and LVH. They found no significant association when the cohort was analysed together, whereas a significant association was, however, reported between the *ACE* D-allele and interventricular septal thickness in individuals with the R403Q mutation in *MYH7* (Tesson et al., 1997). In the present study, we did not find significant differences in effect of the I/D polymorphism between the

three HCM mutation groups, but we did, however, find that the effect of the rs4303 T-allele on mPWT was higher in the R92W$_{TNNT2}$ group, when compared to the R403W$_{MYH7}$ group, but lower in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group. In addition, we identified two *ACE* haplotypes, namely haplotypes 3 (CTDT) and 4 (CGIC), that had significantly different effects on mPWT, the CWT score, as well as PC1.

That said, we report a significant association between the *ACE* rs4356 C-allele and a significant CWT score increase of 0.74 mm after adjustment for all the relevant HCM hypertrophy confounders. Interestingly, this polymorphism falls in the intron 18 to 3' UTR region that was suggested to most likely harbour the functional mutation responsible for positive I/D association signals (Sayed-Tabatabaei et al., 2006). Haplotype association analyses did, however, not yield significant results. One possible explanation for this could be that the other three non-associated SNPs in the haplotype, viz. rs4298, rs4303 and rs4340, mask the effect of the rs4356 C-allele when combined into a single haplotype.

There is a clear link between *ACE* activity and cardiac hypertrophy (Buck et al., 2009; Huang et al., 2007a; Schunkert et al., 1997); moreover, smaller clinical trials report favourable cardiovascular outcomes for HCM patients on ACE-inhibitors (Kyriakidis et al., 1998). However, due to the heterogeneous designs and outcomes of previous studies on the effect of *ACE* polymorphisms on hypertrophy in HCM and the fact that most studies only focussed on the I/D polymorphism, it is difficult to comment on the use of *ACE* polymorphisms for risk stratification in HCM patients. A large, carefully controlled study aimed at genetic variation across the entire *ACE* region with adequate adjustment for known hypertrophy confounders might shed some light on this matter.

A homologue of *ACE,* designated as ACE2, removes the C-terminal phenylalanine from Ang II, to form Ang-(1-7), which is a ligand of the G-protein-coupled receptor Mas and which was only discovered in 2002 (Crackower et al., 2002). This protein is thought to be essential for cardiovascular functions as ACE2-knockout mice develop progressive Ang II-mediated age-dependent cardiomyopathy, which is associated with increased oxidative stress, as well as pathological hypertrophy (Oudit et al., 2007).

Furthermore, results from animal models show that ACE2 deletion does not affect blood pressure, but rather combats adverse cardiac remodelling, as it accelerates cardiac hypertrophy as well as the progression from hypertrophy to cardiac failure (Yamamoto et al., 2006). On the other hand, chronic *ACE2* inhibition resulted in an accumulation in cardiac Ang II, which

increased LVH and fibrosis in transgenic hypertensive rats (Trask et al., 2010). This is complemented by the findings that *ACE2* overexpression protected against Ang II-induced cardiac hypertrophy and fibrosis (Huentelman et al., 2005), while recombinant human ACE2 reduced Ang II-induced LVH in wild type mice and partially prevented the development of dilated cardiomyopathy in pressure-overloaded mice (Zhong et al., 2010).

Lieb et al. reported that the minor alleles of four SNPs in the X-linked *ACE2* gene (rs4646156, rs879922, rs4240157 and rs233575) were associated with interventricular septal thickness, as well as LVM_{index} in unrelated males from a German population from the MONICA Augsburg survey (Lieb et al., 2006). These four SNPs showed high pairwise LD and a common haplotype, consisting of the minor alleles of these SNPs, was associated with a modestly increased LVM_{index} and interventricular septal thickness after adjustment for age, body mass, antihypertensive medications and systolic blood pressure. While these findings were restricted to hemizygous males and not replicated in females, Lieb et al. reported that another SNP outside this LD block, rs2285666, associated significantly with decreased LVM_{index} in females, but not males. In terms of functionality, this possibly suggests that neither of the four SNPs within the common haplotype, viz. rs4646156, rs879922, rs4240157 or rs233575, are, in fact, functional and that this region rather points to a nearby functional variant.

Later, Wang et al. reported that the T-allele of rs2106809 and C-allele of rs6632677 conferred an increased risk for HCM in males, but not females, from a study of 261 Chinese HCM patients and 600 healthy controls (Wang et al., 2008). In addition, a TC haplotype from these SNPs associated significantly modestly increased interventricular septal thickness again in males, but not females, independent of age, body mass and blood pressure. The authors did, however, not investigate the four SNPs that associated significantly with hypertrophy in males from the Lieb et al. study, as these SNPs were either not polymorphic or had very low MAFs in the Chinese population (Wang et al., 2008).

A previous study from our lab on a smaller subset of the present HCM cohort reported that the G-allele significantly increased LVM by 18.7 g, mIVST by 1.9 mm and mPWT by 0.7 mm (Van der Merwe et al., 2008). These findings were replicated in the present larger cohort with slightly smaller effect sizes as the G-allele of this polymorphism was found to increase LVM by 13.7 g, mPWT by 0.623 mm, mIVST by 1.59 mm and mLVWT by 1.68 mm. However, the effect of the G-allele of rs879922 on mPWT was 1.20 mm lower in the $R92W_{TNNT2}$ group when compared to the $A797T_{MYH7}$ group, as shown in Table 3.16. Unfortunately, we could not do haplotype-based

analyses as, as mentioned previously, there are currently no methods available to construct phased haplotypes from X-linked markers in extended families.

The rs879922 polymorphism is located in an intron without any obvious functional role. The possibility then exists that it might be in LD with a functional polymorphism towards the 3' end of the gene as rs879922 resides in a haplotype block spanning a large portion of the 3' end of *ACE2* in both the HapMap CEU end YRI populations (Van der Merwe et al., 2008).

However, there exists a third protein, namely cardiac chymase that is responsible for Ang I to Ang II conversion in the heart. Cardiac chymase is produced from mast cells in the heart and is able to locally generate cardiac Ang II from Ang I, while previous studies have reported that CMA accounts for 80% of the Ang II generated in human ventricles (Urata et al., 1993). This has significant physiological implications, as CMA activity is not inhibited by ACE inhibitors (Guo et al., 2001), which could impact on the efficacy of ACE inhibition as a treatment option in cardiac hypertrophy.

CMA activity is increased in the hearts of cardiomyopathic hamsters (Shiota et al., 1997; Shiota et al., 1998). Moreover, Koga et al. reported increased LVM and blood pressure levels in transgenic mice expressing human CMA, while CMA inhibition increased survival rate, coupled with a regression of cardiac hypertrophy and end-diastolic left ventricular pressure, in a hamster model of myocardial infarction (Hoshino et al., 2003).

Previously, Gumprecht et al. reported that a -1903 G/A transition in the 5' untranslated region of *CMA1* (rs1800875), together with the *ACE* I/D polymorphism associated with an increased risk of LVH development in Caucasian type-2 diabetics (Gumprecht et al., 2002). However, this polymorphism was not associated with the extent of LVH in a cohort of 50 unrelated HCM patients and 50 healthy controls (Pfeufer et al., 1996). In addition, Ortlepp et al. found no significant evidence for association between rs1800875 and LVM or interventricular septal thickness in a HCM family cohort after adjustment for age, sex and the presence of hypertension (Ortlepp et al., 2002).

Our results complement these findings, as we did not find any evidence for association between the rs1800875 or rs1885108 polymorphisms and hypertrophy indices in the present cohort (Figure 3.22), which is supplemented by a lack of association between haplotypes of these SNPs and the investigated hypertrophy traits (Table 3.19). While CMA appears to have an impact on cardiac function *in vivo*, we cannot confirm that genetic variation in this gene contributes to LVH

development in HCM. There is, however, still a possibility that genetic variants in this gene could contribute to LVH in a different population and these results should be verified in an independent, preferably larger cohort, to allow definitive conclusions on this matter.

4.5.4 Angiotensin II receptors

Ang II exerts its main hypertrophic effects through the AT_1R s. Binding of Ang II to these receptors activates a number of cellular pathways involved in cardiac hypertrophy, including vasoconstriction, aldosterone release and growth stimulation (Dostal and Baker, 1992; Hoffmann et al., 2001). The essential role of the AT_1R in the heart is further illustrated in transgenic mice overexpressing the AT_1R selectively in cardiomyocytes, which exhibited an increase in cardiac mass, coupled with cardiomyocyte hyperplasia at birth and died within the first weeks after birth (Hein et al., 1997). More recently, Ainscough et al. developed transgenic mice with cardiomyocyte-specific inducible human AT_1R gene expression. Low levels of AT_1R expression in cardiomyocytes from the start of adolescence increased cardiomyocyte growth in these animals, which lead to cardiac hypertrophy in adulthood, which was not associated with changes in blood pressure or heart rate (Ainscough et al., 2009).

Large clinical trials have concluded that AT_1R antagonists reduce LVH and other associated cardiac morbidities in hypertensive cohorts (Dahlof et al., 2002b; Okin et al., 2003). In addition, a recent consensus document on the current research priorities in HCM identified the AT_1R as a potential target for intervention in HCM that warrants further investigation (Force et al., 2010). This is based on four small clinical trials that demonstrated improvements in cardiovascular morphology and function in patients with non-obstructive HCM receiving AT_1R-inhibitors (Araujo et al., 2005; Kawano et al., 2005; Penicka et al., 2009; Yamazaki et al., 2007). A study by Penicka et al. reported that the AT_1R-antagonist candesartan resulted in a regression of LVH, coupled with an improvement of left ventricular function and exercise tolerance in patients with non-obstructive HCM (Penicka et al., 2009). This group also noted that the magnitude of LVH regression was related to the specific HCM causal mutation, with *MYH7* mutation carriers showing the greatest response to the AT_1R-antagonist (Penicka et al., 2009).

An A/C transversion at position 1166 in the 3' untranslated region of *AGTR1* (rs5186) has received much attention as a potential risk factor in cardiovascular genetic association studies. This polymorphism was previously associated with a number of cardiovascular outcomes (Coto et al., 2010; De Denus et al., 2008; Ortlepp et al., 2002; Xu et al., 2010a). However, studies on the relationship between this polymorphism and LVH yielded discrepant results as some studies

report significant associations with LVM (Kelly et al., 2011; Smilde et al., 2007), while others do not (Castellano et al., 1996; Hamon et al., 1997; Kuznetsova et al., 2004).

A study by Wang et al. investigated the effects of *AGTR1* C-512T, A1166C and L191L (rs5182) polymorphisms on the progression of blood pressure and LVM in a longitudinal study on European American and African American youths (Wang et al., 2006). In the single variant association analyses, none of the variants showed a significant association with LVM. However, haplotype analysis identified a haplotype (-521C, 191L and A1166) associated with a significant decrease of 12.9 g in LVM when compared to the most common reference haplotype (–521T, 191L and A1166) (Wang et al., 2006).

In HCM, most studies only focussed on the A1166C polymorphism. Osterop and co-workers found that the 1166C-allele associated significantly with an increase in LVM in a cohort of 104 HCM patients, independent of age, gender, peak left ventricular outflow gradient, plasma renin, and the *ACE* I/D polymorphism (Osterop et al., 1998). Similarly, Coto et al. reported a significant association between the 1166C-allele and increased left ventricular wall thickness in HCM patients (Coto et al., 2010). On the other hand, Funada et al. reported that when analysed alone, the A1166C polymorphism did not influence LVH, but when analysed in combination with the *ACE* I/D polymorphism, the two SNPs associated significantly with left ventricular end-systolic dimension and ejection fraction, but not with interventricular septal thickness or mean wall thickness (Funada et al., 2010).

The A1166C polymorphism is located in the 3' UTR of *AGTR1*; studies aimed at unravelling the functional mechanism of this polymorphism came up short-handed as this polymorphism did not affect AT_1R affinity or density (Danser and Schunkert, 2000; Paillard et al., 1999), nor did it affect plasma Ang II levels (Miller et al., 1999). A recent *in vitro* study demonstrated that the 1166A-allele allows the binding of hsa-miR-155 micro-RNA, which is able to repress mRNA expression post-transcriptionally, which, in turn, downregulates the expression of only the A1166, and not the 1166C-allele. This results in increased translation of *AGTR1* in C-allele carriers (Sethupathy et al., 2007). However, it is uncertain at this stage whether *AGTR1* and hsa-miR-155 are concomitantly expressed in the heart. As no definite functional mechanism has been demonstrated to link this polymorphism to AT_1R functionality, this polymorphism is most probably not *the* functional variant driving positive association signals.

In the present study we investigated the effects of an intron 1 polymorphism, rs2640539, the rs3772627 in the approximate "middle" of the gene, as well as the rs5182 polymorphism at the

3'-end of the gene. One possible limitation is that we did not investigate the rs5186 polymorphism; however, we did assess the neighbouring rs5182, which is in tight LD with rs5186 (Abdollahi et al., 2007; Su et al., 2007). When we analysed haplotypes of all three variants, we found that a haplotype of all three minor alleles, CAA, associated with an increase in mLVWT of 2.29 mm, as well as an increase of 1.16 mm in the CWT score (Table 3.22). However, in the single variant analysis, only rs2640539 showed significant evidence of association (Figure 3.26). The C-allele of this polymorphism resulted in an increase in mIVST of 1.15 mm, as well as an increase of 1.22 mm in mLVWT.

A previous detailed LD analysis of *AGTR1* yielded two distinctive LD blocks, with a definite break in LD between the 5'- and 3'-ends of the gene (Abdollahi et al., 2007). In the present study we also found a similar LD pattern with little evidence for LD between the 5' rs2640539 and the more 3' rs5182. This definite break in LD prompted Abdollahi et al. to speculate that the 5' and 3' ends of *AGTR1* might have independent functional effects in addition to potential epistatic effects (Abdollahi et al., 2007).

In light of the larger effects of the combined haplotype in the present cohort, one can argue in favour of this theory. The possibility exists that variants from both intragenic loci might contribute to hypertrophy development, possibly even in a context-specific manner. In the present study, we saw a significant difference in effect of the minor allele haplotype CAA in the R403W$_{MYH7}$ group, compared to the A797T$_{MYH7}$ group, while the converse major allele haplotype, ACC, had significantly different effects in the in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group. It is, however, difficult to further substantiate this theory as most previous studies only focused on 3' variants within this gene, while HCM-mutation context-specificity has not previously been explored in *AGTR1*.

While we provide additional information for the involvement of this gene in HCM hypertrophy, more research is needed to pinpoint the functional variants responsible for these associations, perhaps within the context of different disease populations and ethnicities to clarify whether a single or multiple loci in this gene affects hypertrophy development.

Contrary to the AT$_1$Rs, the AT$_2$Rs are associated with cardioprotective effects (Van Kesteren et al., 1997b). Studies in adult rat hearts suggested that AT$_2$Rs have antihypertrophic effects on the heart that counterbalance the hypertrophic effects of the AT$_1$Rs (Booz and Baker, 1996; Mukawa et al., 2003). In addition, AT$_2$R blockade in Ang II-treated rats results in an amplified left ventricular growth response to Ang II (Bartunek et al., 1999), while AT$_2$R-knockout mice

show higher left ventricle/body weight ratios, as well as left ventricular end-diastolic and left ventricular end-systolic dimensions after myocardial infarction, when compared to wild type mice (Oishi et al., 2003).

Previous studies have identified a +1675 G/A polymorphism in exon 2 of the X-linked *AGTR2* gene as a possible modifier of cardiovascular phenotypes. This polymorphism is located at a lariat branch-point in the first intron, 29 bp before exon 2, in a region that is essential for transcriptional activity (Erdmann et al., 2000; Warnecke et al., 2005). This polymorphism was thought to affect pre-mRNA splicing (Nishimura et al., 1999), but later investigations demonstrated that it modulates AT_2R protein expression, but not mRNA splicing (Warnecke et al., 2005). This provides a possible mechanism for action, as increased AT_2R protein levels may be protective in LVH development (Warnecke et al., 2005).

The +1675 A-allele was significantly associated with increased LVM, as well as relative left ventricular wall thickness in young, mildly hypertensive males (Schmieder et al., 2001). Similarly, Herrmann et al. reported a significant association between the +1675 A-allele and LVM in males from the GLAEOLD cohort (Herrmann et al., 2002). However, this association was not replicated in the similar, but larger GLAECO cohort (Herrmann et al., 2002).

In contrast to these findings, Alfakih et al. reported an association between the +1675 G-allele and increased LVM_{index} as determined with MRI (Alfakih et al., 2004), which is credited with being a more accurate and reproducible measure of LVM than 2D- and M-mode echocardiography, although echocardiography is still widely used to estimate LVM, as it is more readily available as a clinical tool (Myerson et al., 2002).

In accordance with the latter study, we find a significant association between the +1675 A-allele and a decrease of 0.9 mm in mIVST as shown in Figure 3.28. In a previous investigation on a smaller subset of the present cohort, we found significant evidence for association between the +1675 G/A polymorphism and PC1 (Carstens et al., 2011), which was not replicated in the present cohort. This smaller group did, however, also show a significant association between the +1675 G/A polymorphism and mIVST, which is in accordance with the present findings (Carstens, N; M.Sc). The fact that we did not find a significant association with PC1 in the present cohort might relate to the possibility that the larger cohort provided improved statistical power to detect true effects. Another possibility is that it might be a reflection of the particular pattern of LVH in the newly added patients, as cardiac hypertrophy might be localised to the IVST in some HCM patients. PC1, on the other hand, was constituted from 16 cardiac wall

thickness measurements at three levels in the heart, which would require hypertrophy at more than one segment to be significantly elevated.

We did, however, not find any evidence for association between rs11091046 (+3123 A/C) and any of the investigated hypertrophy traits, as shown in Figure 3.28, which stands in contrast to the findings of Deinum et al. (Deinum et al., 2001). This group reported an association between the +3123 C-allele and decreased LVM_{index} in unrelated female, but not male, HCM patients, independent of plasma renin, the +1166 A/C *AGTR1* variant or the I/D *ACE* variant. This study did, however, not account for differences in the primary HCM causal mutation, which may have had confounding effects on the association. For instance, the effect of the +3123 C-allele on mIVST was 2.69 mm higher in the $R403W_{MYH7}$ group, compared to the $A797T_{MYH7}$ group, in the present study. In addition, this group did not investigate the +1675 G/A polymorphism, so another possibility is that the positive association signal originated from LD with the +1675 G/A variant, which is known to affect AT_2R protein expression, as the +3123 A/C and +1675 G/A variants were in LD in this cohort.

4.5.5 CYP11B1/B2 locus

Studies on perfused rat hearts and neonatal cardiomyocytes demonstrated increased cardiomyocyte contractile force and hypertrophy in response to aldosterone administration (Barbato et al., 2004; Dooley et al., 2011; Rossier et al., 2010; Sato and Funder, 1996). In fact, aldosterone directly stimulated cardiac hypertrophy in neonatal rat ventricular cardiomyocytes (Okoshi et al., 2004). This hypertrophic response was associated with increased alpha- and beta-myosin heavy chain mRNA levels and the activation of ERK1/2, JNK, and protein kinase C, while MR inhibition suppressed this effect (Okoshi et al., 2004).

Increased serum aldosterone concentration was associated with significant increases in LVM, posterior wall thickness and relative wall thickness in a cardiovascular risk cohort of 1575 patients without overt heart failure (Edelmann et al., 2011), as well as a population based sample of 615 middle-age subjects (Schunkert et al., 1997), while suppression of aldosterone was associated with a reduction in LVM_{index} after adjustment for blood pressure change in the hypertensive ALLAY cohort (Pouleur et al., 2011). Myocardial aldosterone and *CYP11B2* mRNA levels is furthermore elevated by 4- to 6-fold in HCM patients, compared to healthy donor hearts (Tsybouleva et al., 2004).

The -344T/C polymorphism (rs1799998) resides in a putative SF-1 binding site and the -344 C-allele increases SF-1 binding four-fold *in vitro*, which points toward a possible mechanism

whereby this polymorphism could influence gene expression (White et al., 1999). However, a later study confirmed the -344 C-allele increase in SF-1 binding *in vitro*, but also demonstrated that that SF-1 failed to stimulate *CYP11B2* expression *in vivo* (Bassett et al., 2002). This polymorphism in the 5' promoter region of the *CYP11B2* was significantly associated with plasma aldosterone levels in a cohort of 216 patients with essential hypertension, after adjustment for age and urine Na^+-excretion (Pojoga et al., 1998), as well as LVM in 84 healthy Caucasians, independent of sex, body size, blood pressure, physical activity, smoking, and ethanol consumption (Kupari et al., 1998).

However, no evidence for association was found between this polymorphism and serum aldosterone, LVM_{index} or other echocardiographic measures of LVH in 1445 young Caucasian adults from the third MONICA survey (Schunkert et al., 1999b). In addition, a later meta-analysis reported that the -344T/C variant was not significantly associated with LVM or interventricular septal wall thickness in a pooled sample of 2157 unrelated subjects, although a smaller subset of hypertensive subjects (n = 332) homozygous for the T-allele showed a 6.9% increase in LVM, compared to CC-homozygous subjects (Sookoian et al., 2008).

Mayosi et al. investigated the effect of this promoter variant, as well as five other *CYP11B2* polymorphisms (singularly and in combined haplotypes) on heart size, in 955 members from 229 British Caucasian extended families recruited through hypertensive probands (Mayosi et al., 2003). Haplotype analysis revealed a relatively common haplotype with a frequency of 22.4% that associated significantly with an increase in septal wall thickness in this cohort. In a single variant association analysis, which was confirmed using measured haplotype analyses, the G5937C variant associated with septal wall thickness, while the I2C and A4550C variants associated with left ventricular cavity size. The estimated effects of these polymorphisms were, however, comparatively modest as the G5937C variant contributed to 2.4 % of the variability in septal wall thickness, while the I2C and A4550C variants contributed to 2.0 and 3.4% of the variability in left ventricular cavity size, respectively (Mayosi et al., 2003).

Mayosi et al. further speculated that these associations could be due to or influenced by genetic variation in the neighbouring *CYP11B1* gene. More extensive haplotype analyses of the entire CYP11B1/B2 locus later revealed a high degree of LD between markers in these two genes (Ganapathipillai et al., 2005; Keavney et al., 2005), which was followed by studies that showed that aldosterone synthesis is influenced by genotypes at *CYP11B2,* as well as *CYP11B1* (Imrie et al., 2006; Alvarez-Madrazo et al., 2009).

To the best of our knowledge, this is the first study to investigate the influence of the entire CYP11B1/B2 locus on LVH phenotypes in HCM, whereas previous investigations in HCM largely focussed on the influence of the -344T/C polymorphism on cardiovascular phenotypes. In a study on 142 unrelated HCM patients, Patel et al. found that *CYP11B2* –344T/C genotypes did not influence LVM or interventricular septal thickness (Patel et al., 2000). In contrast to the study by Tsybouleva et al., Chai et al. found that plasma and left ventricular tissue levels of aldosterone in 79 unrelated HCM patients were not significantly different from those in age-matched controls (Chai et al., 2006). In addition, they reported a significant association between the *CYP11B2* -344T-allele and LVM_{index}, as well as interventricular septal thickness in men, but not in women (Chai et al., 2006).

When analysed alone, we did not find any evidence for association between the -344T/C polymorphism or the other investigated CYP11B1/B2 polymorphisms and LVH in the present cohort (Figure 3.30). Two haplotypes were, however, found to associate significantly with hypertrophy traits. Haplotype 8 associated with a 3.23 mm increase in mLVWT, as well as a 1.73 mm increase in CWT score and a 0.11 increase in PC1. This haplotype was, however, only observed in 2.7 % of the cohort, while haplotype 10, which was observed in only 1.9% of the cohort, associated with a 2.34 mm increase in mPWT. The effect of haplotype 8 was, however, significantly different between the $R403W_{MYH7}$ and $A797T_{MYH7}$ groups, as well as between the $R92W_{TNNT2}$ and $A797T_{MYH7}$ groups, which points to a strong context-specific effect underpinning the association of this haplotype in the entire cohort. Further research in larger cohorts are needed to confirm this differential effect, due to the low frequency of haplotype 8 in the cohort, which resulted in relatively small test groups for this particular analysis.

The results reported here are, therefore, in keeping with previous reports that LVH is influenced by variation across the entire locus. The effect sizes estimated for our study are, however, larger than that observed for the Mayosi et al. cohort (Mayosi et al., 2003), notwithstanding that the haplotypes from this study were constructed from one more variant than the previous haplotype. In contrast to the haplotype described by Mayosi et al., which was observed in 22.3 % of that cohort, haplotype 8 had a frequency of only 2.7% in the present cohort, which is consistent with the general trend seen in the effect of genetic variation on complex phenotypes, where common variants are generally associated with smaller effects than rarer variants.

Moreover, we report a high degree of LD between CYP11B1 variants, which extends from rs4310186 within *CYP11B1* to the first *CYP11B2* polymorphism, rs3097 (Table 3.24). Intermediate D' values were, however, observed between the remaining *CYP11B2* and *CYP11B1*

variants demonstrating incomplete LD between these two genes, which is in contrast to previous studies that reported complete LD across the entire locus in a Caucasian population (Ganapathipillai et al., 2005).

4.5.6 Mineralocorticoid receptor and 11 β-hydroxysteroid-dehydrogenase type 2

Aldosterone exerts its main effects on cellular Na^+ homeostasis and cardiac hypertrophy when bound to the MR to form the MR/aldosterone complex, which activates aldosterone-induced early and late response gene transcription and signalling cascades to mediate these effects via its downstream effectors (Fuller and Young, 2005). Studies on animal models demonstrated that aldosterone is able to directly induce cardiomyocyte hypertrophy through its binding to the MR (Le Menuet et al., 2004; Yoshida et al., 2010), while several lines of evidence demonstrate the potential benefits of MR-blockade on cardiac hypertrophy. MR-blockade with eplerenone resulted in a regression of LVH and related attenuation of heart failure in rats with salt-sensitive hypertension the absence of an antihypertensive effect (Nagata et al., 2006). Likewise, the MR antagonist spironolactone attenuated LVH in uremic rats (Michea et al., 2008) and transgenic rats overexpressing human the MR (Stas et al., 2007) without a significant reduction in blood pressure.

In a transgenic mouse model of human HCM, Tsybouleva et al. provided convincing evidence that aldosterone affects the relationship between sarcomeric dysfunction and the HCM cardiac phenotype as the MR blocker spironolactone significantly improved diastolic function, while reducing myocyte disarray and interstitial fibrosis in these mice (Tsybouleva et al., 2004). Furthermore, overexpression of the human MR in mice resulted in dilated cardiomyopathy in two independent studies (Le Menuet et al., 2001; Ouvrard-Pascaud et al., 2005), which was coupled with significant increases in heart rate, while blood pressure levels remained unchanged. Ang II furthermore induced LVH and diastolic dysfunction without affecting systolic function in transgenic mice with cardiomyocyte-specific overexpression of the human MR, without affecting blood pressure (Di Zhang et al., 2008). Moreover, cardiomyocyte-restricted MR deficiency prohibited adverse cardiac remodelling following myocardial infarction and pressure overload (Fraccarollo et al., 2011; Lother et al., 2011).

In randomized clinical trials, MR antagonists reduced the risk of ventricular remodelling, myocardial fibrosis and sudden cardiac death, independent of the antagonist's blood pressure lowering effects (Pitt et al., 1999; Pitt et al., 2003; Pitt et al., 2005). MR blockade is currently under investigation as a possible experimental therapy option in HCM, as small preliminary studies showed beneficial cardiovascular effects of the MR blocker spironolactone (Marian, 2009).

There is currently a paucity of studies on the effect of genetic variation in *NR3C2* on both overload-induced and blood pressure-independent hypertrophy and the present study is the first to our knowledge to investigate the effect of *NR3C2* SNPs on LVH in HCM. We report an association between the rs745019 G-allele and a 10.19 g increase in LVM, as well as an increase of 0.443 mm in mPWT, while the rs1403142 G-allele, as well as rs13150372 A-allele was significantly associated with respective decreases in mPWT of 0.541 mm and 0.626 mm. As a high degree of LD exists between these three polymorphisms (Table 3.28), this possibly indicates the existence of a functional variant within that LD block that has a marked effect on mPWT.

Haplotype analyses did not reveal common haplotypes, but rather a large range of low frequency haplotypes, as evident in Table 3.30, which is most likely a reflection on the incomplete LD in this fairly large gene. Five haplotypes showed a significant association with the CWT score. Haplotypes 2, 13 and 21 were associated with increases in CWT of 1.96 mm, 4.03 mm and 5.11 mm, respectively, while haplotypes 6 and 15 resulted in respective 2.24 mm and 2.94 mm decreases in CWT. Interestingly, these haplotypes all share the rs745019 A-allele, the rs7699349 C-allele, as well as the rs13118022 G-allele; haplotypes associated with a decrease in CWT also contained the rs13150372 A-allele, while haplotypes associated with an increase contained the G-allele of this polymorphism. In addition to the association with the CWT score, haplotypes 2, 6 and 13 also associated with PC1, while haplotype 2 also associated with mLVWT and haplotype 15 with LVM. The relatively large haplotype effect sizes reported here are again consistent with the current theory that rarer variants exert greater effects in complex phenotypes.

Notwithstanding epistatic effects, the possibility then exists for multiple LVH-modifying loci within *NR3C2*, when one takes the low levels of LD and the size of this gene into account. More research is certainly warranted to explore the full contribution of genetic variation in *NR3C2* to LVH in HCM in light of the present findings, as well as the results from previous studies that suggest an important link between the MR and cardiovascular phenotypes *per se* and within the context of HCM, as discussed earlier in section 1.12.

The MR, which is able to bind both aldosterone and cortisol with equal affinity, is protected from cortisol occupancy under normal circumstances by 11β-HSD2, which degrades cortisol to corticosterone, which is then unable to bind to the MR (Farman and Bocchi, 2000). AME, a rare form of congenital hypertension, is caused by null-mutation in this gene and patients with AME

suffer from severe hypertension, hyperkalaemia and low aldosterone levels, as well as associated end-organ complications, such as renal or cardiovascular damage (Dave-Sharma et al., 1998).

Transgenic mice with cardiomyocyte-specific overexpression of human 11β-HSD2 were normotensive, but developed LVH, fibrosis and heart failure (Qin et al., 2003). However, *HSD11B2-null* heterozygote mice, expressing only half of the wild type enzyme levels, develop salt-sensitive hypertension, coupled with an increase in heart:body weight ratio, which could be partially reduced by the MR-blocker spironolactone (Bailey et al., 2011). Spironolactone, additionally, modulated MR and 11β-HSD2 expression levels in a rat model, which improved adverse left ventricular remodelling (Takeda et al., 2007). Little is, however, known about the involvement of this gene in LVH development in humans, although one study reported a correlation between urinary 11β-HSD2 activity and LVM in patients with essential hypertension (Glorioso et al., 2005).

This gene falls into a region of high LD, while the whole gene is itself located within one LD block in all four HapMap populations, and comparatively few polymorphic markers have been reported on dbSNP for this gene. We, therefore, only chose one haplotype-tagging SNP for investigation. However, we did not find any statistically significant evidence for association between this *HSD11B2* rs5479 variant and any of the investigated hypertrophy traits. We can consequently not exclude the possibility of additional, yet undetected, variants in *HSD11B2* that might have an impact on LVH solely on the basis of the present findings.

4.5.7 Epithelial sodium channel subunits

The ENaCs, consisting of three homologous α-, β-, and a γ-subunits, are regulated by the MR/aldosterone complex and these channels are important regulators of intracellular Na^+, as they are responsible for the eventual electrodiffusion of Na^+ through epithelial cells upon RAAS stimulation. The cardiovascular effects of the ENaCs are evident from Liddle syndrome, which is caused by gain-of-function mutations in ENaC subunit genes; these mutations lead to decreased ENaC degredation, resulting in excessive Na^+-absorption, K^+-wasting, systemic hypertension, as well as an elevated incidence of early cardiovascular disease and LVH (Hansson et al., 1995; Jeunemaitre et al., 1997; Rossi et al., 2011).

ENaC mutations have been implicated in numerous hypertensive phenotypes, while the ENaC inhibitor amiloride has well-documented antihypertensive effects (Spence, 2010; Su and Menon, 2001). ENaC inhibition has, in addition, been linked to a number of cardiovascular benefits (Teiwes and Toto, 2007). ENaC inhibition in combination with a thiazide-diuretic caused

significant reductions in both coronary mortality and sudden cardiac death in elderly patients with hypertension, that could not be achieved through diuretics alone (Hebert et al., 2008).

Studies on salt-sensitive and spontaneously hypertensive rats demonstrated that amiloride can reduce LVH and other cardiovascular complications, independent of its effect on blood pressure and changes in serum K^+ levels (Ji et al., 2003; Mirkovic et al., 2002). However, blood pressure regulation and Na^+ balance appeared normal in heterozygous α-ENaC-knockout mice, in spite of varying salt diets, which is attributable to increased RAAS activity through a compensatory upregulation of AT_1Rs (Wang et al., 2001). The AT_1R antagonist candesartan was found to increase the abundance of ENaC β- and γ-subunits, while decreasing the quantity of ENaC α-subunits, which is the rate-limiting factor for assembly of mature ENaC complexes, in salt-restricted rats (Beutler et al., 2003). In addition, previous studies reported altered expression of renal ENaC subunits in obese rats, as well as rats with chronic heart failure; this was partially reversed by candesartan treatment (Lutken et al., 2009; Madala Halagappa et al., 2008; Zheng et al., 2011).

The association of the ubiquitin ligase Nedd4-2 with the ENaCs, leads to ubiquitination and subsequent removal of the ENaCs from the plasma membrane, which demonstrates that Nedd4-2 is essential to ENaC activity and regulation (Staub et al., 2000; Zhou et al., 2007). Nedd4-2-knockout mice had elevated blood pressure levels, coupled with impaired ENaC activity, which was exaggerated by a high salt diet (Shi et al., 2008); these animals ultimately developed cardiac hypertrophy and systolic dysfunction (Shi et al., 2008).

In the present cohort, we found no statistically significant evidence for association between SNPs in the ENaC α-subunit gene, *SCNN1A*, and LVH as evident from Figure 3.40. However, two *SCNN1A* haplotypes associated with LVM. The most common haplotype, ATTAG, was present in 17.4 % of the cohort and associated significantly with a 15.9 g decrease in LVM. Haplotype 10 (GGTCA), on the other hand, was present in only 1.8 % of the cohort, but was associated with a 36.9 g increase in LVM.

Interestingly, these haplotypes contain different alleles for all the investigated SNPs, except for the rs7973914 T-allele. In addition, a haplotype containing the same alleles as haplotype 1, except for a C-allele at rs7973914, ATCAG, did not associate with LVM. Likewise, a haplotype with a haplotype containing the same alleles as haplotype 10, except for a C-allele at rs7973914, GGCCA, also showed no statistically significant evidence for association. This SNP did, however,

not associate significantly with LVH in the single SNP analyses, which either points to a context-dependent effect of this SNP, or an epistatic effect of all the investigated variants.

We also found a significant association between the G-allele of rs8044970 in *SCNN1B* and a significant decrease of 1.66 mm in mIVST, as well as a significant decrease of 1.28 mm in mLVWT. In addition, four haplotypes were found to associate significantly with hypertrophy traits. Haplotype 5 was present in 5.9% of the cohort and was associated with a 0.083 mm increase in mPWT as shown in Table 3.37. However, this significant association is possibly driven by the effect of this haplotype in R92W$_{TNNT2}$ carriers, as this haplotype had a 1.39 mm increased effect on mPWT in the R92W$_{TNNT2}$ group, compared to the A797T$_{MYH7}$ group (Table 3.36). Haplotype 9 was significantly associated with a 0.94 mm decrease in mPWT, as well as a 1.09 mm decrease in CWT score and a 0.068 decrease in PC1.

Haplotype 19 was found to associate significantly with all the investigated hypertrophy traits except LVM; this haplotype decreased mIVST by 2.80 mm, mLVWT by 2.58 mm, mPWT by 1.22 mm, CWT score by 1.48 mm and PC1 by 0.093. Haplotype 20, on the other hand, resulted in a 3.88 mm increase in mIVST and a 3.63 mm increase in mLVWT. Both these haplotypes then had relatively large effects, which is probably a reflection on their low frequency, and again is consistent with the theory that rarer variants exert greater effects in complex phenotypes.

In contrast to *SCNN1A* and *SCNN1B*, we found no statistically significant evidence for association between *SCNN1G* SNPs, or related haplotypes, and LVH in the present cohort. We did, however, find that three of the four *SCNN1G* had strong differences in effect between the different HCM mutation groups, even though the combined effect did not reach statistical significance, which point to possible context-specific effects.

4.5.8 Na^+/K^+-ATPase subunits

Another vital downstream effector of the MR/aldosterone complex is the Na^+/K^+-ATPase, which is responsible for the exchange of Na^+ and K^+ ions across plasma membranes. This transmembrane protein consists of an α- and a β-subunit (Kaplan, 2002), where the α-subunit confers the catalytic activity of the enzyme and contains binding sites for Na^+, K^+ and ATP (Shull et al., 1985), while the β-subunit modulates the pump function. The β-subunit is also essential for α-subunit expression, as well as for integration of the latter subunit and its stability within the endoplasmic reticulum (Rajasekaran et al., 2005).

Na^+/K^+-ATPase knockout mice also suggest different functions for α- and β-subunits in the heart. Heterozygous Na^+/K^+-ATPase α-knockout mice showed altered contractility (James et al., 1999), while homozygous Na^+/K^+-ATPase β-knockout mice exhibited ventricular hypertrophy (Magyar et al., 1994). Homozygous knockout mice with cardiomyocyte-specific Na^+/K^+-ATPase β-inactivation exhibited mild hypertrophy, coupled with reduced contractility and ventricular function (Barwe et al., 2009).

Previous studies on animal models of cardiac hypertrophy reported altered expression of Na^+/K^+-ATPase α- and β-subunit isoforms in hypertrophied ventricles (Baek and Weiss, 2005; Trouve et al., 2000; Xie et al., 1999; Yamamoto et al., 2009; Zwadlo and Borlak, 2005), while several other studies reported Na^+/K^+-ATPase isoform shifts in cardiac hypertrophy (Charlemagne et al., 1994; Charlemagne and Swynghedauw, 1995; Kim et al., 1994; Zahler et al., 1996).

Research on human failing hearts demonstrated that Na^+/K^+-ATPase isoform expression is altered in failing compared to non-failing human hearts (Muller-Ehmsen et al., 2001; Schwinger et al., 1999; Shamraj et al., 1993). Myocardial biopsies from patients with aortic valve disease demonstrated that myocardial hypertrophy was also associated with a reduction in Na^+/K^+-ATPase concentration (Larsen et al., 1997). Endomyocardial biopsies from patients with impaired cardiac function, furthermore, showed a 40% decrease in total Na^+/K^+-ATPase concentration, while such a decrease in Na^+/K^+-ATPase concentration correlated with a decrease in heart function (Schwinger et al., 2003).

Partial inhibition of the cardiac Na^+/K^+-ATPase with ouabain and other related cardiac glycosides has positive inotropic effects on the myocardium (Akera and Ng, 1991; Huang et al., 1997). Ouabain also induced hypertrophic growth in cultured neonatal rat cardiomyocytes, coupled with increased expression of TGF-β (Huang et al., 1997). The hypertrophic response that follows Na^+/K^+-ATPase inhibition was also associated with p42/44 MAPK and ROS-dependent pathways (Kometiani et al., 1998; Xie et al., 1999).

Later, Fedorova et al. demonstrated that LVH development and subsequent transition to heart failure in Dahl salt-sensitive rats on a high Na^+ diet was associated with sensitivity to ouabain and shifts in left ventricular Na^+/K^+-ATPase isoform composition (Fedorova et al., 2004). In a mouse model of pressure overload hypertrophy, mice overexpressing an ouabain sensitive Na^+/K^+-ATPase α1-subunit showed severe myocardial hypertrophy four weeks after aortic banding, compared to ouabain resistant mice (Wansapura et al., 2011).

In the present study, we investigated the Na^+/K^+-ATPase α1, α2, β1 and β3 subunits as these subunits showed convincing evidence for cardiac expression (Malik et al., 1998; Schwinger et al., 1999; Wang et al., 1996). While we found no significant evidence for association between either of the investigated *ATP1A1* SNPs, viz. rs850609 and rs10924074, and LVH, we found that the combined haplotype of the two minor alleles of these variants resulted in a decrease in mPWT of 1.48 mm, which suggests that the individual effects of these alleles might be too small to detect, and that some form of epistatic interaction exists between these alleles. Interestingly, the T-allele of rs850609 was found to have an increased effect of 1.40 mm on mPWT in the $R92W_{TNNT2}$ group versus the $A797T_{MYH7}$ group, which possibly point to a context-specific effect. These two SNPs are located in introns, with no obvious functional effects, and were in complete LD with each other in the present cohort, so it is therefore not possible to pinpoint the location of the variant responsible for the positive association from these results alone.

As with *ATP1A1*, we also found no association between the investigated *ATP1A2* variants and LVH when considered alone, whereas two haplotypes showed significant evidence for association. The relatively common AAA haplotype, which is compiled of all three major alleles, resulted in an 11.1 g decrease in LVM, as well as a 0.97 mm decrease in mIVST. On the other hand, the rarer haplotype of all three minor alleles, TGG, resulted in a 44.1 g increase in LVM. As all three major or minor alleles are required to have a statistically significant effect on LVM, this might point towards weak individual, but stronger compound, SNP effects.

Contrary to these genes, we found significant evidence for association with *ATP1B1* SNPs and haplotypes. When considered alone, the T-allele of rs1200130 was associated with a 12.5g increase in LVM, a 0.39 mm increase in mPWT, a 0.38 mm increase in CWT score, as well as a 0.024 mm increase in PC1. The effect of this allele was, however, not statistically significant when combined with the other two investigated SNPs, except for within the TAG haplotype that was associated with a 0.83 mm increase in mPWT. On the other hand, the combined haplotype of all three major alleles, CGG, which was present in 14.5% of the cohort, associated significantly with a 20.2 g decrease in LVM.

The investigated *ATP1B3* polymorphism had strikingly different effects between the different mutation groups, although the average effect did not amount to statistical significance. This strongly points to context-specific effects, but it is still unsure at this stage if this variant truly significantly affects LVH development.

4.6 Optimal selection

Previous studies found that increased LVH correlated with an increased risk for cardiovascular morbidity and mortality (De Simone et al., 2009; Gupta et al., 2010; Lai et al., 2011; Okin, 2009; Verma et al., 2008); most noted is the Framingham LVH risk scores for cardiovascular outcome prediction (Pencina et al., 2009). It is, therefore, imperative to identify factors that confer an increased risk for LVH development before its occurrence. Risk prediction with the use of genetic markers has become quite attractive as constantly improving genotyping technologies offer the promise of relatively quick and inexpensive clinical tools. In addition, the outcomes of single gene- and genome-wide association studies have greatly expanded risk prediction from a handful of mutations with severe effects to a multitude of variants with modest individual effects.

There is, however, some grounds for caution here, as genetic risk prediction has had some success in some diseases (such as breast cancer and type 2 diabetes), but not all (for example, Crohn's disease) (Jostins and Barrett, 2011). This can be attributed to a number of factors such as inadequate adjustment for relevant confounders, weak individual effects, as well as allelic and locus heterogeneity of risk variants across populations (Jostins and Barrett, 2011). Genetic risk prediction in a complex phenotype such as HCM LVH is even further complicated by the individual causal mutations that each confers somewhat different cardiac phenotypes.

Investigating more than one gene in a pathway provides certain benefits over single variant analyses, especially when individual effects are relatively weak (Cordell and Clayton, 2005; Delles et al., 2008). By considering the compound effect of a number of variants in a pathway, we can evaluate greater effect sizes, which enhance risk stratification, while also taking epistatic effects into account. This is particularly relevant in the RAAS, as this system is comprised of a complex interplay between a number of contributing factors, while single variant analyses might oversimplify RAAS involvement in cardiovascular phenotypes.

Ortlepp et al. investigated a set of five polymorphisms, collectively termed the "pro-LVH" polymorphisms, which was previously suggested to influence the HCM phenotype, in 26 individuals with HCM from a single family with the same HCM-causal mutation (Ortlepp et al., 2002). This included the *ACE* I/D, *AGT* M235T, *AGTR1* A1166C, *CYP11B2* -344T/C and *CMA* -1903 G/A polymorphisms. After adjustment for age, sex and the presence of hypertension, this group found that each of the five polymorphisms associated significantly with LVM. In addition, they found a direct correlation between the number of pro-LVH" polymorphisms present in an individual and the degree of LVH (Ortlepp et al., 2002). This group could, however, not test

whether each variant was associated with LVM independent of the other "pro-LVH" polymorphisms due to the small sample size of the cohort.

Later, Perkins et al. assessed the effect of the *ACE* I/D variant, as well as a composite "pro-LVH" score (0-5), derived from the presence or absence of the five "pro-LVH" variants as a proxy for the pooled effect of these variants, in 389 unrelated HCM patients (Perkins et al., 2005). They found a significant association between left ventricular wall thickness and the *ACE* DD genotype in the patient subset with *MYBPC3* HCM mutations, but not in patients with *MYH7* or as yet unidentified HCM-causal mutations. In addition, they only found significant evidence for association between the "pro-LVH" score and left ventricular wall thickness in the subgroup of patients with as yet unidentified HCM mutations (Perkins et al., 2005).

More recently, Kaufman et al. investigated the effect of these polymorphisms on LVH in a paediatric HCM cohort (n = 65) (Kaufman et al., 2007). They found a positive correlation between the number of "pro-LVH" genotypes and an increase in LVM, independent of age, sex, race and a positive family history of HCM. At baseline, they found a higher LVM_{index} in patients with two or more "pro-LVH" genotypes. In a follow-up investigation on 40 of these children after a median of 1.5 years, they found that patients with two or more "pro-LVH" genotypes had an increased LVM_{index}, as well as interventricular septal thickness (Kaufman et al., 2007).

These studies therefore show that the collective effect of a number of RAAS polymorphisms might be a better predictor of LVH than single polymorphisms, but the five variants used in these studies might not be adequate or even effective in LVH prediction across all ethnicities and cohorts. In fact, previous studies have shown that the utility of LVH risk prediction scores can vary considerably between different ethnic populations (Lai et al., 2011; Riddell et al., 2010).

In the present study we did not find any statistically significant evidence for association between the five "pro-LVH" variants and LVH. We therefore used stepwise selection to identify a subset of variants that served as better LVH predictors in the present cohort. To this end, we initially identified a set of 12 RAAS variants that associated significantly with at least one hypertrophy trait in the present cohort, which is the same criterion for inclusion used in the original Ortlepp study. Thereafter, we further reduced this subset of variants to a set of nine markers that predicted an increase in at least one of the four selected hypertrophy indices, viz. LVM, mIVST, mLVWT and PC1, independent of known hypertrophy covariates and the other significant variants.

We found that our set of "pro-LVH" polymorphisms had far superior predictive value for LVH in the present cohort. If an individual were to possess the risk alleles for all nine of these polymorphisms, we would expect a 127.80 g increase in LVM, as well as a 13.97 mm increase in mIVST, a 14.67 mm increase in mLVWT, as well as a 7.85 increase in PC1. This is in stark contrast to the combined effect of all five "pro-LVH" polymorphisms, which resulted in only a 27.40 g increased LVM, a 2.15 mm increased mIVST, a 2.22 mm increased mLVWT, as well as a 1.49 increase in PC1.

There is, however, a very slim chance that an individual would possess all nine of these alleles (or even all five of the "pro-LVH" risk alleles) and we did not observe such an individual in the present cohort. It is, furthermore, perhaps to be expected we would observe larger effects as we used four more polymorphisms than the previous studies. Even so, we find that our alleles had a greater average effect per variant in our cohort, compared to the average effect of the "pro-LVH" variants, which is indicative of a better predictive value per variant. The average effect per allele on LVM was 14.20 g for our set of polymorphisms, whereas the average "pro-LVH" allele predicted a 5.50 g increase on LVM. Moreover, the average allele in our set of polymorphisms predicted an increase of 1.55 mm, 1.63 mm and 0.87 in mIVST, mLVWT and PC1, respectively. However, the average "pro-LVH" allele only predicted increases of 0.43 mm, 0.44 mm and 0.30 in mIVST, mLVWT and PC1, respectively.

It is, however, important to note here that our set of alleles might not be an accurate predictor of hypertrophy development in a different HCM population. There might be a number of equally valid sets of "pro-LVH" polymorphisms, each unique to a particular population. The set of polymorphisms that impact significantly on hypertrophy development in a given population might be influenced by the LD structure in that population, as well as the molecular mechanisms that contribute to LVH development in that particular population, where we expect some mechanisms to overlap between populations, while others could be unique to a particular population.

4.7 Closing thoughts and future directions

Instead of merely evaluating the effect of the presence or absence of certain alleles, we have provided a quantitative measure of their effect, which we feel will be more suited to risk prediction as an allele can be significantly associated with a particular disease trait, while only exerting a comparatively modest effect on that particular phenotype, as is frequently the case in complex phenotypes.

As a whole, we also found that significant effects were not restricted to the commonly studied genes in the more proximal part of the RAAS, and that the MR and downstream RAAS effector genes, such as the *ATP1B1* gene, can also provide significant insight into the future risk of LVH development.

Suffice to say, these variants are also in no manner linked to LVH in a simple, deterministic way. There are a multitude of environmental factors and other pharmaceutical or therapeutic interventions that could impact on the eventual cardiac phenotype of an individual. It is at this stage likely that there will be multiple and equally valid sets of "pro-LVH" variants within the RAAS and other pathways across different population groups. Moreover, previous studies demonstrated that sex, height, BMI and systolic blood pressure conferred a strong independent risk for increased LVM (Meijs et al., 2010) and there is consequently a clear need for adequate adjustment for known hypertrophy confounders, such as the primary disease-causing mutation in HCM, to filter the true risk contributors from those with relatively little or no predictive value on LVH development in a particular population.

While our set of polymorphisms serves as a relevant and accurate predictor of LVH in this cohort, this might not be the case in a different ethnic background and it is therefore vital to take this into account when designing future studies. Also, risk profiles might be different for different LVH indices as evident from the present study, where we found that certain RAAS allele subsets had larger effects on LVM than mLVWT, for instance. Clarification on the best echocardiographic measure for use in genetic association studies as an indicator of cardiovascular morbidity and mortality risk in HCM will provide a standard against which, as well as a context within which, these studies can be evaluated to educate future study designs aimed at identifying LVH risk variants in order to maximise their applicability and use.

Research such as the present rather provides the basis on which future studies can build improved risk profiles for LVH development within the context of HCM and ultimately in all patients with a risk of cardiac hypertrophy. Stepwise selection provides a valuable tool with which one can evaluate a set of loci, while adjusting for non-genetic covariates or the effects of other investigated markers, allowing researchers to screen for a subset of markers that best predicts the variability in LVH (Schaid, 2004). As a caveat, we did not use haplotypes in our stepwise selection analyses as we are not able estimate haplotypes for the X-linked genes at this stage. However, haplotype-based predictive models may provide advantages over single-SNP

approaches by simultaneously taking epistatic effects into account and facilitating the detection of effects driven by cis-interactions among nearby SNPs (Kang et al., 2011).

4.8 Conclusion

In the present study we identified a number of RAAS variants that had significant effects on HCM hypertrophy, whether alone or within the context of a multi-variant haplotype. Our results are consistent with previous studies that reported relatively modest effects of RAAS variants in cardiac hypertrophy. We add to these findings by reporting generally larger effects with RAAS gene haplotypes, than with single polymorphisms, while the largest of these effects seemed to be found with rarer variants, which is perhaps not surprising considering the general trend in the contribution of genetic variation to complex phenotypes (Frazer et al., 2009).

The present study should be viewed as "hypothesis generating" and variants identified here as plausible hypertrophy modifiers serve as a valuable starting point for future research. Moreover, our findings suggest that the eventual hypertrophic phenotype of HCM is indeed modulated by the compound effect of a number of RAAS modifier loci, where each polymorphism makes a modest contribution towards the eventual phenotype. However, RAAS involvement in HCM hypertrophy still has several partially answered, or even unanswered, questions. For now, HCM modifier studies appear to have converged on a very limited list of "pro-LVH" polymorphisms in the proximal part of the RAAS, while the contribution of additional RAAS modifiers of the HCM hypertrophic phenotype has received little attention to date. Our findings suggest that genetic variation in renin and renin-associated genes, as well as the MR and downstream RAAS effectors such as the Na^+/K^+-ATPase and ENaCs, also has plausible hypertrophic effects in HCM. This is the first RAAS investigation, to our knowledge, to provide clear quantitative effects for a subset of RAAS variants indicative of a risk for LVH development that are representative of the entire pathway.

The ultimate aim in research projects such as the present is to identify effective targets for antihypertrophic therapies and to build an accurate, population-specific genetic risk profile for use as a clinical tool to assist in HCM risk stratification and treatment. A position of strength in this regard will essentially rely on the successful integration of knowledge gained from a range of approaches, including animal models, clinical trials, as well as carefully designed and properly executed genetic association studies. For now, the primary challenge will be to understand and appreciate the complexity of the HCM hypertrophic phenotype and to develop studies and analysis methods that increase the likelihood of identifying real effects, while

minimizing false positives, with recognition for the inherent shortcomings of these particular approaches.

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APPENDIX I

PAGE PROOFS FOR CHAPTER IN ANGIOTENIN: NEW RESEARCH (SEE FOOTNOTE 1)

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APPENDIX II

SOLUTIONS AND BUFFERS

1. DNA EXTRACTION SOLUTIONS

Cell lysis buffer

Sucrose	0.32 M
Triton-X-100	1%
$MgCl_2$	5 mM
Tris-HCl	10 mM
H_2O	1 l

3 M Sodium Acetate

Sodium Acetate (Merck (Pty) Ltd, RSA)	40.81 g
H_2O	50 ml

Adjust pH to 5.2 with glacial acetic acid (Merck (Pty) Ltd, RSA) and adjust volume to 100 ml with ddH2O

Na-EDTA solution

NaCl (Merck (Pty) Ltd, RSA)	18.75 ml of 4 mM stock solution
EDTA (B & M Scientific)	250 ml of 100 mM stock solution

Mix well

Phenol/Chloroform

Phenol (saturated with 1x TE) (Merck (Pty) Ltd, RSA)	50 ml
Chloroform (Merck (Pty) Ltd, RSA)	48 ml
8-hydroxyquinone (Merck (Pty) Ltd, RSA)	2 ml

Mix well, store at 4˚C

Chloroform/octanol (24:1)

Chloroform (Merck (Pty) Ltd, RSA)	96 ml
Octanol (Merck (Pty) Ltd, RSA)	4 ml
Mix well, store at 4˚C	

TE-buffer (10x stock solution)

TrisOH	0.1 M (pH 8.00)
EDTA	0.01 M (pH 8.00)
H_2O	150 ml
Mix well	

2. ELECTROPHORESIS STOCK SOLUTIONS

SB Buffer (20x stock)

Di-sodium tetraborate decahydrate	38.137g/mol
Add ddH_2O to a final volume of 1 l	

Bromophenol blue

Bromophenol blue (Merck (Pty) Ltd, RSA)	0.2 % (w/v)
Glycerol	50%
Tris (pH 8.00)	10 mM

Ethidium Bromide

Ethidium Bromide	500 mg
ddH_2O	50 ml

APPENDIX III

PUBLICATION OF SINGLE SNP ASSOCIATION RESULTS FOR AGTR2

http://www2.jraas.com/content/12/3/274.refs