THE INFLUENCE OF *ACE* (I/D) POLYMORPHISM IN CARDIOVASCULAR DISEASE

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A thesis submitted in accordance with the regulations of the University of London for the degree of MD (Res)

April 2008

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To my grandparents, parents and sister

DECLARATION

All the work presented in this thesis is my own, or has been carried out as part of a collaboration in which I played a major part. All collaborative work has been acknowledged as follows:

1) Genomic DNA from participants of HIFMECH, NPHSII and HCM studies had been isolated previously by co-workers in the Centre for Cardiovascular Genetics laboratory, UCL.

2) *ACE* I/D genotypes were obtained in NPHSII by Jutta Palmen (Technician) and in the HCM cohort by Kah Wah Li (Technician).

3) The majority of the statistical analysis was performed by Jackie Cooper, statistician within the Centre for Cardiovascular Genetics.

4) ECG, echocardiography and cardiopulmonary exercise testing for the HCM study was carried out by cardiac physiologists at St George's Hospital, London, and Heart Hospital, London.

No part of this thesis has been submitted for a degree, diploma or other qualification at any other University.

Amal Muthumala

ACKNOWLEDGEMENT

I would like to thank both my supervisors:

Professor Steve Humphries, for providing me with the opportunity to pursue a higher degree in science and his assistance, support, guidance and enthusiasm throughout my research.

Dr. Perry Elliott, for allowing me the chance to work at the Heart Hospital, and to gain an important insight into the management of patients with cardiomyopathy and heart failure. I would also like to thank him for his continuous encouragement and guidance both in clinical medicine and research.

I would like to thank all members of the CVG laboratory and the cardiomyopathy service at the Heart Hospital throughout my time as a research fellow. In particular, I would like to thank Jasmine Matin and Ka Wah Li for guidance in laboratory techniques. I would also like to thank David Gable, Andrew Smith, Andy Thompson, Fotios Drenos and Birgit Dorfmeister from the CVG, and Rami Harb, Sanjay Kohli and Steve Page from the Heart Hospital, for their support.

The financial support of the British Heart Foundation and Shire Human Genetic Therapies is gratefully acknowledged.

I would like to thank my friends for their help and encouragement. Thankyou finally to my parents and my sister for always being there for me.

Publications arising from the work in this Thesis

Muthumala A, Gable DR, Palmen J, Cooper JA, Stephens JW, Miller GJ, Humphries SE. Is the influence of variation in the ACE gene on the prospective risk of type 2 diabetes in middle aged men modified by obesity? *Clin Sci (Lond). 2007 Dec;113(12):467-72.*

Muthumala A, Montgomery H, Palmen J, Cooper JA, Humphries SE. Angiotensin-Converting Enzyme Genotype Interacts With Systolic Blood Pressure to Determine Coronary Heart Disease Risk in Healthy Middle-Aged Men. *Hypertension. 2007 Aug;50(2):348-53.*

Muthumala A, Cooper J, Humphries SE on behalf of the HIFMECH Study Group. European differences in the association between ACE I/D polymorphism and incidence of MI may be explained by gene-lipid interaction. *Atherosclerosis. 2006 Dec;189(2):474-7.*

Abstract

The renin-angiotensin system (RAS) is involved in maintenance of cardiovascular function and has been implicated in coronary heart disease (CHD) and type 2 diabetes (T2D). Pharmacological inhibition of the RAS improves cardiovascular disease outcomes. Angiotensin Converting Enzyme (principal component of RAS) levels are significantly associated with the *ACE*(I/D) polymorphism. Large studies have demonstrated a mild effect of this polymorphism on CHD risk. However the presence of RAS in diverse tissues implicated in cardiovascular diseases justify the hypothesis that genetic polymorphisms encoding proteins in this system 'modify' the risk of such diseases in the context of harmful stimuli or affect disease progression.

This thesis therefore tested the hypothesis that the *ACE*(I/D) polymorphism 'modifies' risk of CHD and T2D, and affects progression of Hypertrophic Cardiomyopathy (HCM). Using a study of 532 myocardial infarction (MI) survivors and 574 controls (HIFMECH), an interaction between the *ACE* polymorphism and lipid levels on MI risk in Northern compared to Southern Europeans is demonstrated: the MI odds with high ApoB levels in D allele carriers was much greater in the North than the South. In a prospective cohort of 3052 healthy men (NPHS2), an interaction between *ACE* and systolic blood pressure (SBP) on CHD risk is presented where D allele carriers were protected against CHD at lower SBP, but at higher SBP were more susceptible. In the same study, *ACE* variants modified the risk of T2D in obese individuals, with obese D allele carriers having a substantially higher T2D risk compared to obese II homozygotes. From a retrospective study of 541 patients with HCM a possible effect of the polymorphism on left ventricular remodelling is demonstrated.

Thus the work in this thesis offers strong evidence that the *ACE* gene modifies risk of CHD and T2D in the presence of known risk factors.

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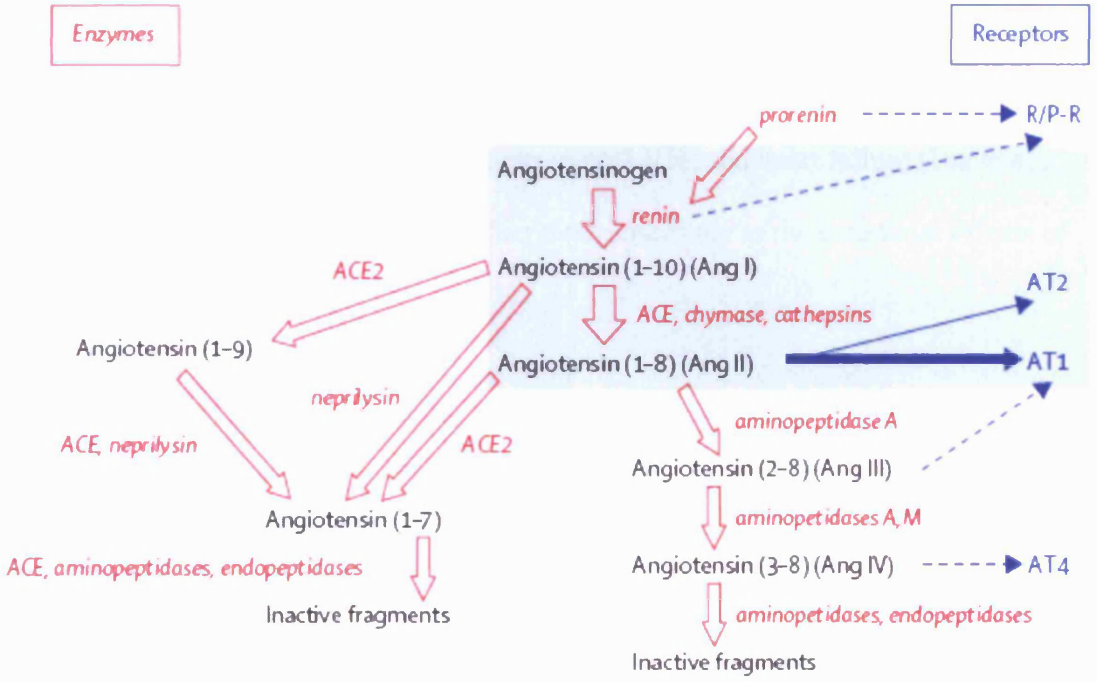
Renin Angiotensin System (RAS)

Renin was first identified by Tigerstedt and Bergman at the end of the 19th Century. It was subsequently shown to be a protease, converting the α_2 globulin, angiotensinogen to the decapeptide, angiotensin I (Ang I). The function of endocrine RAS was initially thought to be only maintenance of cardiovascular homeostasis. It has since become clear that local tissue RAS also exist, where they have paracrine, autocrine and intracellular roles. Circulating ACE contributes less than 10% of total body ACE, with the largest proportion of ACE located in tissue compartments (Cushman and Cheung, 1971). RAS components have been found in various tissues including cardiac (Danser, 1996), vascular endothelium (Dzau, 1993), brain (Hilbers et al., 1999), lung (Pieruzzi et al., 1995), kidney (Harris and Cheng, 1996), adipose tissue (Schling et al., 1999), pancreas (Sernia, 2001) and skeletal muscle (Reneland and Lithell, 1994).

Angiotensin I Converting Enzyme (ACE) (158 kDa) plays a key role in the RAS through acting as a dipeptidyl carboxypeptidase. By removing the C-terminal dipeptide, it converts Ang I (decapeptide) to Ang II (octapeptide) and inactivates bradykinin (BK). ACE can exist in two forms: a somatic form containing two homologous zinc-binding catalytic domains, and a truncated testicular form containing only the C-terminal catalytic domain necessary for fertility. ACE is anchored to the plasma membrane of endothelial or epithelial cells by its C-terminus, which is subsequently cleaved by ACE secretase to yield circulating ACE (Wei et al., 1991). The complexity of RAS has increased through the discovery of ACE2 (homologue of ACE) which converts Ang I to

inactive Ang(1-9) and converts Ang II to vasodilator Ang(1-7) (Vickers et al., 2002). Figure 1.1 is a diagram outlining the RAS.

Figure 1.1 (from (Schmieder et al., 2007))



Ang II acts via at least two different types of G-protein-coupled receptors – the Ang II type 1 (AT_1R) and type 2 (AT_2R) receptors. The systemic function of the RAS acting via AT_1R is to maintain cardiovascular homeostasis. It is activated by the sympathetic nervous system, volume loss and salt loss. Persistent activation of RAS however causes oxidative stress, inflammation, hypertrophy and fibrosis affecting vascular smooth muscle cells, endothelial cells, cardiac myocytes and the extracellular matrix (reviewed in (Mehta and Griendling, 2007)). As well as affecting the cardiovascular system, the presence of local RAS can cause fibrosis in lung (Marshall et al., 2000) and kidney (Mezzano et al., 2001), and inflammation in adipose tissue (Engeli et al., 2000). The AT_2R is thought to oppose the actions of the AT_1R (Matsubara, 1998). The detrimental role of ACE in cardiovascular disease is highlighted by the evidence that ACE

inhibitors have been shown to improve morbidity and mortality in cardiovascular disease and heart failure (Yusuf et al., 2000; SOLVD Investigators, 1991).

The RAS is intimately connected to the Kallikrein-Kinin system. Bradykinin (inactivated by ACE) act upon β_1 and β_2 G-protein coupled cell-surface receptors (termed BDKRB1 and BDKRB2, respectively). Kinins cause vasodilatation (via nitric oxide and prostaglandin release) and are thought to be protective against atherosclerosis (Su et al., 2000), and left ventricular hypertrophy (LVH) and heart failure (Liu et al., 1997). Reduced kinin degradation may therefore contribute to the beneficial effects of ACE inhibition (Linz et al., 1995).

ACE (I/D) polymorphism

The *ACE* gene (Figure 1.2) located on the long arm of chromosome 17 (17q23), is 21kb long and contains 26 exons (Sayed-Tabatabaei et al., 2006). The human *ACE* gene has close homology with a wide range of other species, and its structure may be the result of gene duplication (Hubert et al., 1991). The NCBI database lists more than 160 polymorphisms, the majority being single nucleotide polymorphisms (SNP). 34 are in coding regions of which 18 lead to missense mutations (Sayed-Tabatabaei et al., 2006). Transcription of testicular ACE mRNA differs from the somatic ACE mRNA in that somatic form is transcribed from the promoter on the 5` end (leading to transcription of all exons) and the testicular form is transcribed from an internal promoter in intron 12 (Howard et al., 1990).

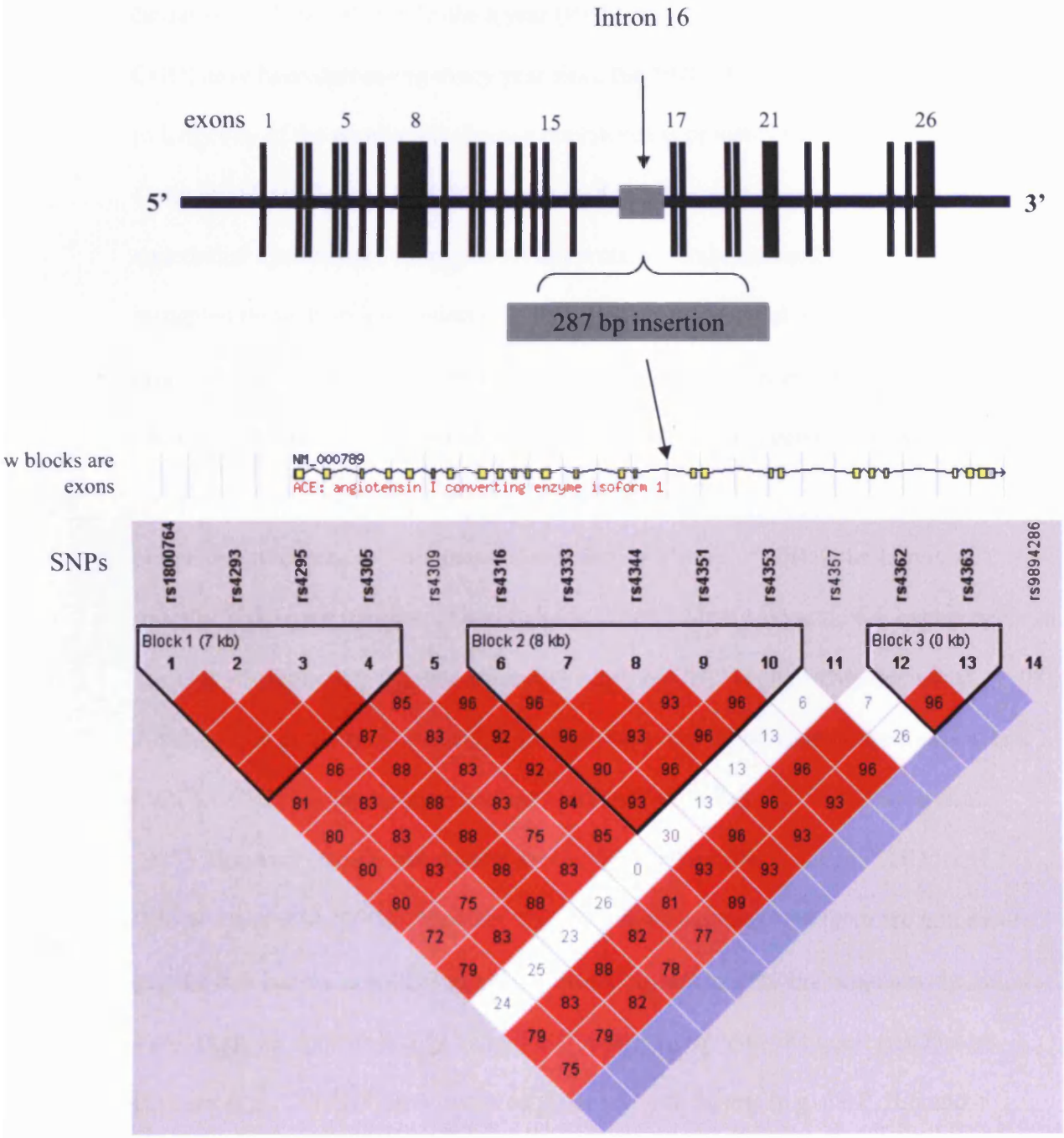
ACE levels are under considerable variation between individuals, with evidence of strong heritability (Cambien et al., 1988). In 1990 a common variant in *ACE* (Figure

1.2) - the insertion (I) or deletion (D) of a 287 bp fragment (Alu repeat) in intron 16 was shown to be associated with differing serum and tissue ACE activity, with the insertion associated with lower levels (Rigat.B. et al., 1990). Studies have demonstrated that the polymorphism is responsible for 18% to 47% of variance of tissue and plasma ACE levels in western Caucasian populations (Danser et al., 1995; Costerousse et al., 1993; Rigat.B. et al., 1990). This association is ethnic specific, being replicated in Japanese (Yamamoto et al., 1997) and Mexican American cohorts (Kammerer et al., 2004) but with more modest effects reported in black cohorts (Forrester et al., 1997; Cox et al., 2002). In non-black populations, the *ACE* (I/D) polymorphism is most probably in strong linkage disequilibrium (LD – term explained later) with a functional polymorphism directly responsible for genetic variation of ACE levels.

There has been an intensive work to find the functional polymorphism. The presence of two possible ACE determining quantative trait loci has been suggested with one being in strong LD with the I/D polymorphism and the other in the 5' region (Villard et al., 1996). In view of the close LD operating over the chromosomal region where the gene is located, ACE activity has been associated with a small number of haplotypes that exist within the gene (Keavney et al., 1998). Despite all the efforts, the precise functional polymorphism is yet to be determined. It is most likely in the region between intron 18 and 3'UTR (Zhu et al., 2000) however there remains evidence for a contribution from 5'UTR region (Villard et al., 1996) and possibly a trans element on chromosome 4 (Kammerer et al., 2004). Regardless, in caucasians, *ACE*(I/D) polymorphism is in strong LD with the majority of polymorphisms in the putative region (Payne et al., 2007) and remains a useful marker of ACE levels, and therefore can be used as a genetic tool to explore disease processes in which RAS has been

implicated. AngII levels have not been used in evaluating the *ACE*(I/D) polymorphism in view of the difficulties of measuring it.

Figure 1.2 Schematic Representation of *ACE* (not to scale), with reference to I/D polymorphism and a LD plot demonstrating the strong LD within the gene. Darker boxes represent stronger LD. The D' (term explained later) for any two SNPs is presented in the box representing their intersection. No number indicates a D' of 1 i.e 100% LD



Coronary Heart Disease

Cardiovascular disease (CVD) remains the largest cause of death in the industrialised world, with increasing prevalence in developing countries adopting western lifestyles (Zipes and Libby P et al, 2005). In the UK it is responsible for more than 200,000 deaths a year, and coronary heart disease (CHD) – itself the largest cause of death, causes more than 100,000 deaths a year (BHF, 2007). Death rates from CVD (and CHD) have been decreasing every year since the 1970s, however in view of the increase in longevity of the population, disease prevalence is probably increasing (BHF, 2007). Coronary Heart Disease (CHD) is a progressive inflammatory disorder involving endothelial dysfunction, dysregulated lipoprotein metabolism and coagulation which is instigated through an interaction of genetic and environmental risk factors. The principal modifiable risk factors are cigarette smoking, hypertension, type 2 diabetes and dyslipidaemia. Non-modifiable risk factors include age, gender and family history.

Numerous studies have demonstrated that family history of CHD is an important vascular risk factor (Goldbourt and Neufeld, 1986). Most premature CV events occur in families where there is a positive family history of CHD (Pohjola-Sintonen et al., 1998). A substantial proportion of this influence is due to the inheritance of the classical risk traits for CHD as demonstrated in twin studies (Feinleib et al., 1977; Austin et al., 1987). However, family history is a strong independent risk factor for CHD itself (Lloyd-Jones et al., 2004) (Hawe et al., 2003) demonstrating that there are unmeasured genetic risk factors as well. Supporting this is the fact that the Framingham algorithm cannot explain approximately 1 in 5 CHD events using 'conventional' risk factors (Cooper et al., 2005). Genes involved in 'novel' risk factors (e.g. CRP, IL6 and fibrinogen), working through CHD mechanisms such as inflammation and innate

immunity, may be responsible for family history of CHD. Though now there is less consensus for CRP and inflammatory markers being independent CHD risk factors (Casas et al., 2006b), so genes in alternative plausible pathways such as cell senescence or those yet to be discovered may be the cause for family history being an independent risk factor.

Gene-association studies in CHD

One powerful way to identify culprit genes in complex diseases like CHD is through knowledge of the underlying biological pathways that underpin the disease. Specific genes that are known to code for enzymes or structural proteins that are in these pathways (which lead to the formation of causal traits e.g elevated cholesterol or higher blood pressure or a pro-inflammatory state) are therefore ideal 'candidate genes'. Common variants within these genes which are associated with a change in function of protein or altered trait level can then be typed (using standard molecular techniques) in a cohort of cases and controls. If the frequencies differ statistically significantly between these two groups then the variant is associated with the phenotype in question.

Over the last 20 years there have been vast numbers of gene association studies – over 3500 in CHD alone (Casas et al., 2006a). Though many have reported a positive association between genotype and phenotype, only a minority of these associations have been consistently replicated. There are several reasons for this (reviewed in (Hirschhorn et al., 2002) and (Colhoun et al., 2003) - a common one being that the underlying genetic effect is mild to modest at most, therefore if the cohort is small to medium sized, it may be underpowered to detect an effect with a high degree of statistical

certainty. Frequently the initial positive study also overestimates the initial effect (the so-called 'winners curse'), therefore subsequent studies need even more subjects for confirmation of a significant result.

Another reason why initial results may be a false positive is because of the publication bias that exists toward positive association studies, and those which refute associations tend not to be published. A further problem is that some study designs include subjects from different ethnicities; this can introduce error because both the allele and phenotype in question may vary in frequency or prevalence by ethnicity.

In a substantial proportion of cases, the candidate polymorphism genotyped is not itself functional but is in LD with the functional polymorphism. LD or allelic association, is a population-based concept describing a situation in which a certain allele at one locus occurs in the presence of a specific allele at a second locus more frequently than expected by chance alone. Thus the candidate variant can act as a marker for the functional variant. It is usually measured as D' ranging from 0 – no association between the variants to 1 – 100% segregation of both variants together. Therefore errors can be introduced if the candidate variant is not in strong LD with the functional variant, or if there is ethnic admixture where the LD between candidate and functional variant changes.

Complex diseases are the product of several genes and environmental factors interacting together and therefore an observed gene association study which is a consequence of interaction with the genetic milieu and environmental setting of the particular cohort, will only have a reduced chance of replication in another cohort unless the studies are exactly similar. A related problem is that in seeking these interactions, multiple testing

increases the likelihood of false-positives. Individual SNP association studies also do not capture the full variation across the gene in interest. Tagging SNPs that combine SNPs from those that are in close LD is one method of accomplishing this (Wootton et al., 2006) however there are specific disadvantages with this, including reliance of strong LD across specified region, that the phenotype should be determined by many common genetic variants (and not rare ones), and the subsequent determination of functionality from the positively associated SNPs.

From these criticisms have come guidance for doing gene association studies – ideally they should be very large (in the order of more than a thousand subjects) in order to pick up mild effects when the rare allele frequency is in the order of at least 10% (Casas et al., 2006a). Another important suggestion is that meta-analysis should be used to pool the results of association studies (thereby increasing the power) in order to demonstrate mild to moderate effects with good statistical power. Meta-analyses that include from upwards of 2800 subjects have demonstrated CHD risk effects of 11 genotypes with a magnitude between 1.1 to 1.3 (Casas et al., 2006a). An important criticism of meta-analyses is that they continue the problem of positive publication bias but the pooling of only large studies is a substantial advantage. To reduce problems from ethnic admixture and confounding from differing gene-environment and gene-gene interactions the cohort should ideally be homogeneous with the same ethnicity and same gender.

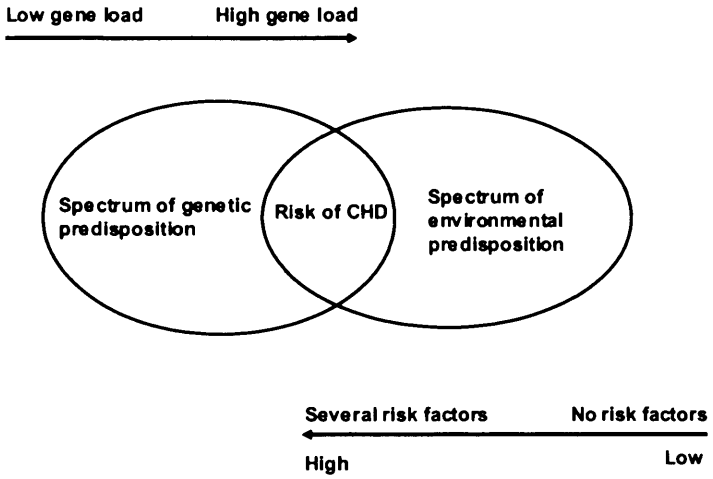
Most studies are cross-sectional which are easier to perform and have the advantage of recruiting large numbers of cases as well as controls. Prospective gene association studies have several advantages in preventing confounding from survivor bias and retrospective patient recall of lifestyle factors. Through also enabling environmental factors to be measured at baseline, important gene-environment interactions can be

explored. Problems with this type of study include the cost and logistics of follow-up, together with the need for even larger numbers and substantial follow up required to achieve statistically significant associations in view of event rate in diseases like CHD being relatively low (Casas et al., 2006a).

Gene environment interaction (GEI)

Gene environment interactions is a concept that is being increasingly used to examine and explain the effect of plausible disease causing or disease modifying genes. In the presence of a harmful stimulus e.g smoking or hypertension a gene variant (or its product) would increase the risk of disease e.g CHD however in the absence of this stimulus, possession of the gene variant leads to no increased risk of disease. Alternatively the disease risk comparison between the two alleles /genotypes varies by the presence of the stimulus. This is biologically conceivable in that pervasive environmental factors like smoking and hypertension switch on (or modify) different genes (or gene clusters) at a transcriptional or at a translational level altering function at a cellular and tissue level (Spira et al., 2004)(Grayson et al., 2007). In view of the complexity of polyphenotypes involving interactions between several risk 'genes' and several environmental risk 'factors', it is convenient to envisage a scenario when an individual with a higher 'genetic' risk enters a high risk environment, leading to a substantially increased disease risk (Figure 1.3).

Figure 1.3 (from (Talmud, 2007))



Examples of Gene environment interaction (GEI) on CHD risk

In view of the difficulty of determining GEI (as explained above), so far only a small number of such associations has been found for CHD. Several have been determined in the Northwick Park Heart Study2 (NPHS2) which is a prospective study of 3052 healthy middle aged men followed since 1990 looking at development of CHD (Miller et al., 1995). Examples which have been found so far using this study have been included in Table 1.1.

Table 1.1 Results of gene environment interaction studies on CHD in NPHS-II (adapted from Casas 2006)

Gene	Variant[1]	Genetic model	Main genetic RR (95%CI)	Main RR (95%CI) of smoking or hypertension	RR (95%CI) in smokers or hypertensives with risk genotype[4]	Candidate System	Reference
Smoking							
LPL	D9**N**	N9 vs D9	2.33 (1.08-5.03)	1.89 (1.28 – 2.79)[2]	10.38 (4.72-22.81)	Lipid levels. Plaque foam cell development	(Talmud et al., 2000)
APOE	E3/E2/**E4**	E4 vs E3	0.82 (0.59-1.15)	1.73 (1.10-2.73)[2]	2.79 (1.59 - 4.91)	Levels of atherogenic lipoprotein	(Humphries et al., 2001)
MMP3	**5A**>6A	6A vs 5A	1.91 (1.11-3.28)	1.99 (1.30 – 3.06)[2]	4.01 (1.57-10.24)	Plaque matrix deposition and plaque receptor	(Humphries et al., 2002)
PECAM	**R**670G	RR vs GG	1.07 (0.75-1.94)	1.65 (1.15-2.35)[2]	2.16 (1.16 – 4.01)	Monocyte ingress into plaque	(Elrayess et al., 2004)
Hypertension							
AGT2R	1657**A**>G	A vs G	1.0 (0.75 –1.34)	2.08 (1.51-2.88)[3]	2.30 (1.49 – 3.55)	Inflammation and vascular function + Cardiac growth in response to hypertension	(Jones et al., 2003)
BDKRB1	-699**G**>C	GG vs CC+GC	1.0 (0.66-1.51)	2.04 (1.44-2.89)[3]	1.88 (1.11 –3.19)		(Dhamrait et al., 2003)
BDKRB2	**+9**/-9bp	+9+9 vs -9-9	0.75 (0.32-1.75)	1.68 (0.86-3.29)[3]	2.21 (0.86-5.70)		(Dhamrait et al., 2003)
LPL	S447**X**	SX+XX vs SS	1.06 (0.76-1.48)	1.35 (1.19-1.53)[3]	1.83 (1.20-2.77)	Lipid levels. Substrate delivery	(Talmud et al., 2007)

1: Risk allele shown in bold. 2: Relative risk of smoking on CHD. 3: Relative risk of hypertension on CHD. 4: Relative risk of CHD in subjects carrying the candidate gene-variant and the environmental risk factor (smoking or hypertension) compared with subject who do not carry the gene-variant and the environmental risk factor

Gene–smoking interaction on CHD risk

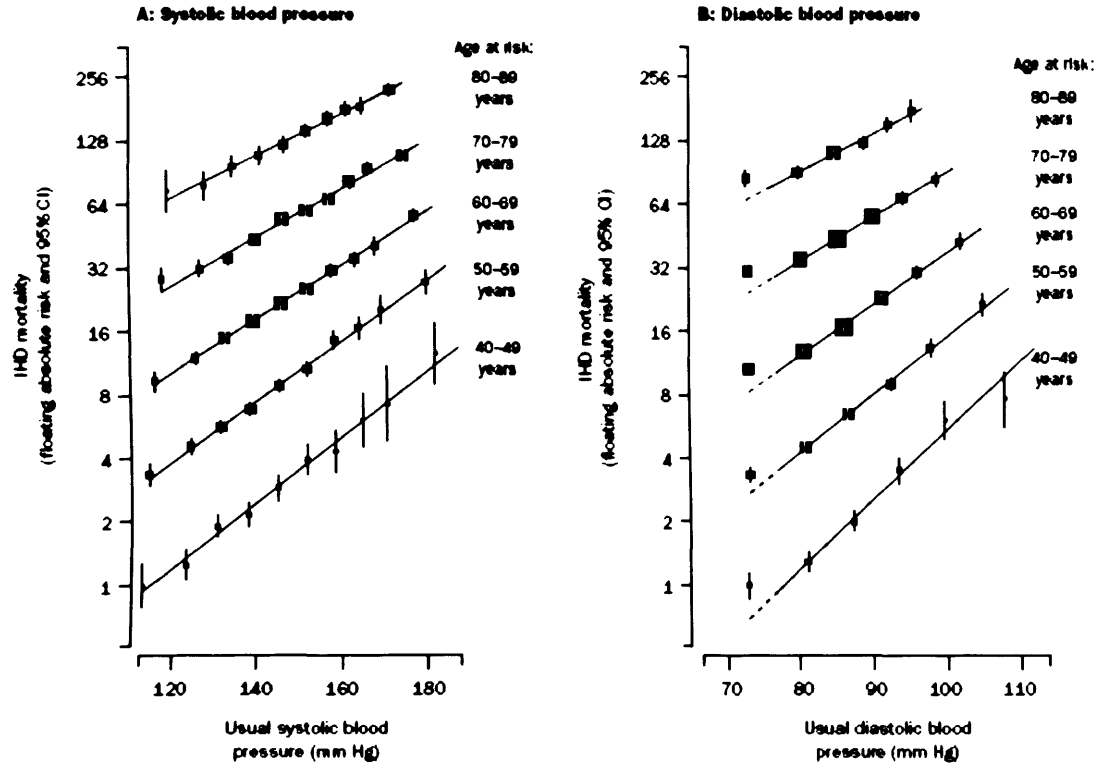
Table 1.1 demonstrates that smoking interacts with genes involved in lipoprotein metabolism, and vascular remodelling. This reflects the varied pro-atherosclerotic processes affected by smoking: endothelial dysfunction (Blann, 1992), up-regulation of adhesion molecules necessary for monocyte and platelet attachment (Lu and Creager, 2004); impact on lipid metabolism with reduced HDL and increased LDL and triglycerides (Eliasson et al., 1997). Interestingly the effect of the *LPL* D9N interaction was independent of its effect on triglycerides, but this association has not been examined in any studies. The *APOE*-smoking interaction (Humphries et al., 2001) has been most studied with subsequent replication in the Framingham Offspring study (Talmud et al., 2005).

Gene–hypertension interaction on CHD risk

Essential hypertension, a complex phenotype itself resulting from multiple gene-environmental interactions, exerts an effect on a wide range of tissues including the kidney, heart, vascular tree and brain. It is an independent cause of CVD (including CHD and stroke) and renal failure, and is strongly associated with the development of left ventricular hypertrophy (LVH) and heart failure (Chobanian et al., 2003) (Levy et al., 1988).

There is a continuous and linear association between both systolic and diastolic blood pressure and CHD and stroke mortality which exists down to 'normal' blood pressures from 115/75mmHg (Prospective Studies Collaboration, 2002). This is illustrated for CHD in Figure 1.4.

Figure 1.4: Ischaemic heart disease (IHD) mortality rate in each decade of age versus usual blood pressure at the start of that decade (Prospective Studies Collaboration, 2002).



Key pathophysiological abnormalities in hypertension include inappropriate regulation of extracellular osmolality (and volume) mediated by the kidneys (Guyton, 1990) and the presence of generalised membrane abnormalities (Orlov et al., 1999). Altered sodium handling (Guyton, 1990), compounded by sympathetic nervous system and RAS activation lead to sodium and fluid retention and promote the development of hypertension (Mark, 1996; McConnaughey et al., 1999). Increased peripheral arterial resistance due to vascular remodelling, a hallmark of hypertension causes an impairment of autoregulation (or control of tissue perfusion) which is important in the development of target organ damage (Mulvany, 1999).

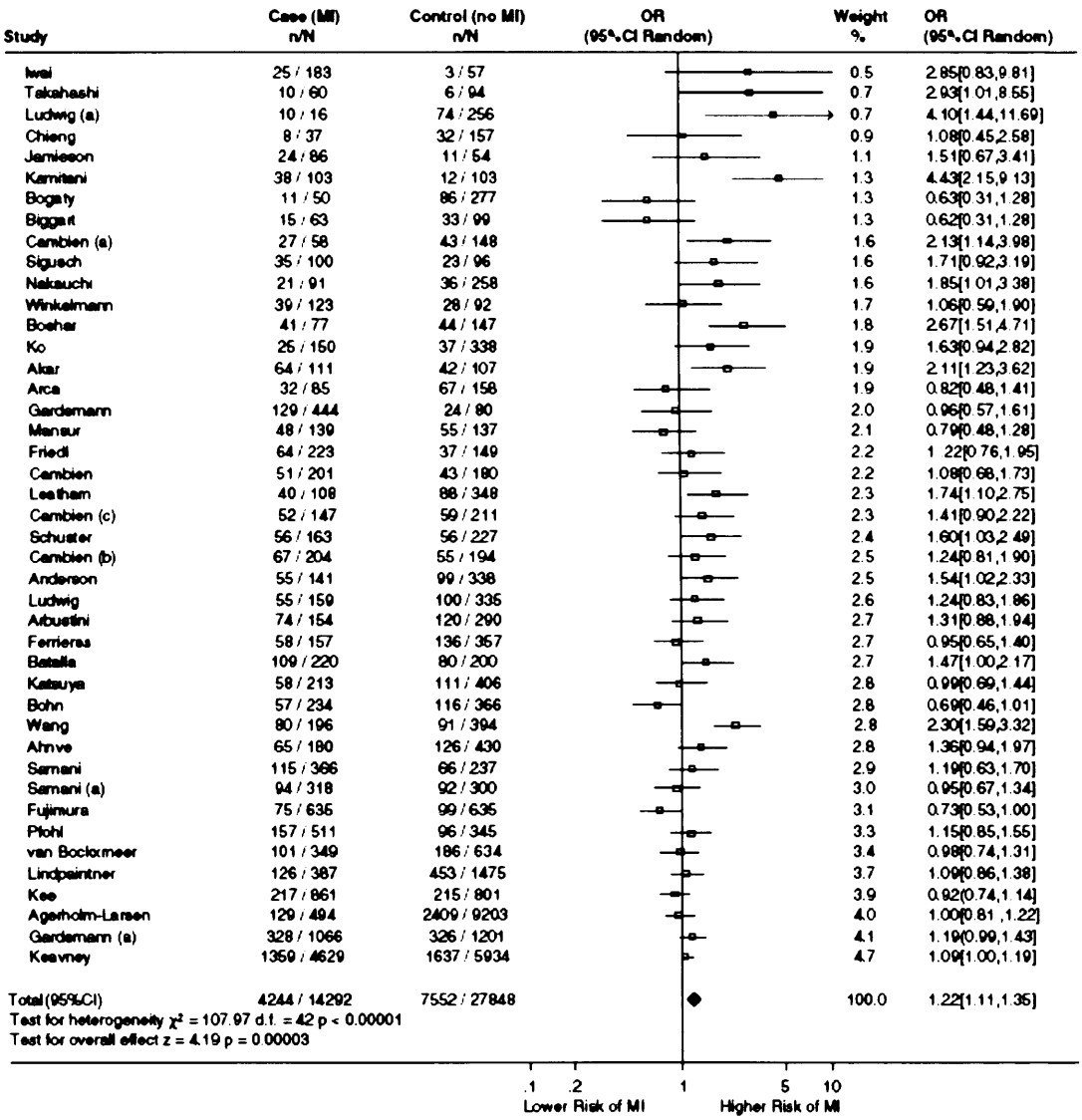
Given the spectrum of tissues affected by hypertension with the presence of systemic abnormalities, it is very likely that gene expression is changed (causing changes in

harmful enzyme or receptor activity) and GEI is thus possible in predisposing certain individuals or groups to a greater CHD risk. From table 1.1, gene variants involved in lipid metabolism and RAS combine with blood pressure to raise CHD risk. It is conceivable that lipoprotein metabolism genes interact with hypertension in view of the clustering of hypertension and dyslipidaemia in the metabolic syndrome (Eckel et al.,). Genes involved in RAS are particularly ideal candidates to modify the detrimental effects of hypertension as the RAS is heavily implicated in the development of hypertension (McConnaughey et al., 1999) and many of its components are present in tissues where end organ damage occurs.

Role of *ACE* (I/D) polymorphism in CHD

Cambien initially reported an association of the D allele with risk of myocardial infarction (MI) (Cambien et al., 1992). Since then many small studies have confirmed the D allele and DD genotype MI risk but large scale cohort studies have shown no significant association (Keavney B. et al., 2000). A meta-analysis looking at the *ACE* polymorphism concluded that the risk of MI associated with carrying the DD genotype was 1.22 [95% CI 1.11 -1.35] (Morgan et al., 2003) as illustrated in Figure 1.5.

Figure 1.5: Impact of sample size on random effects odds ratios (OR) for myocardial infarction (MI) (ACE DD genotype vs II or ID). CI (confidence interval) (Morgan et al., 2003)



Emerging evidence for *ACE*(I/D) polymorphism as a modifier in CHD

Since the advent of large prospective gene association studies, the *ACE* gene has been shown to alter the development of polyphenotypes (e.g. CHD, CVD, hypertension) through interaction with other stimuli such as smoking, age and blood pressure (Sayed-Tabatabaei F.A. et al., 2005). Interaction between smoking and the *ACE* polymorphism, has been implicated in an increase in CHD mortality (Sayed-Tabatabaei F.A. et al.,

2005), and an increase in risk of hypertension both in younger age groups (Schut et al., 2004a). An interaction between the *ACE* D allele and hypertension has been found to lead to an increase in risk of heart failure (Schut et al., 2004b).

Influence of variation in the *ACE* gene on CHD is highly plausible in that RAS is expressed in the vascular endothelium and myocardium, and that Ang II can play an important role in driving atherosclerosis (Harrison et al., 2003; Ferrario and Strawn, 2006) and cardiac hypertrophy (Li et al., 2002; Wenzel et al., 2001). The impact of specific CHD risk factors namely hypertension and lipids on ACE (and other RAS components) have been described:

Hypertension:

Hypertension and RAS are strongly linked. Activation of RAS may precede the others development but it is likely they potentiate each other. There is overwhelming evidence from animal models that AngII is involved in vascular remodelling that is central to the vascular damage in hypertension. In large artery remodelling, AngII induces smooth muscle cell hypertrophy, collagen deposition, endothelial dysfunction and cytokine secretion (Albaladejo et al., 1994; Kakar and Lip, 2007). In small artery remodelling, RAS is clearly involved with AngII induced changes mediated by MAPKs and ROS generation via activation of NADPH (Touyz et al., 2002; Virdis et al., 2004).

RAS is heavily implicated in the development of atherosclerosis specifically with ACE expressed in foam cells and lymphocytes of human carotid atherosclerostic lesions (Fukuhara et al., 2000). Through animal models of atherosclerosis for the most part, AngII has been shown to cause endothelial ROS production with subsequent endothelial dysfunction (Wang et al., 2001; Anderson et al., 2000), SMC hyperplasia (Daemen et

al., 1991), matrix deposition (Castoldi et al., 2003), and increased vascular permeability (Tamarat et al., 2002). Greater ACE activity has been demonstrated from coronary lesions of patients with acute coronary syndromes compared to lesion from patients with stable CHD suggesting that RAS is associated with a more vulnerable plaque (Hoshida et al., 2001).

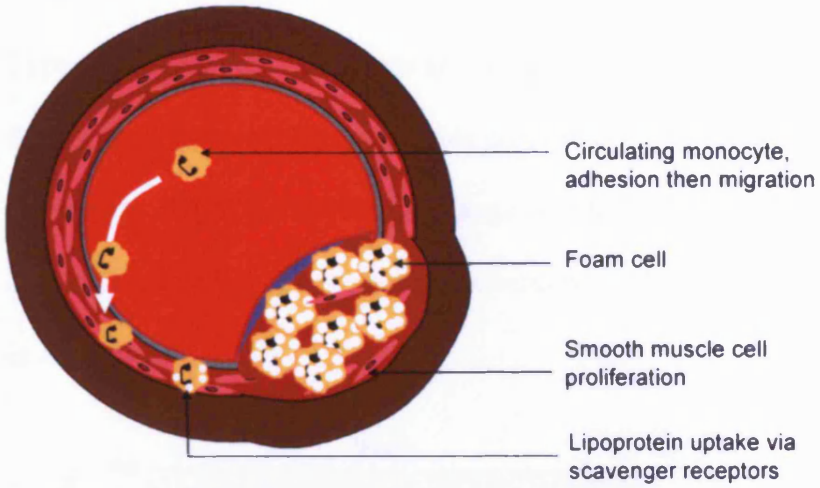
It is therefore very clear to see why, in individuals who have greater plasma and tissue levels of ACE (i.e. those that carry the D allele of I/D polymorphism), hypertension will affect susceptible tissues to a greater extent, thereby raising CHD risk. RAS also plays an important role in cardiac hypertrophy through cardiomyocyte hypertrophy (Li et al., 2002) and myofibroblast remodelling affecting interaction with the extracellular matrix (Siwik et al., 2001). Furthermore, the *ACE* D-allele is associated with a greater left ventricular (LV) growth response to a variety of stimuli (Estacio et al., 1999; Montgomery et al., 1997; Lechin et al., 1995) including hypertension (Iwai et al., 1994), and given that exuberant LV growth is independently associated with excess CHD risk (Levy et al., 1988; Haider et al., 1998), an interaction of *ACE* with hypertension in determining CHD risk would appear entirely logical. ACE inhibitors reduce CHD events (Yusuf et al., 2000; Fox et al., 2003) in high risk individuals through reducing blood pressure but also through other putative anti-atherosclerotic mechanisms. This also supports evidence for raised ACE (and RAS) levels being pathogenetic in hypertension but also having further impact on CHD mechanisms and surrogates including atherosclerosis and LVH.

Lipids:

Lipids play a central role in the development of atherosclerosis and are an independent risk factor for CHD (Wilson et al., 1998). Their deposition in the arterial endothelium enables the expression of adhesion molecules such as PECAM 1 and ICAM 1 which promote the sticking and transmigration of leucocytes into the arterial wall (Frostegard et al., 1991; Ross, 1999). The trapped LDL undergoes further modification including oxidation which perpetuates the inflammatory process by causing macrophages to become foam cells (Lusis, 2000).

There is evidence that hypercholesterolaemia increases plasma AngII concentrations (Daugherty et al., 2004). AngII is also involved in LDL oxidation and uptake (Keidar et al., 2001). It has therefore been suggested that there is a positive feedback relationship with high cholesterol levels increasing AngII and AngII increasing lipid uptake (Keidar et al., 2001). It is therefore plausible that there may be a positive interaction between elevated lipid status and *ACE* I/D polymorphism on CHD risk. Interestingly, Cambien et al (Cambien et al., 1992) initially reported an *ACE* genotype DD interaction with plasma ApoB levels and BMI, finding a risk ratio of 3.2 with low ApoB (< 1.25g/L) and low BMI (< 26kg/m^2). This was difficult to explain biologically and has not been confirmed by others (Keavney B. et al., 2000).

Figure 1.6: Diagram of atheromatous plaque formation (Zipes and Libby P et al, 2005)



Therefore *ACE*(I/D) polymorphism is an excellent candidate modifier gene to study in CHD in view of the wide distribution of ACE in culprit tissues and its key role in disease development especially in the presence of known risk factors.

Type 2 Diabetes

Type 2 Diabetes (T2D) is a huge and increasing burden on morbidity and mortality throughout the world. The worldwide prevalence was estimated to be 2.8% in 2000 (Wild et al., 2004) but in view of changes in lifestyle and diet, and particularly a rise in obesity, the number of people with diabetes will double between 2000 and 2030 (Wild et al., 2004).

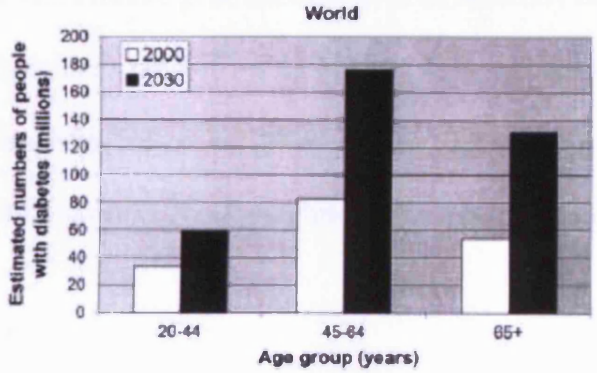


Figure 1.7: Estimated global number of adults with diabetes by age-group in 2000 and 2030 (Wild et al., 2004)

In the UK there are currently over 2 million people with diabetes (Diabetes UK, 2006). Patients with T2D have a 2-4 fold higher incidence of CHD (Garcia et al., 1974). There is also a higher risk of stroke, amputation, kidney disease and blindness (Klein, 1995). The pathogenesis of T2D is due to impaired insulin action or insulin resistance and impaired insulin secretion (which occurs later). T2D and CHD are thought to be closely linked with shared aetiological mechanisms including inflammation (Pradhan and Ridker, 2002). The clustering of components of the metabolic syndrome including obesity, dyslipidaemia, hypertension and insulin resistance underlie both diseases (Pradhan and Ridker, 2002; Haffner, 2002).

There is also a substantial genetic component to T2D as evidenced by family studies (Meigs et al., 2000), twin studies (Barroso, 2005) and variation in frequency between ethnic groups (Simmons et al., 1991). As in polyphenotypes like CHD, T2D is probably conferred by a number of genes each with modest effect, interacting with their environment. Again like CHD there have only been a small number of SNPs so far that have demonstrated a consistent T2D risk effect confirmed by meta-analyses (reviewed in (Gable et al., 2007a)). Though no meta-analysis has as yet been published, the *TCF7L2* intronic gene variants have consistently been shown to influence T2D 'risk' with an odds ratio of 1.4 for heterozygote and 1.8 for homozygote risk allele (Humphries S.E. et al., 2006), with a high population-attributable fraction of approximately 20% (Humphries S.E. et al., 2006; Groves et al., 2006).

GEI in T2D

In terms of GEI, data has been understandably limited in view of the difficulty evaluating these relationships. Obesity is the most likely environmental factor to be studied in view of it being the single most important risk factor for T2D (Gable et al., 2006). Obese individuals have a greater CHD risk (Lamarche, 1998), and there is evidence that adipose tissue (especially when located centrally) is pro-inflammatory, secreting tumour necrosis factor-alpha and Interleukin-6 (IL-6) (Kern et al., 1995; Fried et al., 1998) thereby actively worsening insulin resistance and increasing T2D risk.

An interaction between *PPARG* Pro12Ala and obesity on T2D risk has been determined with the Ala allele conferring protection only in those individuals with lower body mass index (under 34 kg/m2) (Florez et al., 2007a). An interaction between a nonsynonymous

polymorphism in *APM,* (+276G>T) and obesity on T2D risk has been demonstrated with an increased risk associated with +276T carriers in the obese men but not in the lean men (Gable et al., 2007b). An interaction between lifestyle intervention and *TCF7L2* rs7903146(C>T) on T2D risk has also been reported but was not statistically significant (Florez et al., 2006).

In view of T2D being an arbitrary definition for a state of glucose intolerance (or insulin resistance), interactions have been sought with insulin resistance and other T2D surrogates e.g *KCNJ11* E23K SNP has been shown to effect insulin secretion in patients with impaired glucose tolerance (Florez et al., 2007b).

Role of *ACE* (I/D) polymorphism in T2D

There is good evidence that RAS plays a role in the pathogenesis of T2D. They exist in tissues controlling glucose metabolism i.e adipose tissue, skeletal muscle and pancreas (Karlsson et al., 1998). Ang II is also diabetogenic through a number of mechanisms, including interfering with insulin signalling, having a pro-inflammatory effect on tissue beds causing endothelial dysfunction, inhibiting adipocyte differentiation, and causing beta cell dysfunction via oxidative stress (Hattori et al., 2005; Leung and Carlsson, 2005; Engeli et al., 2003). A meta-analysis has shown ACE-inhibitors or Angiotensin II Receptor blockers (ARB) can delay the development of T2D in high-risk individuals (Scheen, 2004).

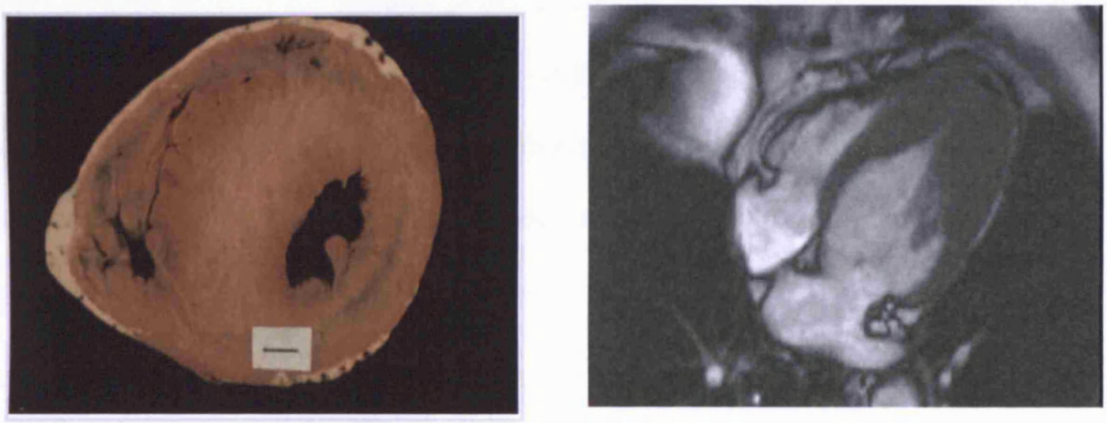
The *ACE* I/D polymorphism has been widely studied on T2D risk with varying results. The D allele has been associated with a higher T2D risk [OR=1.52,p=0.02] in a case-

control study (Stephens et al., 2005). In those with T2D, D allele carriers have poorer outcomes, with a higher risk of myocardial infarction and renal disease (Kennon et al., 1999), and a worse response when starting on an intensive hypoglycaemic medication regime (Salonen et al., 2006). Paradoxically the D allele has been associated with a higher insulin sensitivity (Panahloo et al., 1995). Combined SNP interactions or haplotype effects have been reported in diabetic nephropathy (Osawa et al., 2007; Ng et al., 2006), and there is a consistent observation of the D allele being specifically associated with diabetic nephropathy (Sayed-Tabatabaei et al., 2006). There is however no consensus between this genotype and T2D risk with negative studies published (Grammer et al., 2006; Schmidt and Ritz, 1997). Despite this, the role of RAS in T2D pathogenesis in several areas, and the observation that ACE inhibition may decrease T2D risk (Scheen, 2004) lends weight to an effect of the *ACE* I/D polymorphism, and therefore robust consideration of GEI should be taken into account. Obesity is the most powerful T2D risk factor and its strong inflammatory component complements RAS activation and therefore it is plausible that there is an interaction between obesity and *ACE* (I/D) polymorphism.

Hypertrophic Cardiomyopathy

Hypertrophic Cardiomyopathy (HCM) is a common genetic heart muscle disorder, inherited in an autosomal dominant manner, characterised by unexplained left ventricular hypertrophy, with a prevalence in the order of 1 in 500 (Maron et al., 1995). The clinical course is highly variable, ranging from asymptomatic throughout life to premature sudden death, heart failure and arrhythmia. It is the most common cause of sudden death in young adults (Maron et al., 1995). Histological features include myocardial disarray and fibrosis. The initial pathogenetic insult in the myocardium is ascribed to a cellular energetic deficit (Ashrafian et al., 2003).

Figure 1.8: Histological specimen and Magnetic Resonance Image of Hypertrophic Cardiomyopathy



There is an overall tendency in HCM for gradual wall thinning and a gradual decrease in systolic performance over time (Thaman et al., 2004; Thaman et al., 2005). Survival rates have however improved over the last 40 years and recent studies demonstrate an annual mortality rate of less than 3%, annual sudden death rate (most common mode of death) of 1% and annual heart failure death (or cardiac transplantation) rate of 0.5% (Elliott et al., 2006). Despite this there are individuals and families who however have

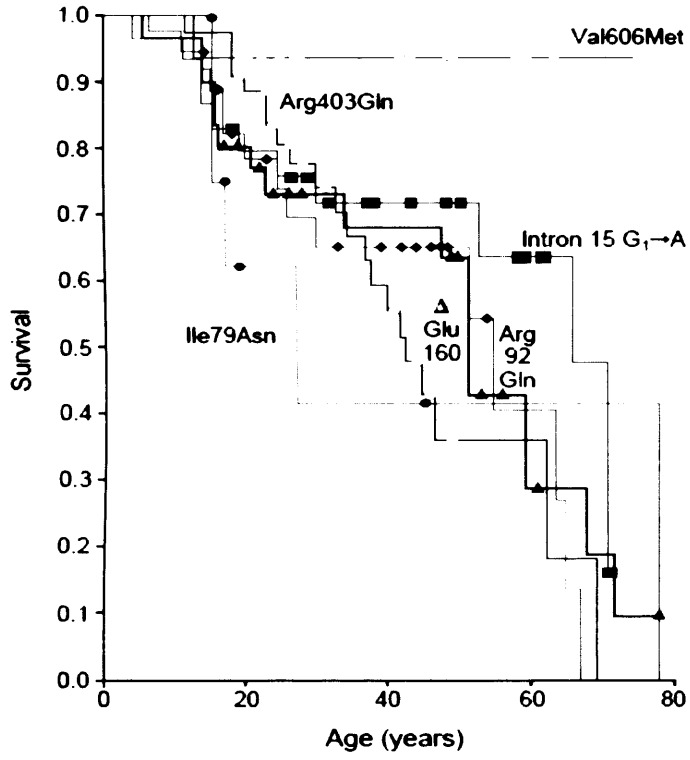
malignant phenotypes with high rates of sudden death, heart failure and stroke (Elliott et al., 2000; Thaman et al., 2005; Kubo et al., 2007).

The first causative mutation, discovered in 1989 occurred in cardiac Beta-myosin heavy chain gene, a sarcomeric protein gene (Geisterfer-Lowrance et al., 1990). The majority of mutations have since been found in sarcomeric protein genes, the commonest being in genes coding for Beta-myosin heavy chain, Myosin binding protein C and Troponin T (Marian, 2002). The remaining causative mutations are in other sarcomeric protein genes, AMP kinase gene (Blair et al., 2001b), mitochondrial DNA (Simon and Johns, 1999) and also occur in triplet repeat syndromes (Phillips and Harper, 1997). Not all disease-causing mutations have been discovered, with screening only detecting mutations in up to 70% of patient cohorts (Ashrafian and Watkins, 2007). HCM not only exhibits genetic heterogeneity as described above, but exhibits allelic heterogeneity with many mutations found in each gene – at least 70 identified in Beta-myosin heavy chain, more than 40 in Myosin binding protein C, and at least 15 in Troponin T (Marian, 2002). The frequency of each causal mutation is low, with many of the mutations not being found elsewhere (Marian, 2001).

Studies have suggested that HCM caused by beta myosin heavy chain mutations cause early onset disease with greater LVH and a higher sudden death rate compared to HCM caused by myosin binding protein C gene mutations (Abchee and Marian, 1997; Charron et al., 1998). Troponin T mutations have been associated with mild LVH but with a high incidence of sudden death (Watkins et al., 1995). Despite this, many studies have demonstrated that though some mutations are associated with specific phenotypes, the phenotypes are not gene specific with some mutations conferring no association with phenotype (Ackerman et al., 2005; Watkins et al., 1992; Watkins et al., 1995).

Figure 1.9 demonstrates the substantial differences in mortality between HCM patients with different Beta-myosin heavy chain and Troponin T mutations. Furthermore even within affected families (with identical mutations) there is definite phenotypic variance (Ashrafian and Watkins, 2007). One reason may be the inheritance of compound mutations may be more common than expected (Blair et al., 2001a). Another explanation for this could be presence of other modifier genes e.g in hypertrophic or fibrotic response, or environmental factors e.g hypertension, which interact with the causative gene to influences the phenotype.

Figure 1.9: Kaplan-Meier survival curves in patients with HCM associated with different Beta-myosin heavy chain and Troponin T mutations demonstrating marked variation in mortality (Watkins et al., 1995)



Modifier genes in HCM

Searching for 'modifier' genes has so far been limited. Using the candidate gene association study approach, results have been conflicting. Polymorphisms in the RAS have been most widely studied since they are ideal contenders – being localised in the myocardium (Danser, 1996), with AngII inducing cardiomyocyte hypertrophy (Sabri et al., 1998) and myofibroblast remodelling (Siwik et al., 2001). In terms of the *ACE*(I/D) polymorphism, the D-allele is associated with a greater left ventricular (LV) growth response to a variety of stimuli (Lechin et al., 1995)(Estacio et al., 1999)(Montgomery et al., 1997) including hypertension (Iwai et al., 1994). Specifically with regard to HCM, the DD genotype has been associated with higher risk of sudden death (Marian et al., 1993) and increased severity of hypertrophy (Lechin et al., 1995; Tesson et al., 1997), accounting for 10-15% of variability of hypertrophy (Lechin et al., 1995). Other studies have demonstrated no association between this polymorphism and HCM (Yamada et al., 1997; Osterop et al., 1998). Other SNPs in the RAS – *AGT* M235T and *AGTR* 1166 A/C have been studied in HCM with mixed results (Ishanov et al., 1997; Brugada et al., 1997; Osterop et al., 1998). SNPs in endothelin 1 and TNF alpha genes have also been associated with severity of hypertrophy in HCM (Brugada et al., 1997; Patel et al., 2000). Results of gene association studies have been summarised in Table 1.2. The varied results are not surprising in view of typical problems associated with such studies including limited sample size and cross-sectional heterogenous cohorts.

Table 1.2 Summary of 'modifier gene' association studies in HCM (from (Marian, 2002))

Gene	Polymorphism	Association with HCM phenotype	Reference
Angiotensin converting enzyme	I/D	DD genotype associated with higher risk of SCD	(Marian et al., 1993)
		DD genotype associated with severity of hypertrophy	(Lechin et al., 1995; Tesson et al., 1997)
		No genotype association with hypertrophy	(Yamada et al., 1997; Osterop et al., 1998)
		DD genotype more common in HCM	(Pfeufer et al., 1996; Yoneya et al., 1995)
		Genotype frequency unchanged	(Yamada et al., 1997)
Angiotensinogen	M235T , T174M	235T allele more common in HCM	(Ishanov et al., 1997)
		Genotype frequency unchanged	(Yamada et al., 1997)
	-6 G/A	No genotype association with hypertrophy	(Brugada et al., 1997; Yamada et al., 1997)
Angiotensin II receptor 1	1166A/C	C allele associated with severity of hypertrophy	(Osterop et al., 1998)
		No genotype association with hypertrophy	(Brugada et al., 1997)
Bradykinin B2 receptor	T21M	M allele found in HCM cases but not in controls	(Erdmann et al., 1998)
Aldosterone Synthase	-344 T/C	No genotype association with hypertrophy	(Patel et al., 2000)
Endothelin-1	8002G/A	A allele associated with severity of hypertrophy	(Brugada et al., 1997)
Tumor necrosis factor α	-308G/A	A allele associated with severity of hypertrophy	(Patel et al., 2000)
Platelet activating factor Acetylhydrolase	994G/T	T allele more common in HCM and associated with increased left ventricular dimension and decreased function	(Yamada et al., 2001)
Insulin-like growth factor 2	820G/A	No genotype association with hypertrophy	(Patel et al., 2000)
Transforming growth factor B1	-509C/T	No genotype association with hypertrophy	(Patel et al., 2000)
Interleukin 6	-174G/C	No genotype association with hypertrophy	(Patel et al., 2000)

Recent studies have looked at polymorphisms in RAS as a candidate system demonstrating a cumulative influence on hypertrophy (Ortlepp et al., 2002; Perkins et al., 2005) though, in the latter study this was only evident in the subgroup with no

previously identified mutation. A further study has now demonstrated this 'additive' effect prospectively in a group of paediatric HCM patients (Kaufman et al., 2007). Association studies are not the only method of searching for 'modifier genes' – a genome wide linkage study has identified the 10p13 locus as having a very strong 'modifier' effect in HCM (Daw et al., 2007). *ITGA8* and *C10orf97* genes located in this region are both plausible candidates, being abundantly expressed in the heart and having cell-matrix adhesion and apoptosis functions respectively.

In summary, there is currently functional evidence and some epidemiological evidence that *ACE* I/D polymorphism (and other polymorphisms in RAS) are associated with a modifying effect on HCM phenotypes.

MATERIALS

Reagents

DNA Extraction: All reagents were supplied by Sigma (Poole, UK).

Polymerase Chain Reaction: PCR primers and *Taq* polymerase were supplied by Gibco-BLR Ltd (Paisley UK). Restriction enzyme (Mlu-I) was supplied by New England Biolabs Inc (UK). dNTPs were supplied by Pharmacia Biosystems Ltd (Milton Keynes, UK).

Gels: 37.5:1 acrylamide:N,N'-methylenebenebisacrylamide was supplied by Protogel, National Diagnostics (Hull, UK). TEMED (NNN',N'-tertramethylethylethylenediamine and ammonium persulphate (APS) were supplied by BDH (Leicestershire, UK).

Commonly used stock solutions

DNA Extraction:

1M $MgCl_2$: 20.33g $MgCl_2$ dissolved in 100ml in dH_2O.

1M Tris pH 7.5: 12.11g Tris, made up to 100ml in dH_2O, Correct pH to 7.5 and autoclaved.

Sucrose lysis mix: 109.54g sucrose, 5ml 1M $MgCl_2$, 10ml 1M Tris pH7.5, 10ml Triton-X-100, made up in 1000ml in dH_2O and stored at 4°C.

0.5M Na_2EDTA: 37.22g EDTA, made up to 200ml in dH_2O. Adjust with NaOH to pH 8.0

10%SDS: 10g Sodium dodecyl sulphate, made up in 100ml in dH_2O.

Nuclear lysis mix: 1ml 1M Tris-HCl pH8.2, 2.34gNaCl, 0.4ml 0.5M Na_2EDTA pH 8.0, 10ml 10%SDS, made up to 90ml in dH_2O.

5M Sodium perchlorate: 70.24g sodium perchlorate, made up to 100ml in dH_2O.

TE buffer pH 7.6: 1.21g Tris, 0.37g EDTA, made up to 1000ml in dH_2O.

MADGE gels: For each gel a solution was made up containing:-

5mls of 10xTBE , 12.2mls 30% acrylamide-bisacrylamide (in a ratio of 19:1), 32.5mls of distilled dH_2O, 150µl N'-tetramethylethylenediamine (TEMED), 150µl 25% ammonium per sulphate

TBE (10x Tris-Boric acid-Ethylenediaminettraacetic acid contained:- 0.9M Tris, 0.9M Ortho-boric acid, 0.2M ethylenediaminetetraacetic acid)

TaqMan assays: All TaqMan assays were obtained from Applied Biosystems (California, USA). TaqMan Absolute QPCR Rox mix and 384 well plates were supplied by ABgene (Surrey, UK).

METHODS

Study samples

HIFMECH: Hypercoagulability and Impaired Fibrinolytic Function Mechanisms Study (HIFMECH)

The HIFMECH study was designed to study genetic and environmental mechanisms contributing to the higher cardiovascular risk in Northern compared to southern Europe (Juhan-Vague et al., 2002). The study sample of Caucasian male first myocardial infarction survivors below 60 years of age were recruited from four European centres (Northern European-Stockholm, London; Southern European-Marseille, San Giovani Rotondo) (n=598). Subjects with familial hypercholesterolemia and insulin-dependent diabetes mellitus were excluded. A selection of randomly-selected age-matched healthy controls was also recruited from each catchment area (n=653). The study was performed in accordance with the guidelines in The Declaration of Helsinki and approved by local ethics committees. Written informed consent was obtained from all subjects. The patients and control subjects were examined in parallel in the early morning after an overnight fast, and a blood sample was also obtained. Post-infarction patients were investigated 3 to 6 months after the acute event.

The Second Northwick Park Heart Study Cohort (NPHSII)

The Second Northwick Park Heart Study (NPHSII) was recruited by the Medical Research Council Cardiovascular group at The Wolfson centre for Preventative Medicine. In brief, 3012 unrelated healthy Caucasian middle-aged male subjects (mean age 56.1±3.5 years) recruited from nine UK general practices, detailed elsewhere were prospectively followed for up to 15 years (Miller et al., 1995). The study was approved by the institutional ethics committees and performed in accordance with the declaration of Helsinki. All subjects gave written informed consent.

Baseline characteristics were ascertained by means of a questionnaire at entry into the study, with SBP recorded twice with a random zero mercury sphygmomanometer after five minutes seated. Exclusion criteria at baseline were a history of MI, cerebrovascular disease, life-threatening malignancy or regular cardiovascular medication with aspirin, anticoagulants or anti-hypertensives. At entry, a 5ml EDTA blood sample was obtained, from which genomic leukocyte DNA was extracted. Time to first CHD event (defined as sudden cardiac death, symptomatic/silent MI (the appearance of a new major Q wave on the follow up ECG, using Minnesota codes 1_1,$1_{2.1\ to}$ $1_{2.7}$, $1_{2.8}$ plus 5_1 or 5_2, or coronary revascularisation) was recorded, yielding only one event/subject (Miller 1995). Cases of T2D at baseline were identified by self report. Exclusion criteria precluded subjects requiring insulin or oral hypoglycaemics from entry into NPHS II. New cases were identified by practice note search for physician diagnosed and treated T2D according to current national guidelines (Gable et al., 2006). The reporting of T2D was done thoroughly and systematically, and after 15 years 288 CHD events occurred in 2782 of the subjects with DNA available for analysis. There were 76 cases of T2D at baseline and by 15 years a further 169 cases had been identified.

Retrospective study of Hypertrophic cardiomyopathy (HCM)

The study cohort consists of 541 unrelated patients diagnosed with HCM, assessed at St George's Hospital, London, and Heart Hospital, London UK between 1988 and 2006. Mean age was 38.5 years (SD 17.4) with 62% being men. 17 individuals were under age of 16.

The diagnosis of HCM was based on the echocardiographic evidence of left ventricular hypertrophy more than 2 SD for age and sex or in accordance with criteria for the diagnosis of familial disease in patients with at least one first degree relative who had an unequivocal diagnosis (Richardson et al., 1996; McKenna et al., 1997). Patients with other cardiac or systemic diseases that can produce hypertrophy were excluded.

Clinical evaluation

Initial evaluation included history, clinical examination, 12 lead ECG, echocardiography, and symptom limited cardiopulmonary exercise testing. A blood sample was also taken from which genomic leukocyte DNA was extracted.

Survival data and clinical status were collected at subsequent clinic visits for patients followed up at these institutions, and by direct communication with patients and their general practitioners when followed up elsewhere. This was obtained in 485 patients with a mean follow up of 6.65 years (range of 0.56 to 19.1 years with no event and 5.14 years with a range of 0.27 to 16.25 years in those with an event).

390 (72%) of the 541 patients underwent serial echocardiography to study changes in left ventricular size, left atrial size, maximal wall thickness and systolic function over

time. Change in NYHA functional class and change in peak VO_2 during follow up was also studied.

Echocardiography

Echocardiography was performed with an Acuson 128 XP/10 (Mountain View, California, USA), GE Vingmed system V (GE Ultrasound Europe, Horten, Norway), or a Hewlett Packard Sonos 1000 (Hewlett Packard, Andover, Massachusetts, USA). Standard views for M mode and two dimensional studies were obtained. Left ventricular end diastolic (LVED) and left ventricular end systolic diameters (LVES) were measured from two dimensional and M mode images obtained from parasternal long axis views. Fractional shortening (FS) was calculated by the formula ((LVED – LVES)/LVED) x 100. Left atrial size (LA) was measured on M-mode.

The magnitude and distribution of left ventricular hypertrophy were assessed in the parasternal short axis view and confirmed from parasternal long axis and apical views. The ventricle was divided into four regions: anterior septum, posterior septum, lateral wall, and posterior walls. Wall thickness was measured at the level of the mitral valve and papillary muscles in each of the four myocardial segments. Maximum left ventricular wall thickness or Max LVWT was defined as the greatest thickness in any of these segments. Patients underwent symptom limited cardiopulmonary exercise testing in accordance with previously published methods (Sadoul et al., 1997). All measurements are quoted in millimeters except FS which is a percentage.

End points for the survival analysis were as follows:

Sudden cardiac death: witnessed sudden death with or without documented ventricular fibrillation, death within one hour of new symptoms, or nocturnal death with no antecedent history of worsening symptoms; Heart failure death: death preceded by signs and symptoms of heart failure or cardiogenic shock; Other cardiovascular death: deaths caused by stroke, pulmonary or systemic embolism, and myocardial infarction; Non-cardiovascular death: deaths caused by known non-cardiovascular and unknown events; Heart transplantation; In patients who had resuscitated sudden cardiac arrest during follow-up or an implantable cardioverter-defibrillator (ICD) as primary prophylaxis the first appropriate shock during follow-up was coded as an outcome in the survival analysis.

All cause mortality was defined as sum of all-cause deaths, cardiac transplantations, VF arrests and ICD discharges. Cardiac mortality was defined as sum of cardiac deaths (all cause minus non-cardiac), cardiac transplantations, VF arrests and ICD discharges. Changes in left ventricular parameters and left atrial size, and change in peak VO_2 during follow up were also studied. *ACE* genotype was obtained as described later in this chapter.

This study was approved by the local research ethics committee and subjects gave their informed consent.

DNA Extraction from Blood – The "Salting Out Method"

Leukocyte DNA for genotyping had previously been extracted for all studies from whole blood using the "salting out" method (Miller et al., 1988). Briefly, the cell lysis is achieved with a sugar lysis buffer followed by nuclear lysis using the defined nuclear lysis buffer. De-proteinisation is performed with sodium perchlorate. The DNA was extracted using chloroform and then precipitated from the aqueous phase using ethanol. The extracted DNA was dissolved and stored in TE Buffer. All samples were carefully logged and entered into a database with a unique identifier in order to preserve the anonymity of individuals in the study. This stock DNA was used to prepare working 96 well arrays for genotyping.

Working DNA was standardized to a concentration of 15ng/µl. This was achieved by calculating the volume of DNA required to be added to 750µl of dH_2O to achieve this concentration on the basis of the absorbance of a 10µl sample of stock DNA. Stock arrays were created in labelled 96-well Beckman's array. These were stored at -20°C. To create working arrays, 100µl of each sample was removed from the stock array, and transferred to another labelled 96-well array.

Polymerase Chain Reaction

In brief, the technique of PCR relies on the fact that the DNA strand is denatured into single strands by heat, and will anneal with primers and nucleotide bases to reform a double strand on cooling. The first step is therefore a short high temperature period to denature the DNA. This initial stage is followed by cooling in the presence of primers

that are complementary to the DNA either side of the sequence to be studied. These primers anneal, and a DNA polymerase adds nucleotides base by base, thus replicating the DNA. The polymerase used is derived from the bacterium *Thermus aquaticus* (*Taq*) and is heat stable. It therefore does not need to be replenished after each cycle of heating and cooling. This technique allows very small initial amounts of DNA to be increased in quantity until there is sufficient DNA for genotyping to be performed.

Sample Preparation for Polymerase Chain Reaction

Following extraction and dispensing into working 96-well arrays, DNA samples were prepared for PCR by centrifuging the DNA working-array at 1500g for 1 minute. This was to ensure that all the DNA dilutions were at the bottom of their respective wells, thus reducing the possibility of cross-well contamination when the array lid was removed.

Two and a half microlitres of each sample was then removed from each array and transferred into a standard 96-well PCR plate (Omniplate, Hybaid) using a Finnipipette multichannel dispenser (Life Sciences, Basingstoke, Hants, UK). Positive and negative controls were utilised to ensure accuracy. Extreme care was taken to ensure that samples were place in the identical orientation as in the original arrays. Loaded Omniplates were then centrifuged at 1500g for 30 seconds to ensure that the DNA was at the bottom of each well, and then dried on a Thermal Cycler block (MJ Tetrad DNA Engine Thermocycler) at 90^0C for 10 minutes.

A bulk-mix of reagents was made up for each PCR, allowing adequate volume for the planned number reactions, with an additional 10% added to ensure that the mix would not run short. PCR primers and *Taq* polymerase (Gibco-BLR LTd., Paisley UK) were kept on ice and added last.

Polymerase chain reaction were performed in a total volume of 20µl. Each reaction contained 1x concentration of polmix (50mM KCl, 10mM Tris-HCl (pH 8.3), 0.2mM dATP, dGTP, dTTP and dCTP) or 1 x NH_3 buffer (16mM $(NH_4)_2SO4$, 67mM Tris-HCl (pH 8.4), 0.01% Tween 20, 0.2mM of each dNTP) and $MgCl_2$, 10pmol of each primer and 0.3U of *Taq* polymerase. Magnesium concentration varied with each PCR.

The PCR mix was added to each well of the PCR plate using an automatic Biohit repeating dispenser (Alpha Laboratories, UK). Each sample was overlaid with 20µl of mineral oil to prevent evaporation. The microtitre plate was then sealed with clear sticky plastic lid and carefully labelled. Plates were centrifuged at 1000rpm for 30 seconds to ensure good mixing of the reaction components in each well. PCR amplification was performed on an MJ Tetrad DNA Engine Thermocycler, using cycle conditions specific to each PCR. A description of the individual primers and conditions for the *ACE* I/D variant can be found at the end of this section.

Detection of DNA (Agarose gels)

In order to check the successful amplification and size of PCR products, agarose gels were utilised. For a 2% gel, 2 grams of agarose was mixed with 100ml of 1×TBE solution containing 10µL of Ethidium bromide (10µg/µL). A microwave oven was used

to heat the mixture and dissolve the agarose. The melted agarose was poured into a plastic gel tray (10 × 14cm) and a comb inserted. Solid gels were placed into an electrophoresis tank containing 750ml of 1xTBE buffer solution. 2µl of MADGE loading dye was added to 5µL PCR product and the entire volume was mixed thoroughly and placed in the separate, submerged wells of the gel. 2µl of a 1Kb ladder (Invitrogen, Paisley UK) was pipetted into the central well in order to size relevant products. All agarose gels were run at 100 volts (v) for a minimum of 30 minutes.

Restriction digestion

Restriction enzymes are derived from bacteria, and cleave double stranded DNA at a particular sequence. The enzyme translocates along the DNA until a particular recognition site is reached, where the DNA is cut. The restriction enzyme is sensitive even to a single base change in the recognition sequence, and thus can be used to detect point mutations and single base polymorphisms. A single base change can either eliminate or create a cutting site for a particular enzyme.

A restriction enzyme digest mix with the recommended buffer system was made up in a 1ml Eppendorf tube on each occasion, containing sufficient enzyme to digest the PCR products in each well of the PCR plate. 5µl of digestion mix was then added to 8µl of each reaction product using a repeater pipette as for the PCR mix. Each omniplate was then centrifuged at 200g for thirty seconds to ensure that the PCR product and restriction enzyme were mixed well. The PCR/digestion mix was then incubated overnight at the recommended temperature.

In view of the *ACE* I/D polymorphism being studied was a deletion polymorphism, specific variants were found after the PCR which could be accurately resolved by Microtitre Array Diagonal Gel Electrophoresis (described below) therefore there was no need in this case to carry out restriction digestion.

Microtitre Array Diagonal Gel Electrophoresis (MADGE)

The DNA fragments produced by restriction enzyme digest were separated by electrophoresis on a non-denaturing polyacrylamide gel, using Microtitre Array Diagonal Gel Electrophoresis (MADGE) (Day et al., 1995). This technique makes it possible to electrophorese all the 96 wells of a standard PCR plate on a single gel, by allowing the samples to run diagonally. Use of MADGE allowed the 96 well DNA array format to be retained throughout the screening process.

MADGE consists of an open arrangement of 8x12 wells each 2mm deep. The wells are arranged at an angle of 71.2 degrees to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used (Figure 2.1). Before making the mix, glass plates of appropriate size (160 x 100 x 2mm) were rigorously cleaned and hand dried. 5 drops of γ-methacryloxypropyltrimethoxysilane ('sticky' saline) were spread across the plates and left to air-dry. Polymerisation of the MADGE mix was initiated by the addition of ammonium persulphate and the solution was mixed and quickly poured into the three-dimensional former. A glass plate was then gently placed over the mould (saline side facing downwards) taking care not to trap any air bubbles. This was then left for fifteen minutes to set, using a small weight to ensure that the glass did not slip whilst the gel was setting. Excess gel was trimmed from the edges of the MADGE

former before the glass plate and attached gel were then prized away from the plastic former.

Gel Staining and Loading

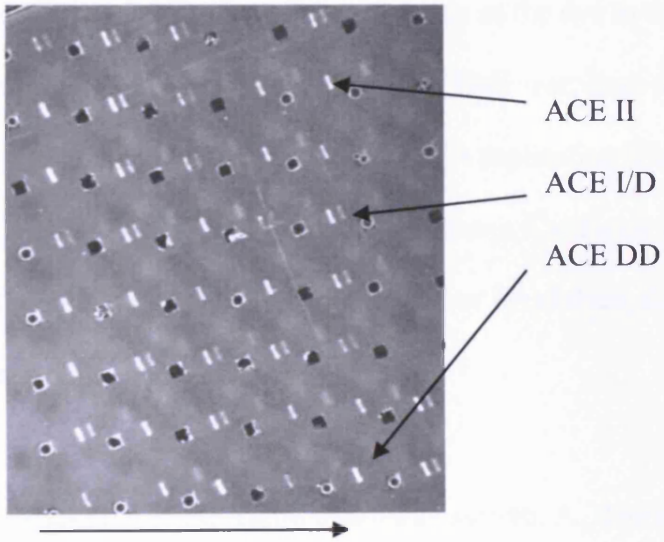
Prior to loading a gel with digested PCR product, each gel was stained with Ethidium Bromide (EtBr). This was achieved by placing them individually in a Stuart box, shielded from direct light, containing 100ml of 1x TBE and 10µl EtBr for 20 minutes.

Whilst the gels were being stained the PCR-digest product was prepared for loading onto the MADGE gel. 2µl of formamide dye (98% formamide, 10mmol/l EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) was added to each well of a new round-bottomed loading tray, followed by 5µl of each digested sample, using a multi-channel pipette to pick up the samples from under the oil in the omniplates. The digested samples were gently mixed, by aspirating the formamide dye–digest mixture up and down several times into the pipette, before dispensing them on the digest plate. After placing the stained MADGE gel into an electrophoresis tank containing 1000ml of 1xTBE, a multi-channel pipette was then used to transfer 5µl of this digest/dye mixture to the wells of the gel. At all times the samples were kept in the same layout as on the PCR tray, allowing each sample to be easily identified without being re-labelled. The gel was electrophoresed at 150 volts for 40 minutes.

Following electrophoresis the gel was viewed and photographed under ultraviolet light using the UVP Gel Documentation System. Care was once more taken to ensure the correct orientation of the MADGE under UV. This was ensured by always placing the

well corresponding to grid-reference "A1" in the bottom left-hand corner of the UV-viewing box. The image thus produced was used for genotyping. An example of typical MADGE gel is shown in Figure 2.1. All genotyping was performed in a double blind fashion using both positive and negative controls. The results were rechecked by two individuals at the time of MADGE imaging and during data entry into the computer database. Any apparent genotype differences were resolved by repeat PCR. Overall there was excellent reproducibility with >95% consistency between observers.

Figure 2.1 *ACE* I/D Genotype Resolved on MADGE



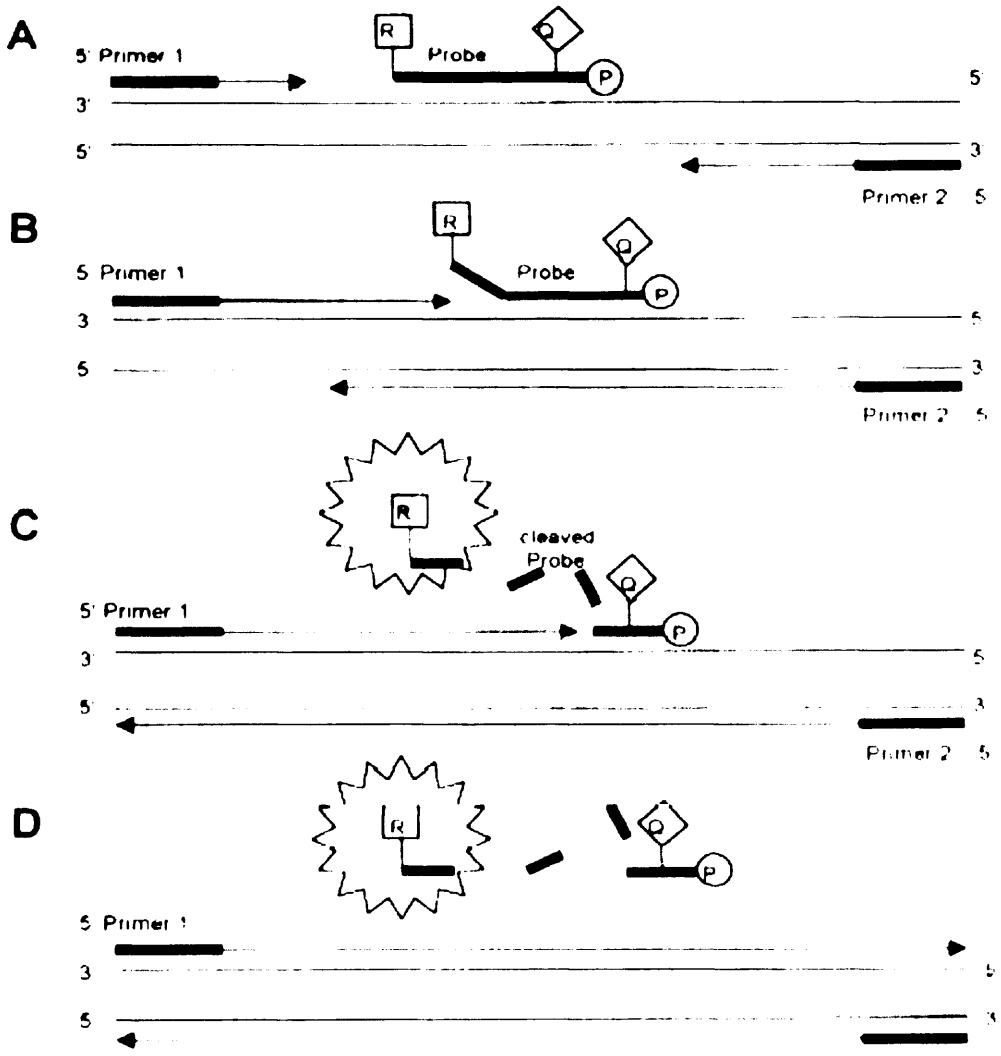
TaqMan® genotyping

TaqMan® assay technology

The principle behind the TaqMan® reaction is described in Figure 2.2. The method involves the inclusion of two fluorescent, dye-labelled probes for each allele of a

specific variant. The allele specific probes each contain a short sequence of DNA, a reporter dye (labelled VIC™ or FAM™ depending upon the allele) at the 5' end, and a non-fluorescent quencher (NFQ) dye at the 3' end. During the PCR process, forward/reverse oligonucleotides as well as the labelled probes, anneal to the DNA of interest (Figure 2.2A). Amplitaq Gold® DNA polymerase is able to replicate the single strand of DNA until it reaches the labelled probe. Any non-specific binding results in a weakened interaction of the labelled probe with the DNA and displacement of the intact probe (no cleavage of the dye from the quencher) (Figure 2.2B). If the probe is entirely complementary (hence allele specific probes) to the annealed DNA sequence, the 5' to 3' exonuclease activity of the enzyme results in the cleavage of the 5' dye from the rest of the probe (Figure 2.2C). The close proximity of the dye to the quencher usually prevents any significant fluorescent emission. However, once the dye is cleaved, fluorescence increases with each round of DNA replication (Figure 2.2D). A 7900HT Sequence Detection machine (Applied Biosystems, California USA) is then able to determine the relative levels of either the VIC or FAM dyes, thereby determining the specific genotype.

Figure 2.2 Schematic of the TaqMan® assay system. A. Represents the annealing of the fluorescent probes and oligonucleotides. B. If the fluorescent probe is not identical to the DNA sequence, the probe is displaced by the Taq. C. Successful annealing leads to 5'-3' exonuclease of the probe.
D. The VIC™ and FAM™ labels fluoresce and are picked up by the Taqman® machine.

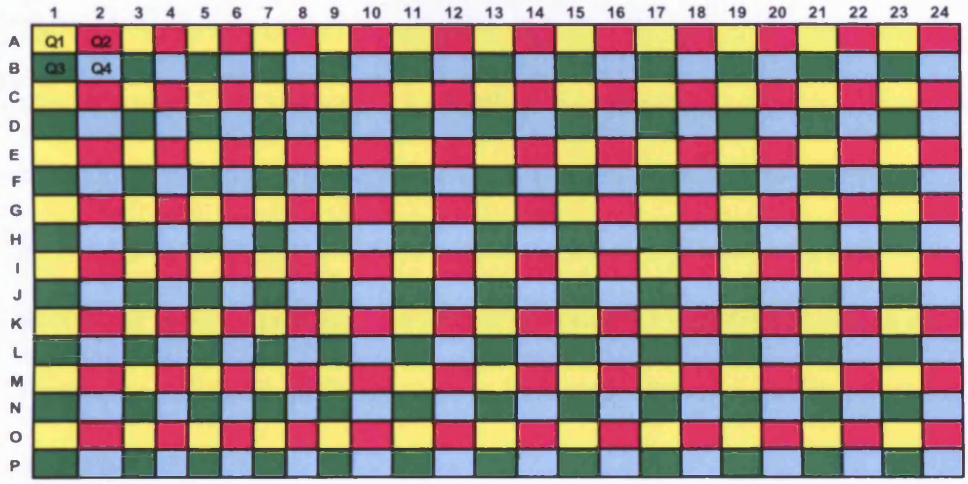


Preparing the DNA

In contrast to traditional PCR and MADGE based technology, the TaqMan® system enables high throughput genotyping in a 384 well format. In order to use this system, DNA was first standardised to an optimal concentration of 1.25ng/μL using the same methodology described earlier. A Biomek 2000 robot (Beckman-Coulter, High Wycombe UK) was used to aliquot 4μL of standardised DNA from a 96 well stock array into a 384 well plate (5ng total) with 16 wells left blank to act as negative controls. A data sheet was also compiled in order to identify each well to patient ID

number (Figure 2.3). The plates were dried out overnight at room temperature in sterile paper bags and stored until use.

Figure 2.3 Schematic of a 384 well plate. Each colour represents an individual 96 well plate as pipetted by the Beckman robot.



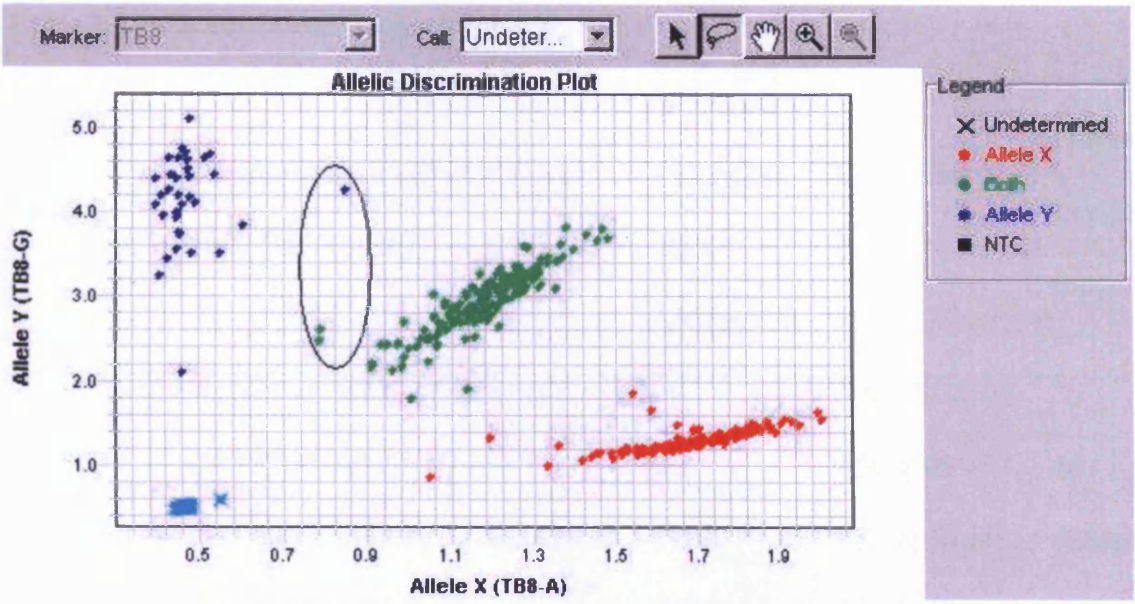
TaqMan master mix

Forward/reverse oligonucleotides and labelled probes were ordered using the 'Assay by demand' service available on the Applied Biosystems website (www.appliedbiosystems.com). Table 2.1 lists the assay successfully designed by Applied Biosystems for genotyping of the SNP (rs4341) which is in complete LD with the *ACE* I/D polymorphism. For each 384 well plate, a master mix was made of the ABgene QPCR Rox mix, the individual assay mix, and distilled sigma water. 4μL of mix was then applied to each well of the 384 plate using a manual Eppendorf 300, 8 channel multi-dispensing pipette and centrifuged (Sigma 4-15) at 200g for thirty seconds. A clear plastic lid (ABgene, Surrey UK) was applied to seal the plate.

Reading and entering of genotypes

A standard two step heat cycle program on a Thermohybaid (Basingstoke, UK) 384 well heated block was used to initiate the PCR reaction (95°C for ten minutes, followed by 40 cycles of 95°C for 15s and 60°C for 1min), and all plates were then read on a 7900HT Sequence Detection machine (Applied Biosystems). The 7900HT Taqman® machine uses SDS software 2.1 (Applied Biosystems) in order to differentiate the different genotypes (Figure 2.4). SDS 2.1 produces an allelic discrimination plot as well as assigning genotypes automatically to an excel file containing patient ID numbers according to array position. To ensure no incorrect inputting of data, a second researcher validated all the genotypes before the data was finally entered into the analysis database.

Figure 2.4 Screen shot of a typical allelic discrimination plot. The three different coloured dots represent each individual genotype: Blue and red represent homozygotes with the green dots representing heterozygotes. Light blue crosses show the negative controls. Those dots which were not tightly clustered (circled in the figure) to a particular group were re-genotyped.



Specific Gene variants

ACE I/D polymorphism (rs1799752)

rs4341 (C>G) - 100% LD with I/D variant

The I/D polymorphism was genotyped in HIFMECH using PCR and MADGE method. In NPHS2 genotypes were determined with the help of Jutta Palmen by PCR and MADGE for approximately 87% of sample and using Taqman genotyping for rs4341 for the remaining samples. One DNA array (94 samples) was genotyped for both *ACE* I/D and rs4341 polymorphisms with 100% concordance. Rs 4341 was genotyped exclusively in the HCM cohort with the help of Kah Wah Li. The details of the Oligonucleotides and PCR conditions can be found in table 2.1. The Taqman oligonucleotides and probes can be found in table 2.2.

Table 2.1 PCR conditions for genotyping by RFLP

Variant	Oligonulceotides (2 FORWARD and 1 REVERSE)	DNA (μl) POLMIX BSA MG(mmol)	Conditions (OC)	Fragment Length (bp)
ACE I/D	FH76 5'- CAT CCT TTC TCC CAT TTC TC -3' FH77 5'- TGG GAT TAC AGG CGT GAT ACAG -3' FH78 3'- ATT TCA GAG CTG GAA TAA AAT T -5'	1.5 NH4 YES 1.5	5 MINS @ 95 45s @ 95 45s @ 54 30s @ 72 (32 CYCLES) 5 MINS @ 72	85 (Deletion) 65 (Insertion)

Table 2.2 Taqman assays design for rs4341

Variant	Primers	Probes		
Rs4341	FOR: CCTTACAAGCAGAGGTGAGCTAA REV: CCATCACATTCGTCAGATCTGGTA	VIC FAM	TCAAGCCATTCAAAC CAAGGCATTCAAAC	NFQ NFQ

Statistical analysis

Statistical analysis was performed using SPSS (version 12.1, SPSS Inc., Chicago) or 'Intercooled STATA' package (version 8.2, STATA Corporation, Texas). The analysis was performed by Jackie Cooper, Fotios Drenios and myself. For gene association analysis, data are reported for those individuals amongst whom high-throughput genotyping was successful.

Deviations from Hardy-Weinberg equilibrium were considered using chi-squared tests. Hardy-Weinberg equilibrium gives the expected genotype distribution based on the observed frequency of the rare allele (q) and common allele (p) as $p^2+2pq+q^2$, where p^2 is the predicted frequency for homozygosity of the common allele, q^2 is the predicted frequency for homozygosity of the rare allele and $2pq$, the heterozygotes. These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection.

Specific details are included in the relevant chapter but in general, allele frequencies, odds ratios and hazard ratios are shown with the 95% confidence interval. Analysis of variance (ANOVA) was used to assess the association between genotypes and baseline characteristics on normally distributed data, or after appropriate transformation. Chi-squared tests were used to compare differences in categorical variables by genotype. No adjustment was made for multiplicity of testing. Whilst making such an adjustment reduces the type I error, it leads to increases in the type II error, and fewer errors of interpretation occur when no adjustment is made. However, analysis always followed an *a-priori* hypothesis or design. In all cases a P value of less than 0.05 was considered statistically significant.

In NPHSII survival analysis with respect to the prospective risk of CHD or type 2 diabetes was carried out using Cox proportional hazards model, 'failure' being the first CHD event or diagnosis of type 2 diabetes. Results are presented as hazard ratios (HR) with their corresponding 95% confidence interval (CI). To allow for differences in baseline data according to age and practice, age was included as a covariate in the model and data stratified by practice.

Chapter 3: **Impact of *ACE* (I/D) polymorphism on Coronary Heart Disease: Interaction with Lipids and Regional Effect**

Introduction

The genetic contribution to CHD is likely to be based on many genetic variants interacting with each other and with environmental influences, with each variant exerting a small effect (Suh and Vijg, 2005). The RAS is a likely candidate system in CHD pathogenesis, with AngII in particular having several strong pro-atherosclerotic properties (Wang et al., 2001; Anderson et al., 2000; Daemen et al., 1991), and RAS inhibition being protective in CHD (Yusuf et al., 2000; Fox et al., 2003). Gene polymorphisms affecting levels (and function) of RAS components (e.g. I/D polymorphism in *ACE* explaining half of the variance of plasma ACE concentration (Rigat.B. et al., 1990)) are therefore ideal tools to study CHD risk.

The impact of the *ACE* I/D polymorphism on risk of CHD has been widely studied since Cambien (Cambien et al., 1992) described a strong association of the D allele with risk of MI. Large cohort studies have been unable to replicate this association (Keavney B. et al., 2000) with a meta-analysis suggesting that the risk of MI associated with carrying the DD genotype was only 1.22 [95% CI 1.11 -1.35] (Morgan et al., 2003). More recently, the *ACE* I/D polymorphism has been shown to modify the risk of complex phenotypes such as CHD, CVD and hypertension in a large prospective study - the Rotterdam study, through interaction with environmental stimuli such as smoking (Sayed-Tabatabaei F.A. et al., 2005; Schut et al., 2004a; Sayed-Tabatabaei F.A. et al.,

2004), age (Sayed-Tabatabaei F.A. et al., 2005; Schut et al., 2004a; Mattace-Raso F.U.S. et al., 2004) and blood pressure (Schut et al., 2004b).

Interaction between smoking and the *ACE* polymorphism, has been reported to lead to (i) a higher CVD mortality at a younger age (Sayed-Tabatabaei F.A. et al., 2005), (ii) higher systolic blood pressure (SBP) and a higher risk of hypertension also in a younger age group (Schut et al., 2004a) and (iii) a significantly greater carotid IMT (Sayed-Tabatabaei F.A. et al., 2004). A relationship between age and this polymorphism has also been reported in association with carotid stiffness (Mattace-Raso F.U.S. et al., 2004) along with an interaction between the *ACE* D allele and hypertension leading to a higher risk of heart failure (Schut et al., 2004b). Thus a likely reason for the many negative association studies in the case of this polymorphism was the failure to evaluate GEI.

Regional differences in CVD mortality exist in Europe, with a higher mortality rate in Northern Europe compared to Southern Europe (Sans et al., 1997; Keys, 1975). CHD differences between populations are only partly explained by differences in classical CHD risk factors, so variation in genetic predisposition and subsequent GEI are likely to be important as well (Kuulasmaa et al., 2000). The aim of this study was therefore to investigate whether the effect of the *ACE* I/D polymorphism on MI risk would differ by geographical location within Europe. The hypothesis was tested that this polymorphism would be interacting with a specific regional environment to determine the regional MI risk.

Methods

The HIFMECH study (Juhan-Vague et al., 2002) was designed to examine genetic and environmental mechanisms contributing to the higher cardiovascular risk in Northern compared to Southern Europe. Caucasian male first MI survivors were recruited from Northern Europe (Stockholm, London) and Southern Europe (Marseille, San Giovanni Rotondo) (n=532) with age matched healthy controls (n=574) from the same locations. For full details see Chapter 2.

ACE genotype using the PCR MADGE method (as described in Chapter 2) (O'Dell S.D. et al., 1995) was obtained in 523 cases and 560 controls.

Statistical analysis

Statistical analysis was performed using SPSS (version 12.1, SPSS Inc., Chicago) and 'Intercooled STATA' package (version 8.2, STATA Corporation, Texas). The analysis was performed by Jackie Cooper and myself. The *a priori* hypothesis was followed, and analysis was carried out in the North and South separately by cases and controls. Differences in baseline variables between North and South (by cases and controls) were analysed by ANOVA if normally distributed (or after appropriate transformation), and by Chi-squared tests if categorical. These differences were adjusted for age in view of the likely confounding effect it could play on each variable. Conditional logistic regression models were used for comparison of variables from cases and controls by region. Deviations of allele frequencies from Hardy-Weinberg equilibrium were considered using chi-squared tests. Allele frequencies are shown with the 95% confidence interval.

Analysis of variance was used to assess the association between genotypes and baseline characteristics on normally distributed data, or after appropriate transformation. Chi-squared tests were used to compare differences in categorical variables by genotype. Odds Ratio for MI in genotype groups were calculated using conditional logistic regression models and subsequently adjusted for important CHD risk factors - age, smoking, cholesterol, triglyceride and BMI. Controls were matched to cases within centres so analysis took account of the centre. Interactions were assessed by standard methods as deviations from multiplicative effects.

Results

For all samples the distribution of genotypes were as expected for Hardy-Weinberg equilibrium. There were no significant differences between any trait in the whole and genotyped sample (Table 3.1).

Table 3.1 Differences in variables between total sample and genotyped sample

		Healthy Controls		Cases	
Variable		North	South	North	South
Age (years)	Total sample	52.7 (5.0) n=253	50.5 (5.6) n=321	53.1 (5.1) n=233	51.0 (5.5) n=299
	Genotyped sample	52.7 (5.0) n=245	50.4 (5.6) n=315	53.1 (5.1) n=232	51.0 (5.5) n=291
BMI* (kg/m^2)	Total sample	25.8 (3.1) n=253	26.4 (3.2) n=322	27.2 (3.4) n=229	26.9 (3.3) n=299
	Genotyped sample	25.8 (3.1) n=245	26.4 (3.2) n=316	27.2 (3.3) n=228	26.9 (3.3) n=291
SBP* / mmHg	Total sample	130.2 (15.8) n=251	126.2 (13.1) n=322	129.5 (17.7) n=228	126.4 (16.2) n=294
	Genotyped sample	130.1 (15.8) n=243	126.2 (13.1) n=316	129.5 (17.8) n=227	126.5 (16.3) n=286
Chol	Total sample	5.71 (0.99) n=233	5.39 (0.94) n=321	5.66 (1.22) n=215	5.18 (1.11) n=286
	Genotyped sample	5.71 (0.99) n=225	5.39 (0.94) n=315	5.67 (1.21) n=214	5.17 (1.12) n=278
TG[1]	Total sample	1.52 (0.60) n=233	1.39 (0.61) n=321	1.99 (0.83) n=215	1.79 (0.72) n=286
	Genotyped sample	1.51 (0.59) n=225	1.39 (0.61) n=315	1.99 (0.83) n=213	1.78 (0.71) n=278
ApoB[+]	Total sample	98.2 (23.5) n=233	94.1 (20.2) n=321	107.7 (28.1) n=215	96.5 (22.4) n=286
	Genotyped sample	98.0 (23.1) n=225	94.2 (20.3) n=315	107.8 (28.1) n=214	96.2 (22.5) n=278

TG Triglyceride
* geometric mean (approx sd)
[1]means and sds calculated from log-transformed data
+means and sds calculated from square root transformed data

P values for all comparisons between total and genotyped sample were non-significant

The baseline characteristics by cases and controls, and genotype distributions in the North and South are shown in Table 3.2. With regard to controls, in the North they had a significantly lower BMI and a higher SBP, cholesterol, triglycerides and apoB than those in the South, with no significant difference in smoking. For cases, in the North they had a significantly higher SBP (borderline), cholesterol, triglycerides and apoB than those in the South. There was a trend for a higher BMI in the North and smoking rate was substantially higher in the South than the North. In terms of case-control differences, in the North the cases had significantly higher BMI, triglycerides, apoB and much higher smoking rates than controls. The reason for the lower (non-significant) SBP and cholesterol in cases may be related to the medications given to MI survivors. In the South, cases had a higher BMI, SBP (trend), triglycerides, apoB (trend), and smoking levels than controls. Again, the lower cholesterol in the cases may be related to medication.

Table 3.2 (overleaf) Baseline characteristics of genotyped sample including *ACE* gene frequencies

Values are mean (SD); n, number of subjects in group; BMI, body mass index; BP, blood pressure; (1) Geometric mean. and SD calculated from log-transformed data. (2) Mean and SD calculated from square root transformed data. (3) values adjusted for age. (4) p-values for continuous variables are from analysis of variance, unless otherwise stated, p-values for categorical variables are from chi-squared tests. (5) p-values are from conditional logistic regression models.

	Controls			Cases			Cases vs Controls	Cases vs Controls
	North	South	North-South difference(3),(4)	North	South	North-South difference(3),(4)	in N (5)	in S (5)
Age (years)	52.7 (5.0) n=245	50.4 (5.6) n=315	<0.0001	53.1 (5.1) n=232	51.0 (5.5) n=291	<0.0001	0.37	0.23
BMI (1) (kg/m²)	25.8 (3.1) n=245	26.4 (3.2) n=316	0.03	27.2 (3.3) n=228	26.9 (3.3) n=291	0.47	<0.0001	0.02
Systolic (1) BP (mmHg)	130.1 (15.8) n=243	126.2 (13.1) n=316	0.001	129.5 (17.8) n=227	126.5 (16.3) n=286	0.05	0.67	0.79
Cholesterol (mmol/L)	5.71 (0.99) n=225	5.39 (0.94) n=315	<0.0001	5.67 (1.21) n=214	5.17 (1.12) n=278	<0.0001	0.66	0.01
Triglyceride (1) (mmol/L)	1.51 (0.59) n=225	1.39 (0.61) n=315	0.02	1.99 (0.83) n=213	1.78 (0.71) 278	0.003	<0.0001	<0.0001
Apo B (2) (mmol/L)	98.0 (23.1) 225	94.2 (20.3) 315	0.04	107.8 (28.1) 214	96.2 (22.5) 278	<0.0001	<0.0001	0.25
Smoking Current+Ex Never	157(62.1%) 96 (37.9%)	201 (62.4%) 121 (37.6%)	0.93	179 (76.8%) 54(23.2%)	259(86%) 41 (14%)	0.004	<0.001	<0.001
Genotype distribution II/ID/DD	58/110/77	46/156/114		50/117/65	44/156/92			
D Allele frequency (95% CI)	0.54 (0.49-0.58)	0.61 (0.57-0.65)		0.53 (0.49-0.58)	0.58 (0.54-0.62)			

ACE genotype

The frequency of the D allele was higher in the South than North in both cases and controls, and there was no significant difference in genotype distribution between cases and controls in either the North or the South (Table 3.2). When the odds (adjusted for age, smoking, cholesterol, triglycerides and BMI) of being a case by *ACE* genotype was calculated, the presence of a D allele conferred a 36 % (p=0.29) higher risk of being a case compared to the II genotype in the North, whereas in the South there was only a 7% higher risk (p=0.81) (Table 3.3). The genotype by North-South interaction was not significant (p=0.48) and in the combined data for North and South there was a 20% (p=0.2) higher risk of being a case when having a D allele. The number required to detect this interaction with 80% power at the 5% level is 1465 cases and the same number of controls.

In order to explain the higher MI risk in the North, interaction between *ACE* genotype and those factors that were significantly higher in controls (and cases) in the North (Table 3.2) - triglyceride, apoB, cholesterol and SBP were examined. With a triglyceride above 1.57mmol/L (median value), the odds of having a MI in the North with a D allele was 2.95 (95% CI 1.50-5.79) vs 2.05 (95% CI 1.08-3.90) in the South. With apoB above 98.5 mg/dL (median value) the odds of having a MI in the North with a D allele was 2.33 (95% CI 1.13-4.83) vs 1.25 (95% CI 0.65-2.40) in the South. For both the triglyceride and apoB however, the interaction term was not significant, and these interactions are depicted in Figure 3.1A and B. The cholesterol interaction was far less clear cut: the D allele with a cholesterol above 5.4mmol/L (median value) conferred a 71% higher MI risk in the North vs 53% in the South (both results non-significant) but MI risk was highest in the South with a cholesterol less than 5.4 with II genotype – 2.87

(95% CI 1.14 – 7.19). Genotype interaction with SBP showed no consistent association and was not significant (not shown). Interaction with smoking was not carried out in view of rates being higher in the South than North.

Table 3.3 Odds Ratios of MI in North and South Europe with particular reference to lipid measures

	Odds of MI in North (95% CI)			Odds of MI in South (95% CI)	
Unadjusted	II	1.00	p=0.40	1.00	p=0.88
OR	ID/DD	1.22 (0.77-1.92)		0.97 (0.62-1.51)	
Adjusted	II	1.00	p=0.29	1.00	p=0.81
OR*	ID/DD	1.36 (0.77-2.42)		1.07 (0.63-1.81)	
Triglyceride	II	1.00	p=0.75	1.00	p=0.88
<1.57	ID/DD	1.09 (0.52-2.27)	(1)	0.88 (0.47-1.65)	(1)
	II	2.31 (0.99-5.35)		2.16 (0.87-5.37)	
>1.57	ID/DD	2.95 (1.50-5.79)		2.05 (1.08-3.90)	
ApoB	II	1.00	p=0.45	1.00	p=0.81
<98.5	ID/DD	1.02 (0.47-2.22)	(1)	1.04 (0.55-1.95)	(1)
	II	1.54 (0.63-3.75)		1.35 (0.57-3.20)	
>98.5	ID/DD	2.33 (1.13-4.83)		1.25 (0.65-2.40)	
Cholesterol	II	1.47 (0.60-3.60)	p=0.36	2.87 (1.14-7.19)	p=0.09
<5.4	ID/DD	1.54 (0.77-3.05)	(1)	1.89 (0.93-3.84)	(1)
	II	1.00		1.00	
>5.4	ID/DD	1.71 (0.89-3.29)		1.53 (0.73-3.17)	

OR – odds ratio calculated using conditional logistic regression model. * adjusted for age, smoking, cholesterol, triglyceride, BMI. (1) p value for interaction with variable. Controls are matched to cases within centres so analysis takes account of centre

Figure 3.1A Odds Ratio of MI by *ACE* genotype in those below and above sample median of Triglycerides

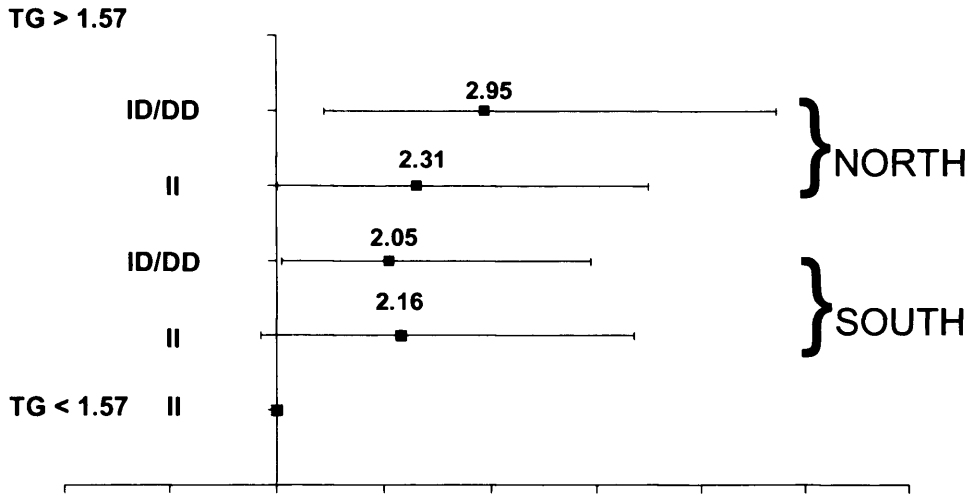
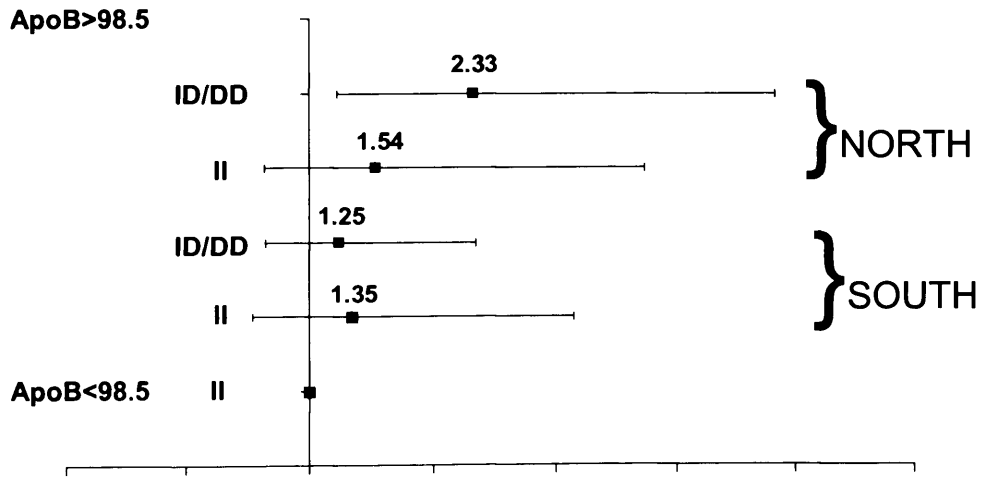


Figure 3.1B Odds Ratio of MI by *ACE* genotype in those below and above sample median of ApoB



Discussion

No statistically significant association between genotype and MI risk was demonstrated however this was not unexpected. A modestly higher odds ratio for MI associated with the D allele was seen in the North , 1.36 (0.77-2.42), which was consistent with the meta-analysis risk estimate of 1.22 (1.11 -1.35) (Morgan et al., 2003). This result supports the view that *ACE* genotype, in conjunction with other factors, is an important but modest contribution to the development of pathological cardiovascular phenotypes. In the HIFMECH study, the controls and cases in the North had higher levels of cholesterol, triglyceride, apoB and a higher SBP than their counterparts in the South. The results here suggest that there is an interaction between one or more of these factors and the D allele in determining risk of MI. Despite the most recent literature of interaction with *ACE* and SBP and smoking, these are unlikely to be playing a role in this sample. Though the SBP was significantly higher in the North than South in controls and cases the actual difference was small (of the order of only 3 - 4mmHg) and the SBP would certainly have been reduced by drugs in cases. Smoking prevalence was higher in the South than North in controls and cases.

The interaction identified here is between the *ACE* D allele, elevated triglycerides or ApoB and North Europe, in that D allele carriers with elevated lipid measures in the North have a substantially (though non-significantly) higher MI risk than D allele carriers with elevated lipid measure in the South. The interaction is more convincing for ApoB where there is also a marked difference in MI risk not only between D allele carriers in North and South, but also between Northern D allele carriers and II homozygotes who have elevated ApoB levels - 2.33 (1.13-4.83) vs. 1.54 (0.63-3.75).

Cambien *et al* (Cambien et al., 1992) initially reported an *ACE* DD genotype interaction with plasma ApoB levels, finding a risk ratio of 3.2 (95% CI 1.7-5.9) with low ApoB (< 1.25g/L) and low BMI (< 26kg/m^2) but this has not been confirmed by others (Keavney B. et al., 2000). The data here supports the opposite, with the D allele risk being statistically significant only in those with high ApoB or triglycerides.

There are possible mechanisms through which this gene-lipid-region interaction may be occurring. The higher AngII (and lower Bradykinin) levels (in plasma and tissues e.g. arterial wall and myocardium) would lead to a pro-atherosclerotic state in D allele carriers through multiple mechanisms, including endothelial ROS production with subsequent endothelial dysfunction (Wang et al., 2001; Anderson et al., 2000) and SMC hyperplasia (Daemen et al., 1991). Elevated lipid components including apoB and triglycerides are robust CHD risk factors (Hokanson and Austin, 1996)(St Pierre et al., 2006) with evidence for causality - excess intracellular triglycerides promoting increased oxidative stress and inflammation (Bakker et al., 2000) and apoB being intimately involved in lipid metabolism (Chapman et al.,1988). The combination of elevated apoB or triglycerides and raised AngII would therefore predispose certain individuals to higher CHD risk. There is also evidence for additional effects between lipid and AngII. Hypercholesterolaemia has been shown to increase plasma AngII concentrations (Daugherty et al., 2004) and AngII is also involved in LDL oxidation and uptake (Keidar et al., 2001). It has therefore been suggested that there is a positive feedback relationship with high cholesterol levels increasing AngII and AngII increasing lipid uptake (Keidar et al., 2001). AngII has also been shown to increase triglycerides (Ran et al, 2004). It is therefore plausible that there may be a positive interaction between elevated lipid status and *ACE* I/D polymorphism on CHD risk.

Why this risk is present in Northern Europeans and not Southern Europeans is probably due to the presence of other gene variants or environmental stimuli (e.g. Mediterranean diet) that differ between the two regions, affording protection in South Europe or harm in North Europe.

This study was underpowered to detect an effect by the *ACE*(I/D) genotype on MI risk by region. The earlier power calculation demonstrated almost three times more cases and controls are needed to confirm or refute if the previous size of effect is correct. This again is no surprise when the modest effect of the polymorphism is considered. The case-control design also introduces survivor bias, in that the reason for a genotype being overexpressed in the cases could be due to it conferring a survival advantage. Subjects from the Northern centres (Stockholm and London) and Southern centres (Marseille and San Giovanni Rotondo) were also not uniform when examined with differing levels of blood pressure and triglycerides. The suggested evidence for *ACE* lipid interaction was confounded by a large proportion of cases being on lipid-lowering treatment. However regional differences in all three lipid measures in cases were highly significant and of similar magnitude when those cases not on therapy were analysed (Table 3.4).

Table 3.4 Differences in plasma lipid concentrations amongst cases not on lipid-lowering drugs according to region in Europe

	Cases NOT on lipid-lowering drugs		
Variable	North	South	N vs S[3,4]
Cholesterol (mmol/L)	5.93 (1.08) n=158	5.30 (1.08) n=198	<0.001
Triglycerides (mmol/L)[1]	2.05 (0.85) n=158	1.81 (0.75) n=198	0.005
Apo B (mg/dL)[2]	115.1 (23.7) n=158	97.7 (21.8) n=198	<0.001

Values are mean (SD) with levels adjusted for age
[1] Means given are the antilog of the mean of log transformed data; [2] Means given are the square of the mean of square root transformed data; [3] Values are adjusted for age; [4] P-values are from analysis of variance

In conclusion, *ACE* genotype may interact with higher triglycerides or apoB in males in the North Europe to influence MI risk. This may account for a part of the difference in CHD mortality between North and South Europe. Further large-scale studies involving this polymorphism in cardiovascular disease, avoiding direct associations but rather targeting interactions with lipids / lipoproteins and other factors, are warranted.

Chapter 4: **Impact of *ACE*(I/D) polymorphism on Coronary Heart Disease: Interaction with Systolic Blood Pressure**

Introduction

As already detailed in the thesis, there is considerable evidence for the involvement of the RAS in the development of atherosclerosis and LVH (Talmud et al., 2007)(Mehta and Griendling, 2007). CHD risk and mortality from heart failure are decreased by ACE inhibitors (Yusuf et al., 2000; SOLVD Investigators, 1991). In view of the significant association between the *ACE* I/D polymorphism and tissue and plasma ACE levels (Rigat.B. et al., 1990; Danser et al., 1995), one might anticipate the *ACE* D-allele to be associated with excess CHD risk. Cambien *et al* were the first to report such an observation (Cambien et al., 1992) however, as in the majority of single polymorphism associations studies further results have been inconsistent, and meta-analysis of published data suggest the impact of *ACE* genotype on MI risk to be modest (relative risk associated with DD genotype of approximately 1.2 (Agerholm-Larsen et al., 2000; Morgan et al., 2003)).

Such inconsistency and weakness of effect may, however, reflect the interaction of *ACE* genotype with environmental factors in determining risk. Indeed, accruing evidence suggests that the *ACE* gene may modulate the development of complex phenotypes through interaction with stimuli such as smoking, where the D-allele has been associated with higher CVD mortality (Sayed-Tabatabaei F.A. et al., 2005). The

previous chapter provided evidence for a possible interaction with lipids in North Europe on CHD risk.

Hypertension is another environmental stimulus that could interact with *ACE*. Hypertension and RAS are intimately linked with RAS activation, probably preceding its development, but both are likely acting in positive feedback. AngII is heavily involved in the vascular remodelling that is a hallmark of the vascular insult in hypertension, inducing smooth muscle cell hypertrophy, collagen deposition, endothelial dysfunction through ROS production, and cytokine secretion (Albaladejo et al., 1994; Touyz et al., 2002; Kakar and Lip, 2007). RAS also plays an important role in cardiac hypertrophy through cardiomyocyte hypertrophy (Li et al., 2002) and myofibroblast remodeling (Siwik et al., 2001). There is also accumulating evidence for AngII in the formation of atherosclerosis causing matrix deposition (Castoldi et al., 2003), and increased vascular permeability (Tamarat et al., 2002).

In view of the systemic effects of hypertension, with diverse tissues being affected, it is not surprising that gene expression is altered, with animal models demonstrating changes in gene expression in several pathways including upregulation of growth promoters, inhibition of growth inhibitors, with upregulation of genes involved in RAS and AngII signalling (Grayson et al., 2007). These gene expression changes (at a transcriptional, translational or post-translational level) may be further magnified by the inherent presence of specific genotypes, which in the context of hypertension are now 'harmful'. It is thus very clear to see why, in hypertensive individuals, or groups who have higher plasma and tissue levels of ACE i.e. those that carry the D allele of I/D polymorphism), CHD risk is made even higher.

In Chapter 1, gene variants in other RAS components (Bradykinin Receptor 1 and 2, and Angiotensin II Receptor Type 2) were discussed that have been shown to combine with blood pressure to raise CHD risk (Jones et al., 2003; Dhamrait et al., 2003). A common functional variant in the gene for lipoprotein lipase (LPL) - the S447X polymorphism has also demonstrated an interaction with SBP on CHD risk (Talmud et al., 2007). In terms of the I/D polymorphism, the D-allele has been associated with a greater LVH in hypertensive subjects (Iwai et al., 1994), and interestingly, an interaction with blood pressure may exist, where the D-allele was associated with a higher risk of heart failure amongst hypertensives (Schut et al., 2004).

We therefore hypothesised that *ACE* genotype might interact with SBP to determine CHD risk. We tested this hypothesis in the Second Northwick Park Heart Study (NPHSII), which offers 15 year prospective follow-up study of middle-aged men healthy at enrollment. In view of this similar pattern of gene-blood pressure interaction with regard to *LPL* S447X, the combined effects of the *ACE* I/D and *LPL* S447X and SBP on CHD risk were also determined.

Methods:

Subjects comprised those recruited from the prospective Second Northwick Park Heart Study (NPHSII), which is described in Chapter 2, and in more detail in (Miller et al., 1995).

DNA was available for 2782 eligible men. Genotypes were determined (as described in Chapter 2) by PCR MADGE method for *ACE* (O'Dell S.D. et al., 1995) in approximately 87% of samples and using TaqMan technology for rs4341 (in complete LD with the *ACE* I/D polymorphism) as described in (Tanaka et al., 2003) for the remaining samples. One DNA array (94 samples) was genotyped for both *ACE* I/D and rs4341 polymorphisms with 100% concordance.

Statistical Analysis was performed using 'Intercooled STATA' (version 8.2, STATA Corporation, Texas) by Jackie Cooper. Baseline characteristics were transformed to a normal distribution as appropriate. Results are presented as hazard ratios (HR) obtained from Cox regression models with their corresponding 95% confidence interval (CI). All models included baseline age as a covariate and were stratified by practice in order to take account of modest differences in the baseline hazard by recruitment site. Interactions were tested as deviations from multiplicative effects. Frequencies were compared by Chi-squared test. Estimated probabilities were obtained from logistic regression models and plotted to illustrate the increase in risk with blood pressure. Additive effects were determined by fitting coding genotype according to the number of rare alleles carried.

Results

ACE genotype data was obtained in 2711 men (97% subjects with DNA), whose baseline characteristics were not significantly different from the whole group (not shown). After 15 years of follow-up, 269 men with *ACE* genotype had developed CHD. Established risk factors were all associated with increased risk of development of CHD (Table 4.1). The distribution of genotypes was as expected for Hardy-Weinberg equilibrium. As shown in Table 1 there was no significant difference between those who developed CHD and those that did not in *ACE* genotype distribution ($p=0.95$) or D allele frequency ($p=0.81$). There was no significant difference in any risk factor between the different genotype groups (Table 4.2), in particular for SBP ($p=0.97$) and DBP ($p=0.82$).

Table 4.1: Baseline characteristics by CHD in NPHSII men with *ACE* genotyping

Characteristics	No CHD N=2442	With CHD N=269	P value
Age (years)	56.0 (3.4)	56.6 (3.5)	0.008
BMI*(kg/m^2)	26.2 (3.4)	26.7 (3.3)	0.01
SBP*(mmHg)	136.7 (18.7)	141.4 (19.2)	<0.0001
DBP (mmHg)	84.3 (11.4)	87.0 (11.4)	0.0003
Smoking [%(N)]	27.3 (666)	37.2 (100)	0.001
Cholesterol (mmol/L)	5.70 (1.01)	6.08 (1.03)	<0.0001
Triglyceride*(mmol/L)	1.77 (0.93)	2.05 (1.06)	<0.0001
Fibrinogen*(g/l)	2.70 (0.52)	2.80 (0.49)	0.003
CRP*(mg/l)	2.40 (2.43)	3.26 (3.37)	<0.0001
Genotype distribution II/ID/DD	579/1194/669	64/134/71	0.95
D Allele frequency	0.518	0.513	0.81

*geometric mean (approx sd)

Table 4.2 Baseline characteristics by *ACE* genotype in NPHSII

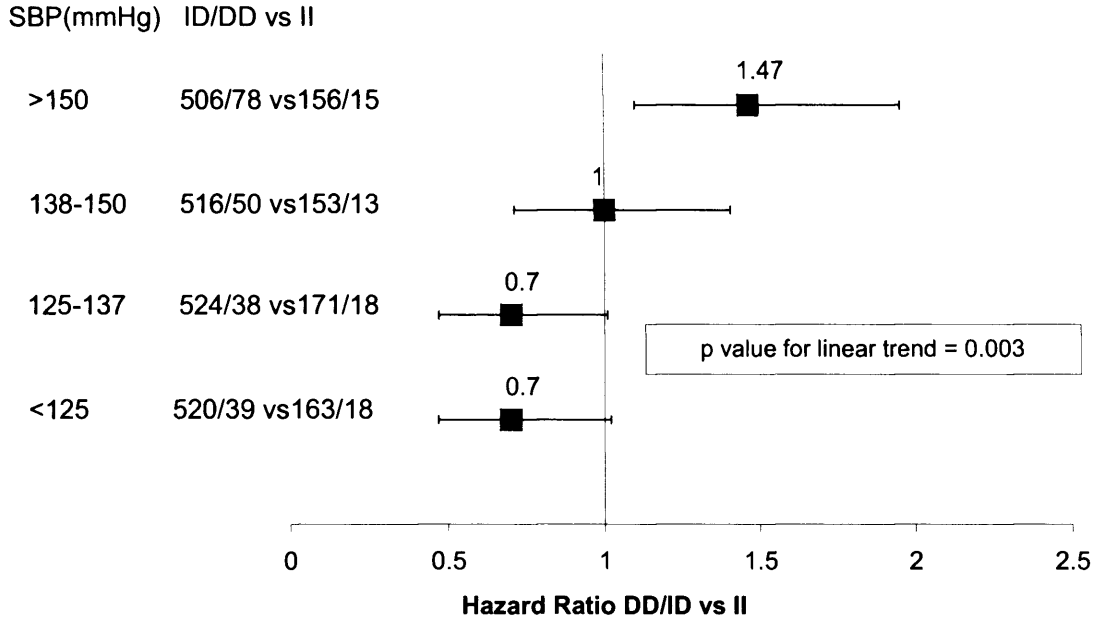
	II N=643	ID/DD N=2068	P value
Age	56.1 (3.4)	56.1 (3.4)	0.94
BMI†	26.2 (3.5)	26.3 (3.3)	0.77
SBP†	137.1 (19.2)	137.1 (18.6)	0.97
DBP	84.7 (12.1)	84.6 (11.2)	0.82
Smoking	29.6 (190)	27.9 (576)	0.40
Cholesterol	5.75 (1.04)	5.73 (1.01)	0.74
Triglyceride†	1.80 (0.97)	1.80 (0.94)	0.98
Fibrinogen†	2.69 (0.52)	2.71 (0.51)	0.37
CRP†	2.36 (2.35)	2.50 (2.57)	0.27

†geometric mean (approx sd)

Since SBP was an established risk factor for development of CHD and there is evidence for *ACE* genotype SBP interaction in other diseases, their interaction on 15 year risk of development of CHD (adjusted for age and practice) was examined. Figure 4.1 demonstrates analysis of CHD risk for D-allele carriers by quartiles of SBP (additive model). In the lower two quartiles there was a trend for a protective effect by the D-allele, with thereafter a stepwise rise in risk for the D-allele, such that in the highest quartile of SBP (>150mmHg), CHD risk of the D-allele carrier was almost 1.5 times that of II homozygotes (genotype SBP interaction for p=0.003). A similar result was seen with the dominant model for D-allele (interaction p value 0.004). When the results were also adjusted for cholesterol, triglycerides and smoking (classical risk factors for

CHD significantly different between cases and controls in NPHSII – see table 4.1), a similar pattern of association was seen (interaction p value 0.004).

Figure 4.1: Hazard Ratio for each additional D allele by quartile of SBP. ID+DD [Number of controls/Number developing CHD] v II [Number of controls/Number developing CHD]. Reference group is specific for each SBP quartile – it is the ratio of number of individuals with II genotype who develop CHD after 15 years to the total number of II controls within the same SBP quartile.



When SBP was examined as a continuous variable (Figure 4.2A), the HR for a 1 standard deviation (SD) increase in SBP was 0.90(0.70-1.15) for IIs and 1.40(1.21-1.61) for ID/DD (genotype SBP interaction p=0.002). Thus, compared to the common D-allele carriers (66.3% of men), who showed the expected effect of increasing CHD risk with increasing blood pressure, the *ACE* II men (23.7% of the group) were protected from this blood pressure risk effect. Additional adjustment for cholesterol, triglyceride and smoking led to a very similar pattern, with the HR for a 1 SD increase in SBP being 0.85[0.66-1.09] for IIs and 1.32[1.14-1.52] for ID/DD (genotype SBP interaction p=0.002).

Figure 4.2A: Probability of CHD by SBP in NPHSII subjects stratified by *ACE* (I/D) genotype. ID+DD [Number of controls/Number developing CHD] = [1863/205] ; II [Number of controls/Number developing CHD] = [579/64]

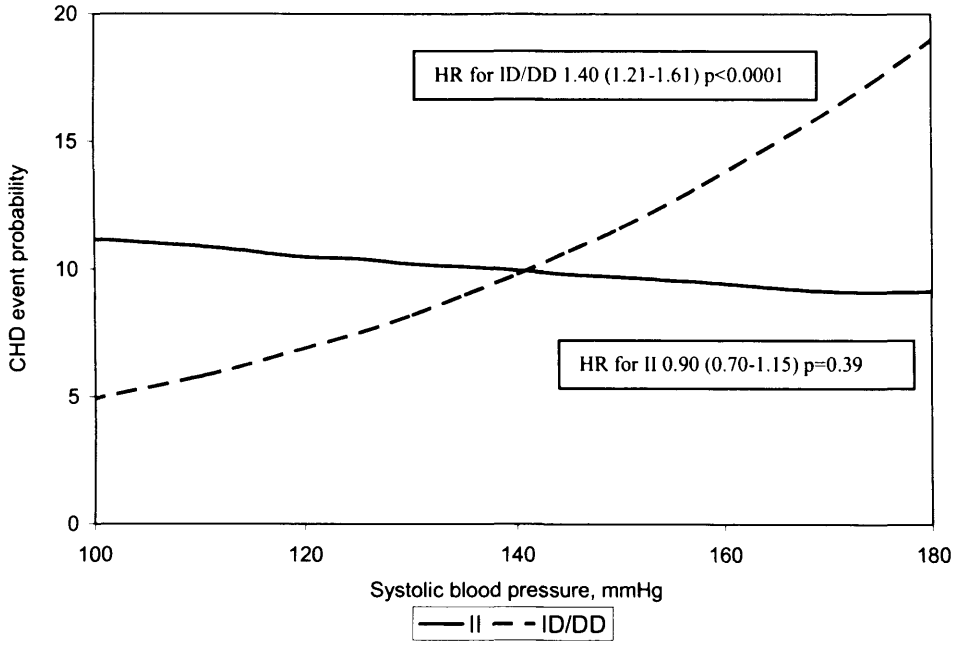
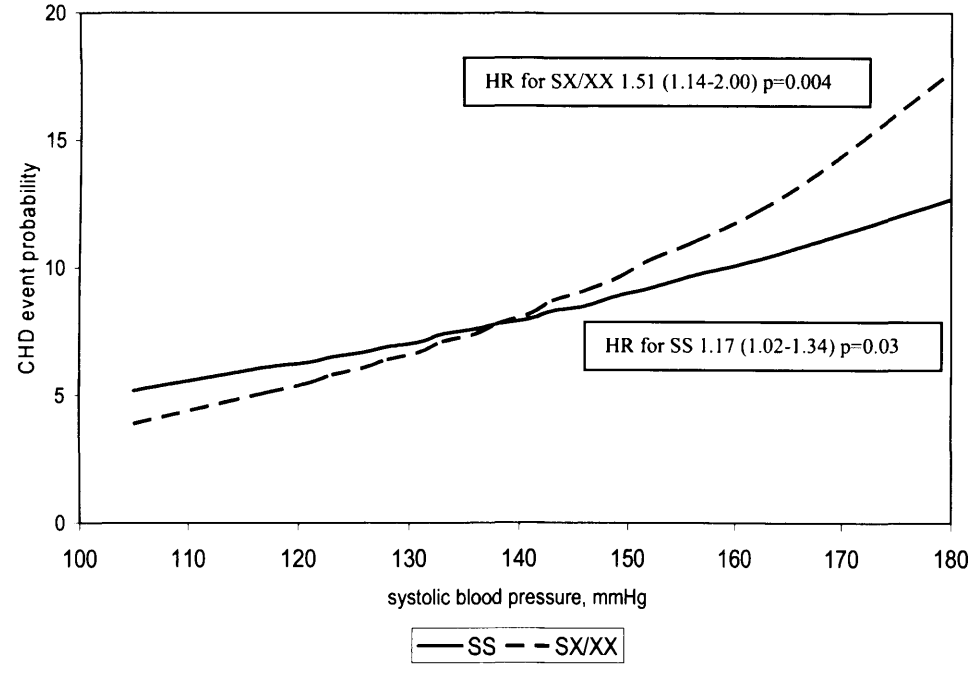


Figure 4.2B: Probability of CHD by SBP in NPHSII subjects stratified by *LPL* S447X genotype. SX+XX [Number of controls/Number developing CHD] = [483/44] ; SS [Number of controls/Number developing CHD] = [2014/175] (Talmud et al., 2007)



Effect on risk of combined genotypes

The combined effects of the *ACE* I/D, *LPL* S447X and SBP on CHD risk were examined. As shown in (Talmud et al., 2007) and in Figure 4.2B, increasing blood pressure had a greater effect on risk in X447 allele carriers than in S447 homozygotes (interaction significant in categorical analysis). 2665 men were successfully genotyped for both polymorphisms. When the men were grouped into *LPL* SS or SX+XX subjects, and stratified by *ACE* II or ID+DD groups (Table 4.3 and Figure 4.2C), the commonest genotype group of the *ACE* D-allele carriers who were also *LPL* SS homozygotes (61.3% of the men), showed the expected effect of increasing CHD risk with increasing blood pressure, with a HR for a 1SD increase in SBP of 1.31 (1.12-1.53) p=0.001. As would be predicted if the genotype risk effects were additive, the largest effect of increasing blood pressure on CHD risk was seen in the *ACE* D-allele carriers who were also *LPL* X-allele carriers (14.9% of the men) who had a HR of 1.78 (1.30-2.45) p<0.0001. By contrast, there was no evidence for a significant effect of increasing blood pressure in those with the *ACE* II and *LPL* SS or *ACE* II *LPL* SX+XX combined genotype, with the HR estimates for both of these groups being significantly lower than for their D-allele carrier counterparts (p=0.02 for both comparisons). These results demonstrate that a rise in SBP only leads to a significant rise in CHD risk with carriage of the *ACE* D-allele, with the risk being even greater in those who also carry the *LPL* X-allele. This association was maintained when also adjusting for cholesterol, triglycerides and smoking (table 4.4). A similar result was obtained when SBP was analysed as a categorical variable (above and below median of 137 mmHg). Carriers of the D allele and X allele had a HR of 0.42 (p=0.02) compared to those with II and SS genotypes in the low risk environment (SBP<137 mmHg), but in those with SBP>137mmHg, D and

X allele carriers had a higher CHD risk compared to their II and SS counterparts with HR 1.60 (p=0.10).

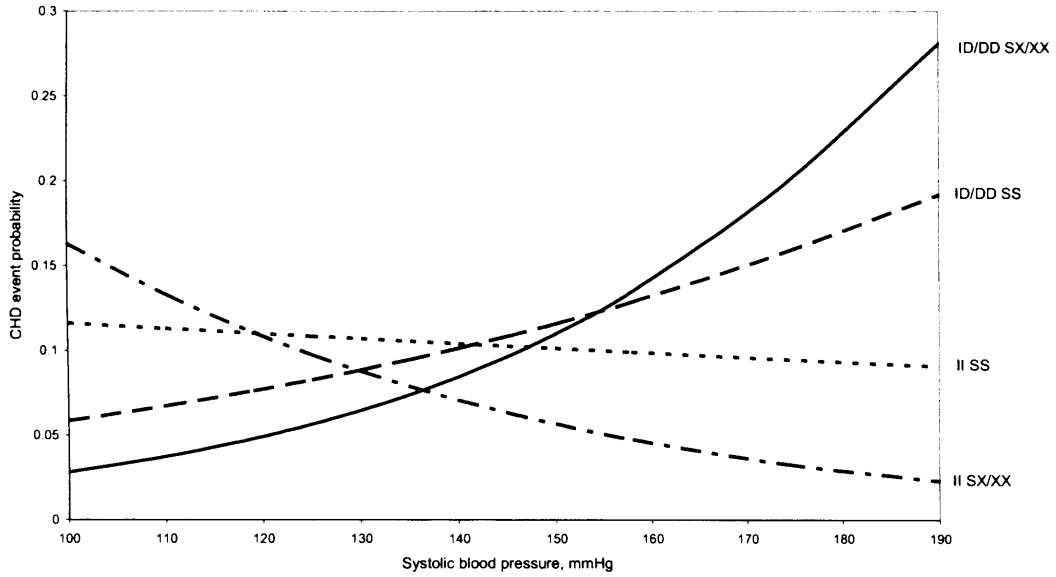
Table 4.3: Interaction of SBP as a continuous variable with *ACE* (I/D) and *LPL* S447X on CHD risk in NPHSII subjects

***ACE* I/D**	***LPL* S447X** (Number of controls with both genotypes / Number of CHD cases with both genotypes)	**HR for 1 SD increase in SBP** (95% CI)	**P value** (adjusted for age and practice)	**Interaction *ACE* (I/D) x *LPL* S447X x SBP**
II	**SS** (460/54)	0.91 (0.70-1.19)	0.49	
	SX/XX (110/9)	0.68 (0.33-1.40)	0.29	
ID/DD	**SS** (1467/167)	1.31 (1.12-1.53)	0.001	P=0.16
	SX/XX (362/36)	1.78 (1.30-2.45)	<0.0001	

Table 4.4: Interaction of SBP as a continuous variable with *ACE* (I/D) and *LPL* S447X on CHD risk in NPHSII subjects with adjustment for age, practice, cholesterol, triglycerides and smoking

***ACE* I/D**	***LPL* S447X** (Number of controls with both genotypes / Number of CHD cases with both genotypes)	**HR for 1 SD increase in SBP** (95% CI)	**P value**	**Interaction *ACE* (I/D) x *LPL* S447X x SBP**
II	**SS** (460/54)	0.87 (0.67-1.14)	0.32	
	SX/XX (110/9)	0.60 (0.29-1.44)	0.17	
ID/DD	**SS** (1467/167)	1.22 (1.04-1.44)	0.02	P=0.11
	SX/XX (362/36)	1.69 (1.23-2.32)	0.001	

Figure 4.2C Probability of CHD by SBP in NPHSII subjects stratified by *LPL* S447X and *ACE* (I/D) genotype. [For number of samples see table 4.2]



Discussion

In view of the increasing evidence for the *ACE* gene acting as a modifier for CVD (Sayed-Tabatabaei F.A. et al., 2005; Schut et al., 2004) and that SBP is a powerful CHD risk factor with a large evidence base for worsening and furthering RAS involvement, the hypothesis was tested that prospective CHD risk by *ACE* genotype would be influenced by SBP. As expected, no overall genotype risk association was found, but analysis by quartiles of SBP revealed a differing genotype CHD risk based on SBP, and possession of the D allele was associated with higher risk when SBP was elevated. There is a precedent for such a proposed interaction: amongst hypertensive subjects, those carrying the *ACE* D-allele have been reported to be at higher risk of heart failure (Schut et al., 2004).

This impact of the *ACE* I/D polymorphism, and its interaction with SBP in determining CHD risk, is likely to depend upon associated differences in tissue ACE activity (Danser et al., 1995; Rigat.B. et al., 1990), and consequent AngII generation. AngII has multiple proatherosclerotic and prohypertrophic properties (Talmud et al., 2007) (Li et al., 2002; Wenzel et al., 2001), with LVH being an independent CHD risk factor (Levy et al., 1988). Indeed the *ACE* D-allele has been associated with a greater LV growth response to a variety of stimuli (Lechin et al., 1995)(Estacio et al., 1999)(Montgomery et al., 1997) and has been associated with hypertensive LVH (Iwai et al., 1994). ACE may also promote atherosclerosis through inactivating bradykinin, a vasodilator with anti-fibrinolytic properties (Strauss et al., 2006). Whatever the mechanism, ACE inhibition reduces CHD event rate amongst patients with vascular disease (Yusuf et al., 2000) and is associated with decrease in LVH in hypertensives (Mathew et al., 2001).

The reason why D allele carriers have a higher CHD risk when hypertensive compared to when normotensive may be due to combination of the increased AngII (and decreased Bradykinin) together with the altered gene expression in the hypertensive state. Hypertension activates vascular RAS itself (Reja et al., 2006; Zhou et al., 2003) with upregulation of genes in RAS and AngII signaling pathway (Grayson et al., 2007). Changes in lipid metabolism gene expression have also been seen, which would also support the *LPL* S447X blood pressure interaction (Grayson et al., 2007).

Whilst the absence of the hypertensive milieu might have accounted for the absence of detrimental impact of the *ACE* D-allele on CHD risk amongst those with lower SBP, the suggested *protective* effect of the D-allele in this group remains unexplained. One might postulate that other factors are at work: the D allele may be associated with improved

insulin sensitivity (Panahloo et al., 1995) and a mouse model has suggested that greater *ACE* expression was associated with less weight gain (Heimann et al., 2005). Rather than a definitive benefit in this situation, it may be more probable that there is a lower risk without the presence of high blood pressure. For AngII to have a damaging effect it may need to be in the setting of hypertension where gene expression has been substantially altered, allowing processes such as endothelial dysfunction or LVH to have already been instigated. In the context of normotension where there is unaltered gene expression with no raised tissue stress, there may be less substrate for raised AngII levels (e.g. lower Angiotensin II Receptor Type I density) therefore lower CHD risk. Whatever, the effect does not seem to have occurred by chance, being evident in both lower quartiles of SBP.

Analysis of the SBP interaction with two gene polymorphisms suggests that only in the presence of a harmful environment (e.g. hypertension) do their common variants become disease-modifying. Further analysis suggests that the combination of the two risk gene variants in the risk environment leads to an even higher risk (but no more than expected). One explanation is that the raised LPL activity from the X allele of *LPL* S447X leads to a greater fatty acid availability and it has been suggested in (Talmud et al., 2007), in the context of myocardial and vascular stress e.g. hypertension, this will be detrimental, leading to vascular dysfunction or greater LVH, again leading to a higher CHD risk. The greater LVH or higher inflammatory stress from both gene variants acting together, may in some patients lead to a multiplicative interaction on risk but this was not demonstrated in this group of healthy subjects. The demonstration in rodents that AngII has also been shown to increase triglycerides (Ran et al., 2004) provides more evidence for interaction between these particular gene variants by delivering

further substrate for LPL. Further studies to examine this in patients, for example with heart failure, would be of interest.

At recruitment no subjects were taking ACE inhibitors, AngII receptor blockers, Beta receptor blockers or statins. Adjustment for anti-hypertensive medication use would be helpful but this data is unavailable for this prospective study based in general practice. Although adjusting for anti-hypertensive use might strengthen the conclusions, the lack of information is unlikely to confound the results. If there was a difference in incidence or treatment of hypertension in men by *ACE* genotype, this would be a potential confounder. This would occur if the *ACE* I/D polymorphism were associated with significant effects on SBP or DBP, however there is no strong evidence for such an association in the literature, and no evidence for a difference in mean SBP or DBP by *ACE* genotype in NPHSII men at baseline. Although there is some published evidence to suggest that subjects with essential hypertension (Arnett et al., 2006) with different *ACE* genotype, may respond differentially to treatment with different anti-hypertensive medications, unless hypertensive subjects with different *ACE* genotype were treated with different medication, which seems implausible, the reported genotype-medication interactions are unlikely to be a confounder of the associations we see with CHD events.

It is interesting to note (from Figure 4.2A) that in men with a SBP of below 140mmHg, when it is likely that no anti-hypertensives were being used in this cohort, the CHD risk of carrying the D allele increased with rising SBP as opposed to carriers of II genotype. This would argue against use of anti-hypertensives confounding the observed results. Furthermore, while those who did develop hypertension may have been treated, it

seems despite this, the CHD risk for individuals with the D allele and II genotype remained distinct, and continued as would be predicted.

It is important to note that treatment after CHD event would not confound these results since survival is only recorded up to the event. Furthermore in the UK, hypertensive middle-aged males asymptomatic for CHD would certainly have been in the minority with regard to adequate treatment, especially throughout the 1990s. The use of anti-hypertensives in this population, especially ACE-inhibitors would have been low. There is certainly no consistent pharmacogenetic interaction between ACE-inhibitors (and other anti-hypertensives) and outcome in hypertensive subjects in a recent comprehensive review (Arnett et al., 2006).

Another assumption is that these associations are explained by differing levels of ACE by *ACE* (I/D) genotype. To confirm this, measurement of plasma ACE levels would have been useful to look for correlation with genotype and levels, and levels and risk, but these measurements had not been made. However, there are strong and consistent published data which demonstrate that ACE levels in subjects with the *ACE* D allele are significantly higher than those with the I allele (Rigat.B. et al., 1990; Danser et al., 1995) and this effect remains the most likely direct mechanism.

It is of note that the mean baseline SBP levels in those that remained CHD free (mean 136.7mmHg for mean age of 56 years) was substantially higher than in the comparative group in the Framingham Heart Study (mean SBP of 129.2mmHg for mean age of 58 years). The explanation for this is not clear, and may be methodological or due to the two groups having different genetic and environmental factors. However the mean SBP

in this group of healthy men is representative of the UK, since compared with national figures (Department of Health, Health Survey of England (2003) http://www.dh.gov.uk/assetRoot/04/09/89/15/04098915.xls), the mean SBP in men in England in age groups 45-54 in 1993 and 1994 (the last 2 years at which recruitment occurred in NPHS2) were 138mmHg and 136mmHg respectively.

In conclusion demonstration of GEI in the pathogenesis of complex phenotypes is increasingly prevalent, especially with the strength of prospective studies (Humphries et al., 2003). Evidence from this study is the first to show interaction between the *ACE* gene and SBP in determining prospective risk of CHD, but replication is required to confirm these findings with appropriate pharmacogenetic evaluation.

Chapter 5: **Impact of *ACE* (I/D) polymorphism on Type 2 Diabetes Mellitus: Interaction with Obesity**

Introduction

As the RAS exists in relevant metabolically active tissues, namely adipose tissue, skeletal muscle and pancreas (Karlsson et al., 1998), it is possible that ACE plays a role in the pathogenesis of T2D. Ang II is known to be diabetogenic, causing insulin resistance through several routes (Ogihara et al., 2002). It interferes with insulin signalling, has a pro-inflammatory effect on tissue beds causing endothelial dysfunction (Hattori et al., 2005), inhibits adipocyte differentiation (Engeli et al., 2003), and causes beta cell dysfunction via oxidative stress (Leung and Carlsson, 2005). Intervention to disrupt the RAS, by the administration of ACE-inhibitors or Angiotensin II Receptor blockers (ARB), has been shown to prevent the development of T2D in high-risk individuals in a meta-analysis (Scheen, 2004). This raises the possibility that individuals with genetically lower levels of ACE may be protected from the development of T2D.

Since plasma and tissue ACE levels are strongly associated with the *ACE* I/D polymorphism (Rigat.B. et al., 1990; Danser et al., 1995), its impact on T2D risk has been widely studied with varying results. The D allele was associated with a higher T2D risk (OR=1.52,p=0.02) in a case-control study (Stephens et al., 2005). In those with T2D, D allele carriers have poorer outcomes, with a higher risk of MI and renal disease (Kennon et al., 1999), and a worse response when starting on an intensive hypoglycaemic medication regime (Salonen et al., 2006). Paradoxically, the D allele has

been associated with a higher insulin sensitivity (Panahloo et al., 1995). There were however a number of studies in which no association has been found between this genotype and T2D (Grammer et al., 2006; Schmidt and Ritz, 1997). The majority of these studies were limited by being cross-sectional and other usual design flaws with gene association studies.

Recently studies have suggested that the *ACE* gene modulates the development of disease phenotypes e.g. CVD and heart failure through interaction with key environmental factors e.g. smoking and SBP (Sayed-Tabatabaei F.A. et al., 2005) (Schut et al., 2004). Evidence from the previous two chapters have suggested interaction of *ACE* both with lipids and with SBP on CHD risk. There have been no reported *ACE* GEI influencing T2D, however combined SNP interactions or haplotype effects have been reported in diabetic nephropathy (Osawa et al., 2007; Ng et al., 2006).

Only a few GEI have been reported so far in T2D: An interaction between *PPARγ* Pro12Ala and obesity on T2D risk has been determined, with the Ala allele conferring protection only in those individuals with lower BMI (under 34 kg/m^2) (Florez et al., 2007a). An interaction between a nonsynonymous polymorphism in *APM*, (+276G>T) and obesity on T2D risk has been demonstrated, with an increased risk associated with +276T carriers in the obese men but not in the lean men (Gable et al., 2007b). An interaction between lifestyle intervention and *TCF7L2* rs7903146(C>T) on T2D risk has also been reported but was not statistically significant (Florez et al., 2006).

For T2D, the major environmental determinant of risk is obesity (Resnick et al., 2000) (Gable et al., 2006). Adipose tissue (especially when located centrally) is pro-

inflammatory, secreting TNFα and IL-6 (Kern et al., 1995; Fried et al., 1998) thereby actively worsening insulin resistance and increasing T2D risk.

The hypothesis that men with the low ACE II genotype would be protected from developing T2D in a prospective 15 year follow up study was therefore examined. In view of the raised inflammatory state in obesity, and AngII being pro-inflammatory, an interaction between obesity and *ACE* genotype in determining prospective T2D risk was also investigated.

Methods:

Subjects were recruited from NPHS2 and detailed elsewhere (Miller et al., 1995) and Chapter 2. Out of the initial 3012 recruits, 76 with T2D were excluded, and in 230, DNA could not be extracted. DNA was therefore available for 2706 eligible men and genotype obtained in 2642 subjects (97.5 %). There were only significant differences in SBP and fibrinogen between the group of 230 and 2706 eligible samples but the absolute differences were small and unlikely to have made an impact (Table 5.1). There were no difference in baseline characteristics between the 64 drop-outs and the 2642 subjects with *ACE* genotype (not shown). Two of those individuals without T2DM and one with T2DM did not have height recorded and so there are missing data on BMI in these three individuals. *ACE* genotypes were determined as stated in Chapter 2 and Chapter 4. Obesity was defined as BMI>30 kg/m^2. A dominant genetic model was assumed based on the results from previous studies (Salonen et al., 2006; Schut et al., 2004; Stephens et al., 2005).

Table 5.1: Baseline characteristics in those with and without DNA

	Without N=230	With N=2706	P value
Age	56.1 (4.0)	56.0 (3.4)	0.65
BMI†	25.9 (3.9)	26.2 (3.3)	0.21
SBP†	133.3 (18.4)	136.9 (18.8)	0.004
DBP	83.1 (11.5)	84.6 (11.4)	0.06
Smoking	33.3%(77)	28.4% (769)	0.11
Cholesterol	5.71 (1.05)	5.73 (1.01)	0.80
Triglyceride†	1.75 (0.88)	1.78 (0.93)	0.63
Fibrinogen†	2.79 (0.56)	2.71 (0.51)	0.05
CRP†	2.38 (2.59)	2.46 (2.51)	0.67

†geometric mean (approx sd)

Statistical Analysis was performed using 'Intercooled STATA' (version 8.2, STATA Corporation, Texas) by Jackie Cooper as detailed in Chapter 4.

Results:

ACE genotype data was obtained in 2642 men. After 15 years of follow-up, 153 men with *ACE* genotype developed T2D. The remaining 2489 men were alive and had not developed T2D. Baseline BMI, obesity, C-reactive protein (CRP), triglyceride, cholesterol and blood pressure were all associated with increased risk of development of T2D (Table 5.2). Alcohol consumption was not different between those who developed T2D and those who did not. The highest risk was that associated with obesity (HR 3.74(2.66-5.26) $p<0.0001$). When a stepwise model was used to determine which of these variables were independently associated, BMI remained the most significant predictor, with an age and practice-adjusted HR for one SD increase, of 1.91 ((1.64-2.24) $p<0.0001$).

There was no significant difference between those who developed T2D and those that did not, in *ACE* genotype distribution ($p=0.48$) or D allele frequency ($p=0.87$) (Table 5.2). Distribution of genotypes was as expected for Hardy-Weinberg equilibrium. HR (adjusted for age and practice) for developing T2D for DD+ID vs II was 0.91 (0.63-1.32) $p=0.63$. To eliminate the possibility of confounding it was confirmed that there were no significant differences in baseline BMI, blood pressure, lipid parameters, age, CRP or alcohol consumption between the different genotype groups (Table 5.3).

Table 5.2: Baseline characteristics by diabetes in NPHSII men with *ACE* genotyping

	No diabetes N=2489	With diabetes N=153	P value
Age(years)	56.0 (3.4)	56.2 (3.3)	0.53
BMI[a](kg/m^2)	26.1 (3.3)	28.6 (3.6)	<0.0001
Obesity[%(N)]	12.3 (307)	33.6 (51)	<0.0001
SBP[a](mmHg)	136.6 (18.7)	142.2 (19.1)	0.0004
DBP(mmHg)	84.5 (11.4)	86.5 (11.3)	0.03
Smoking[%(N)]	28.2 (702)	35.3 (54)	0.06
Cholesterol(mmol/L)	5.72 (1.01)	5.92 (0.98)	0.02
Triglyceride[a](mmol/L)	1.76 (0.92)	2.27 (1.05)	<0.0001
Fibrinogen[a](g/l)	2.70 (0.51)	2.77 (0.54)	0.12
CRP[a](mg/l)	2.41 (2.46)	3.38 (3.24)	0.0004
Alcohol			
% drinkers	80.7	77.8	0.37
Median units/wk	6 [1-16]	5 [1-18]	0.84
[IQR]			
Genotype distribution	584/1232/673	39/68/46	0.48
II/ID/DD			
D Allele frequency	0.518	0.523	0.87
(95% CI)	(0.504-0.532)	(0.465-0.580)	

[a]geometric mean (approx sd); [IQR] – Interquartile Range

Table 5.3 Baseline characteristics by *ACE* genotype in NPHSII

	II N=623	ID/DD N=2019	P value
Age	56.0 (3.4)	56.0 (3.4)	0.99
BMI†	26.2 (3.4)	26.2 (3.3)	0.75
SBP†	137.0 (19.1)	136.9 (18.6)	0.96
DBP	84.7 (12.1)	84.6 (11.2)	0.84
Smoking	29.9 (186)	28.2 (570)	0.43
Cholesterol	5.75 (1.04)	5.73 (0.99)	0.77
Triglyceride†	1.78 (0.96)	1.78 (0.92)	0.99
Fibrinogen†	2.69 (0.52)	2.71 (0.51)	0.31
CRP†	2.34 (2.32)	2.49 (2.57)	0.23
Alcohol			
% drinkers	78.3 (488)	81.2 (1640)	0.11
Median units/wk [IQR]	7 [1-17]	6 [1-15]	0.55

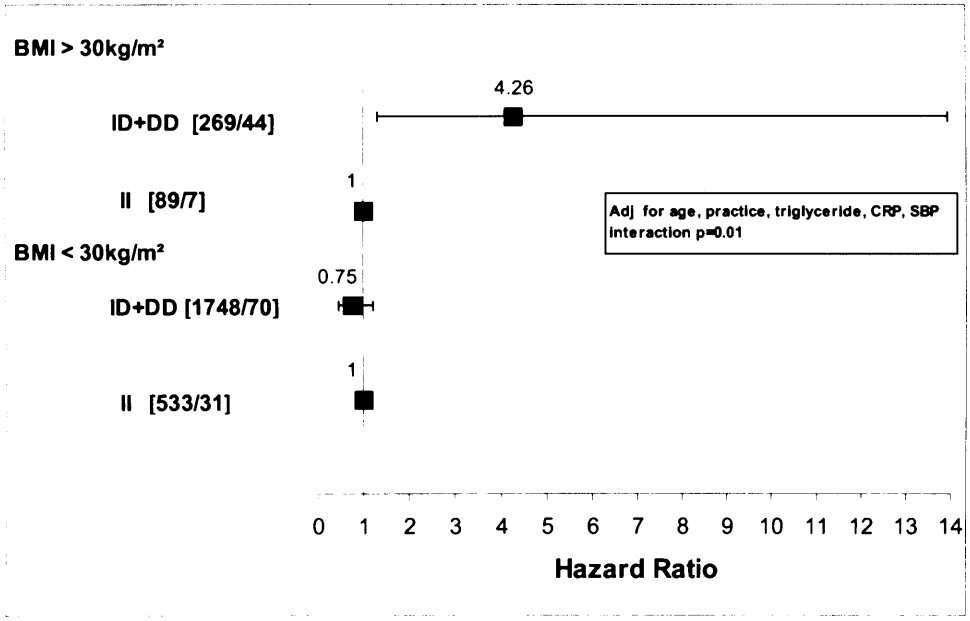
†geometric mean (approx sd)

ACE Obesity interaction on T2D risk

Since obesity was the strongest risk factor for development of T2D, and in view of the potential for escalating the inflammatory burden in obese individuals, the interaction between *ACE* genotype and obesity on 15 year risk of development of T2D (adjusted for age and practice) was examined. In non-obese men, D allele carriers had a non-

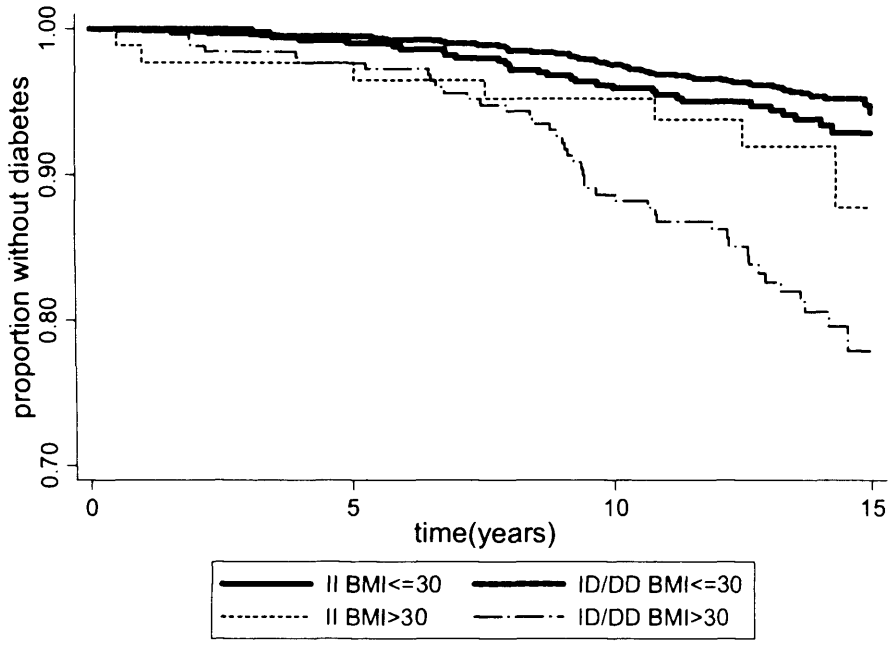
significantly lower risk of T2D - HR ID+DD v II 0.69 (0.45-1.06) while in obese men the association was reversed, HR ID+DD v II 2.11 (0.95-4.69) with a significant genotype-obesity interaction (p=0.02). Figure 5.1 demonstrates that when adjusting for triglycerides, CRP and SBP (three strongest predictors of T2DM (table 5.2) which may also be causal) the risk of T2D in obese men was similarly higher in D allele carriers compared to II homozygotes (HR=4.26(1.30-13.93)), while in lean men there was no genotype difference in risk (HR=0.75(0.46-1.22)), with an overall interaction p=0.01.

Figure 5.1: Hazard Ratio (adjusted for age, practice, triglycerides, CRP and SBP) for T2D in non-obese and obese NPHSII men by *ACE* I/D genotype. [Number of subjects/Number developing T2DM]



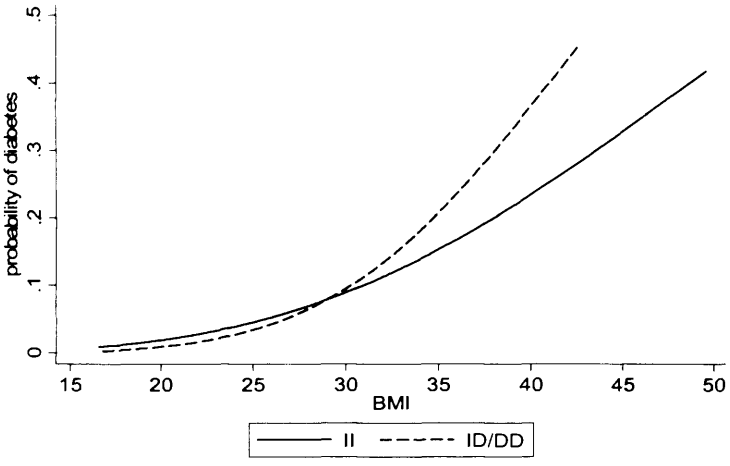
A Kaplan-Meier plot (Figure 5.2) demonstrates that development of T2D in obese subjects occurs much earlier than in non-obese men, with the curve for obese D carriers separating from obese II homozygotes between 7-10 years. It also shows that after 15 years those subjects with the lowest T2D risk are the non-obese D allele carriers.

Figure 5.2: Kaplan-Meier plot for T2D by *ACE* genotype and obesity.



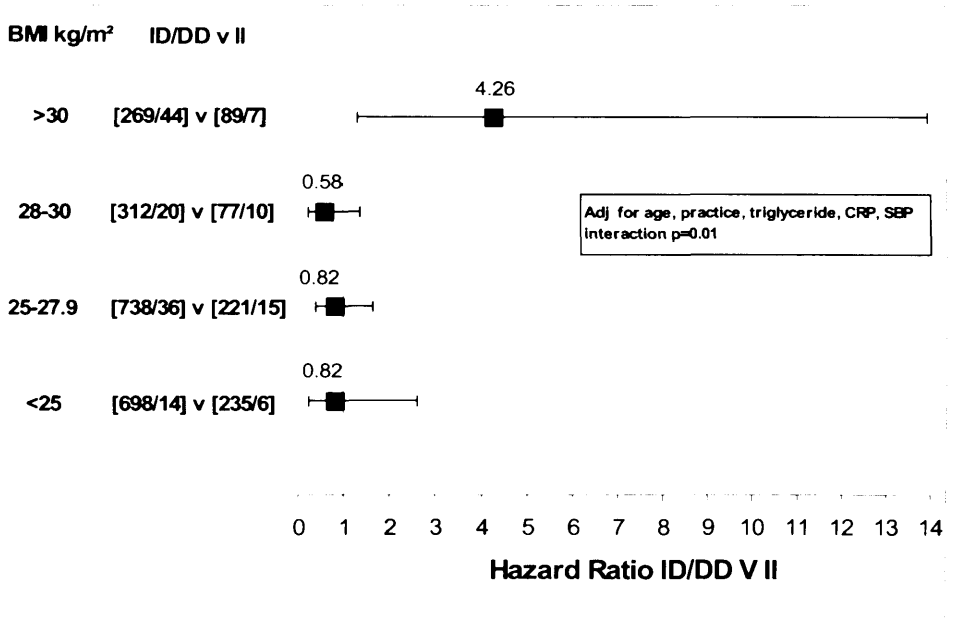
When analysis was carried out using BMI as a continuous variable the HR for a one standard deviation (SD) increase in BMI was found to be higher, but not significantly so with ID/DD vs II HR 2.18 (1.83-2.61) vs 1.73 (1.27-2.36) p=0.20. However there was clear divergence of risk after BMI>30kg/m^2 (Figure 5.3).

Figure 5.3: Graph illustrating divergence of risk after BMI>30kg/m^2 for ID/DD vs II with BMI as a continuous variable



Analysis using BMI in four categories using cut-offs previously reported (Humphries S.E. et al., 2006) showed a non-linear pattern of interaction with a threshold effect evident, with the D allele being associated with risk of T2D (adjusted for age, practice, triglycerides, CRP, SBP) only at high BMI (genotype-BMI interaction p=0.02) as demonstrated in Figure 5.4.

Figure 5.4: Categorical Model for BMI Interaction with *ACE* genotype on prospective risk of T2D. ID+DD [Number of subjects/Number developing T2D] v II [Number of subjects/Number developing T2D]. Reference group is specific for each BMI category – it is the ratio of number of subjects with II genotype who develop T2D after 15 years to the total number of II subjects within the same BMI category. Categories of BMI were as used in a previous analysis (Humphries S.E. et al., 2006).



Discussion:

Based on the protective effect of ACE inhibitors and ARBs on the development of T2D in a meta-analysis of large clinical trials (Scheen, 2004), and experimental evidence for AngII inducing insulin resistance (Ogihara et al., 2002), the hypothesis that *ACE* II subjects, with genetically determined lower ACE levels, would have a lower rate of

T2D development was tested. Furthermore an interaction between obesity and I/D polymorphism on T2D was examined given the strong pro-inflammatory effect of central adiposity and AngII.

An effect was seen, confined to obese men, where D allele carriers had a significantly greater risk than their II counterparts, but in non-obese men there was no difference in genotype risk. Possession of the D allele, therefore, may be increasingly harmful when individuals are obese. Further examination suggested that this was not a linear effect of BMI on risk, but rather the effect only became significant once the individual had a BMI>30 kg/m^2.

The mechanism of the *ACE* gene variant and its relationship with obesity in the pathogenesis of T2D is currently unclear, but is likely to be due to the impact of the different levels of plasma and tissue ACE that will be present constitutively, with the II subjects having roughly 40% lower levels than DD subjects (Danser et al., 1995; Rigat.B. et al., 1990). The higher serum and tissue ACE associated with the D allele would lead to higher AngII levels. The RAS is present in the circulation and several tissues in which glucose metabolism are controlled including skeletal muscle, pancreas and adipose tissue (Karlsson et al., 1998). There is now considerable evidence for AngII promoting the development of T2D through a number of mechanisms (Hattori et al., 2005; Leung and Carlsson, 2005; Engeli et al., 2003). It is likely that the higher AngII levels in D allele carriers would have a bigger impact in the obese state where there is already a greater inflammatory and diabetogenic burden (Kern et al., 1995; Fried et al., 1998).

It is however important to note the wide 95% CI limits for D allele carriers (adjusted for age, practice, triglycerides, CRP, SBP). This would be in keeping with a small effect of the *ACE* (I/D) polymorphism on risk of development of T2D which ties in with conclusions from the DREAM Trial (DREAM Trial Investigators, 2006) where Ramipril did not reduce incidence of T2D in high-risk individuals but suggested a small benefit in glucose metabolism.

Adjustments of the *ACE* risk effects were made for SBP, triglycerides and CRP because with BMI, these were the strongest predictors of T2D development in this cohort. Such adjustments did not materially alter the genotype risk pattern seen. All three factors may be involved in the same causal pathway in T2D pathogenesis. SBP can directly activate the RAS (Reja et al., 2006). Non-esterified fatty acids (from triglyceride hydrolysis) directly inhibits insulin signalling (Wang et al., 2006) and excess intracellular triglycerides promotes increased oxidative stress and inflammation which can cause insulin resistance (Bakker et al., 2000). AngII's properties of affecting insulin signalling and causing oxidative stress would directly affect this. CRP may act together with AngII in promoting insulin resistance again through direct and indirect effects on insulin signalling and oxidative stress (Xu et al., 2007).

There are several limitations to this study. The method of identification of NPHSII men with T2D, by the medical record search, is unlikely to include any false positive diagnosis, but, in the absence of a full recall for fasting glucose testing, some T2D subjects may be misclassified as healthy. This would result in an underestimate of the 15 year incidence of T2D, reducing the ability to detect an effect of the polymorphism, and would not confound the genetic association seen.

The lack of plasma glucose and serum insulin data prevents an exploration of whether these parameters are affected by *ACE* (I/D) genotype, in light of the potential detrimental impact of AngII on insulin signalling and glucose metabolism. Background diet and physical activity data would have also been useful, as they could affect development of T2D.

The incidence of T2D could possibly have been under-estimated in the non-obese subjects as a whole, in view of general practioners being more alert to the presence of T2D in obese rather than non-obese patients. However, this is unlikely in this sample, in view of the thorough, systematic review of patients' notes and it is worth noting that greater than 75% of cases with T2D were from the non-obese group over the 15 years of follow-up. Even if this did take place, it is unlikely it would affect the major conclusions of the studies, because it is implausible that any such under-estimation of T2D would occur by *ACE* (I/D) genotype. All of the 2489 subjects without T2D are still alive since they are 'flagged' with the Office of National Statistics and we receive all death certificates. At recruitment no subjects were taking ACE inhibitors, ARBs and Beta receptor blockers, and although a proportion of those who developed T2D over follow-up may have been prescribed such medications this is unlikely to have confounded the genetic effect on risk observed here, but would rather have the effect of diluting it.

A further limitation is that the study is underpowered to detect associations in which alleles have a small effect. Multiple testing (in this case for interaction and adjustments) may raise the probability that 'chance' is the explanation for the observed associations

and these results may therefore be false positives. A similar prospective study of T2D was not available to us to try and replicate the findings. Re-analysis of a previously published case control study showed a higher risk of T2D in obese D allele carriers compared to II subjects but the genotype-obesity interaction is not significant. There was however limited power (only 60% to detect the previously observed effect at the 5% significance level) to demonstrate such an effect size. Studies in which there are much larger numbers of cases and controls are necessary for firm conclusions.

In conclusion the work presented here supports the findings that variation in *ACE* gene may interact with BMI to increase the risk of T2D. Further replication is certainly required to confirm these findings. The combination of the pro-inflammatory D allele and an already 'at risk' obese state, may well underlie an impaired metabolic profile and a possible increased propensity to T2D.

Chapter 6: **Impact of *ACE* (I/D) polymorphism on Hypertrophic Cardiomyopathy**

Introduction

Hypertrophic Cardiomyopathy (HCM) is a common genetic heart muscle disorder, inherited in an autosomal dominant manner, characterised by unexplained LVH (Maron et al., 1995). The clinical course is highly variable, ranging from asymptomatic throughout life to premature sudden death, heart failure and arrhythmia. It is the most common cause of sudden death in young adults (Maron et al., 1995). There is an overall tendency in HCM for gradual wall thinning and a gradual decrease in systolic performance over time (Thaman et al., 2004; Thaman et al., 2005).

The majority of causative mutations have been found in sarcomeric protein genes (Marian, 2002). HCM exhibits genetic heterogeneity and allelic heterogeneity with many mutations found in each gene. Though studies have demonstrated specific genotype-phenotype associations (Abchee and Marian, 1997; Charron et al., 1998), the majority of phenotypes are not gene specific, with some mutations conferring a mild or essentially no phenotype (Ackerman et al., 2005; Watkins et al., 1992; Watkins et al., 1995). Furthermore, even within affected families (with identical mutations) there is definite phenotypic variance (Ashrafian and Watkins, 2007). One explanation for this could be presence of other modifier genes e.g in hypertrophic or fibrotic response, or environmental factors e.g hypertension, which interact with the causative gene to influences the phenotype.

Searching for 'modifier' genes has so far been limited. Polymorphisms in the RAS have been most widely studied, since they are good contenders to influence the hypertrophic response. The RAS is localised in the myocardium (Danser, 1996), with AngII known to induce cardiomyocyte hypertrophy (Li et al., 2002) and pathological remodelling (Siwik et al., 2001).

Using the candidate gene association study approach, genes encoding RAS components have been shown to modify the phenotypic expression of HCM. With regard to the *ACE* I/D polymorphism, in a study of 206 patients the DD genotype has been associated with higher risk of sudden death (Marian et al., 1993) and increased severity of hypertrophy (Lechin et al., 1995), n=183 (Tesson et al., 1997), n=114 although other studies have demonstrated no association between this polymorphism and HCM (Yamada et al., 1997), n=281 (Osterop et al., 1998), n=104. Other SNPs in the RAS – *AGT* M235T and *AGTR* 1166 A/C have been studied, again with mixed results (Ishanov et al., 1997; Brugada et al., 1997; Osterop et al., 1998). This is not surprising, in view of typical problems associated with such studies including limited sample size and cross-sectional heterogenous cohorts. The hypothesis that the *ACE* I/D polymorphism affect HCM phenotypes in a large prospective cohort was therefore tested.

Methods

The study cohort consisted of 541 unrelated patients diagnosed with HCM, assessed at St George's Hospital, London, and Heart Hospital, London UK between 1988 and 2006. This is described in Chapter 2.

Statistical analysis

Statistical analysis was performed using 'Intercooled STATA' package (version 9.2, STATA Corporation, Texas) and LogXact (Version 8) (Cytel Software Corporation, Cambridge, Mass). The analysis was performed by Jackie Cooper. Baseline associations were analysed by ANOVA when normally distributed (or after appropriate transformation), and by Chi-squared or Fisher's Exact tests when categorical. They are presented as mean (standard deviation (SD)). Deviations of allele frequencies from Hardy-Weinberg equilibrium were considered using chi-squared tests. Allele frequencies are shown with the 95% confidence interval (CI). Survival analysis was undertaken using Poisson regression models. Where the numbers of events were small Exact Poisson regression models were fitted. Results are presented as rate ratios (RR) along with the rate per 1000 person years of follow-up. The change in echo parameters and peak VO_2 (follow-up – baseline) was divided by the time between measurements for each individual to give the change, per year of follow-up. The mean change (standard error SE) by genotype was then estimated from analysis of covariance models which included baseline levels to take account of regression to the mean.

Results

Baseline data

The mean age of the 541 subjects were 38.5 years (SD 17.4). 62.3% (337) were men, 44.6% (241) were on Beta Blockers (minimum length of 6 months) and 8.3% (45) were on ACE inhibitors (ACEI) or Angiotensin 2 Receptor blockers (ARB) (minimum length

of 6 months). *ACE* I/D genotype was obtained in 496 subjects (91.6%). The distribution of genotypes was as expected for Hardy-Weinberg equilibrium, and the D allele frequency was 0.52 (0.49 to 0.56). Genotype association with baseline characteristics is shown in Table 6.1A and B. It demonstrates D allele carriers had smaller LA size, smaller LV cavity (lower LVED and LVES), higher FS, max LVWT and pVO_2. The magnitude of difference was modest however, and non-significant.

Table 6.1A *ACE* genotype association with baseline characteristics (Additive model)

	DD N=141	ID N=238	II N=117	P (trend)
LA	43.8 (7.8)	44.8 (7.8)	45.0 (8.3)	0.22
LVED	44.0 (5.2)	43.7 (6.4)	44.4 (6.4)	0.64
LVES	25.3 (5.1)	24.5 (5.9)	25.7 (6.4)	0.62
FS (%)	42.8 (8.0)	44.4 (8.6)	42.5 (9.1)	0.85
MAX LVWT*	20.1 (4.8)	20.5 (5.2)	20.2 (4.7)	0.94
pVO_2 *	20.8 (7.7)	21.5 (9.2)	20.4 (8.6)	0.83

*log transformed

Table 6.1B *ACE* genotype association with baseline characteristics (Dominant model)

*log transformed

	DD/ID N=379	II N=117	P value	Numbers needed to detect significant difference ($p<0.05$) between genotype groups with 80% power
LA	44.4 (7.8)	45.0 (8.3)	0.47	8101
LVED	43.8 (6.0)	44.4 (6.4)	0.39	4811
LVES	24.8 (5.6)	25.7 (6.4)	0.13	2081
FS (%)	43.8 (8.4)	42.5 (9.1)	0.14	2060
MAX LVWT*	20.3 (5.0)	20.2 (4.7)	0.73	99135
pVO_2 *	21.3 (8.6)	20.4 (8.6)	0.39	3975

Table 6.1B also demonstrates that in order to detect a significant difference in baseline characteristics between the D allele carriers and II homozygotes, a substantial increase in the numbers of individuals are needed.

Survival analysis

56 patients were lost to follow-up leaving 485 patients in the survival analysis. There were 48 events with 34 deaths (7 cardiac failure, 12 sudden death, 6 other cardiac, 9 non-cardiac) and 5 transplants, 5 VF, and 4 ICD shock. Median follow-up was 6.65 years with a range of 0.56 to 19.1 years in those without an event and 5.14 years with a range of 0.27 to 16.25 in those with an event (death/VF/transplant). Table 6.2 demonstrates that those without follow-up were significantly older, had a larger LV cavity and lower Max LVWT and pVO_2 to those with follow-up. This suggests that those who were followed up had a more benign phenotype, which would go against what would normally occur in a referral centre, in which healthier subjects would be discharged, and the more severely affected, remain under follow-up.

Table 6.2 Baseline characteristics in those with and without follow-up

	No follow-up N=56	Follow-up N=485	P value
Age	45.4 (16.7)	37.8 (17.3)	0.003
% male	64.3 (36)	62.1 (301)	0.75
% on beta blockers	35.7 (20)	45.7 (221)	0.16
% ACEI/ARB	5.4 (3)	8.7 (42)	0.61
LA size	44.5 (9.2)	44.6 (7.8)	0.92
LVED	45.6 (5.4)	43.7 (6.2)	0.04
LVES	26.8 (5.6)	24.9 (6.0)	0.03
FS (%)	41.7 (8.7)	43.5 (8.7)	0.14
MAX LVWT*	18.6 (4.9)	20.5 (4.9)	0.005
pVO_2*	23.9 (9.8)	20.8 (8.5)	0.03

*geometric mean (approximate SD)

Potential predictors of mortality including age, use of Beta Blockers and ACE inhibitors (or ARB) which could confound any genotype effect on mortality were investigated. There was no significant effect of age on all-cause or cardiac mortality as demonstrated in Table 6.3.

Table 6.3 Effect of age on survival

death/VF/transplant	**No N=433**	**Yes N=42**	**RR (95% CI)***	**P value**
Mean age (SD)	37.6 (17.2)	39.0 (18.8)	1.06 (0.97-1.16)	0.19
death/VF/transplant/ICD shock	**No N=429**	**Yes N=46**	**RR (95% CI)***	**P value**
Mean age (SD)	37.6 (17.2)	39.2 (18.1)	1.07 (0.98-1.16)	0.15
cardiac death /VF/transplant	**No N=442**	**Yes N=33**	**RR (95% CI)***	**P value**
Mean age (SD)	37.7 (17.2)	38.3 (19.3)	1.05 (0.95-1.16)	0.36
cardiac death /VF/transplant/ ICD shock	**No N=438**	**Yes N=37**	**RR (95% CI)***	**P value**
Mean age (SD)	37.7 (17.2)	38.6 (18.4)	1.06 (0.96-1.16)	0.27

*rate ratio per 5 year increase in age

Use of Beta Blockers was associated with a trend for survival, with regard to cardiac mortality with a HR of 0.69 (0.36-1.33) (p=0.27) compared to those not taking it (Table 6.4). Use of ACE inhibitors (or ARB) was associated with a trend for survival with regard to all cause mortality (minus ICD shocks) and cardiac mortality with a HR of 0.60 (0.12-1.89) (p=0.57) and HR of 0.69 (0.11-2.19) (p=0.77) respectively (Table 6.4). Only 42 individuals were on ACE inhibitors however, therefore very few events occurred in this group.

Table 6.4 Events by Beta Blocker and ACE inhibitor (or ARB) use

Beta blockers	No N=263	Yes N=221	P
No. death/VF/transplant	24	20	
Rate per 1000 person years of fu	13.3	11.8	
RR (95% CI)	1.00	0.88 (0.49-1.60)	0.68
No. death/VF/transplant/ICD shock	25	22	
Rate per 1000 person years of fu	13.0	13.0	
RR (95% CI)	1.00	0.93 (0.53-1.66)	0.82
No. cardiac death/VF/transplant	22	13	
Rate per 1000 person years of fu	12.2	7.6	
RR (95% CI)	1.00	0.63 (0.32-1.24)	0.18
No. cardiac death/VF/transplant/ ICD shock	23	15	
Rate per 1000 person years of fu	12.8	8.9	
RR (95% CI)	1.00	0.69 (0.36-1.33)	0.27
ACE inhibitors (or ARB)	**No N=443**	**Yes N=42**	**P**
No. death/VF/transplant	41	3	
Rate per 1000 person years of fu	13.1	7.9	
RR (95% CI)	1.00	0.60 (0.12-1.89)	0.57
No. death/VF/transplant/ICD shock	44	4	
Rate per 1000 person years of fu	14.1	10.7	
RR (95% CI)	1.00	0.75 (0.20-2.07)	0.80
No. cardiac death/VF/transplant	33	2	
Rate per 1000 person years of fu	10.5	5.3	
RR (95% CI)	1.00	0.50 (0.06-1.96)	0.51
No. cardiac death/VF/transplant/ ICD shock	36	3	
Rate per 1000 person years of fu	11.6	8.0	
RR (95% CI)	1.00	0.69 (0.114-2.19)	0.77

RR Relative risk; fu Follow-up

Of the 485 with survival data, *ACE* genotype was obtained in 446 individuals in which 46 events occurred. There was no significant mortality difference between D allele carriers and II homozygotes, as shown in Table 6.5. Adjustments were made for Beta Blocker and ACE inhibitor (or ARB) use only in view of the possible pharmacogenetic interaction with *ACE* (I/D) genotype on survival, as evident by the trends in Table 6.4,

and from individuals with heart failure (McNamara et al., 2001; McNamara et al., 2004).

Table 6.5 Survival analysis by genotype

	DD/ID N=337	II N=109	P
No. death/VF/transplant	33	9	
(Rate per 1000 person years of fu)	13.3	12.1	
RR (95% CI)	1.00	0.91 (0.38–1.95)	0.98
Adjusted RR (95% CI)*	1.00	0.93 (0.39-2.01)	1.00
No. death/VF/transplant/ICD shock	36	10	
Rate per 1000 person years of fu	14.6	13.6	
RR (95% CI)	1.00	0.93 (0.41-1.91)	1.00
Adjusted RR (95% CI)*	1.00	0.97 (0.43-2.00)	1.00
No. cardiac death/VF/transplant	25	8	
Rate per 1000 person years of fu	10.1	10.8	
RR (95% CI)	1.00	1.17 (0.46-2.68)	0.83
Adjusted RR (95% CI)*	1.00	1.12 (0.42-2.53)	0.92
No. cardiac death/VF/transplant/ ICD shock	28	9	
Rate per 1000 person years of fu	11.3	12.2	
RR (95% CI)	1.00	1.08 (0.45-2.34)	0.98
Adjusted RR (95% CI)*	1.00	1.14 (0.47-2.49)	0.87

*adjusted for Beta Blocker use, ACE-inhibitor (or ARB) use.

Effect on Left ventricular remodelling

Of the original 541, only 390 subjects had echo follow-up (median 6.5 years with a range of 119 days to 20.4 years). Table 6.6 demonstrates that those without echo follow-up were older, and less likely to be on Beta Blockers, which may be consistent with a typical tertiary referral centre practice, where younger symptomatic patients are followed up, but older less symptomatic patients are reviewed by the referring centre. The two groups did not differ in baseline levels of echo parameters.

Table 6.6 Baseline characteristics in those with and without follow-up echo data

	No echo follow-up N=151	Follow-up N=390	P value
Age	42.7 (18.7)	36.9 (16.6)	0.0007
% male	62.3 (94)	62.3 (243)	0.99
% on Beta Blockers	27.2 (41)	51.4 (200)	<0.0001
% ACEI/ARB	5.3 (8)	9.5 (37)	0.11
LA	44.6 (7.7)	44.6 (8.0)	0.99
LVED	44.4 (5.7)	43.8 (6.3)	0.27
LVES	25.7 (6.0)	24.8 (6.0)	0.13
FS (%)	42.5 (8.9)	43.6 (8.7)	0.19
MAX LVWT*	19.9 (4.9)	20.5 (5.0)	0.15
pVO_2*	21.7 (9.3)	20.9 (8.4)	0.37

*geometric mean (approximate SD)

Table 6.7 shows that in those with echo follow-up there was a highly significant propensity for LV remodelling with increase in left ventricular dimensions, drop in fractional shortening and thinning of LV wall.

Table 6.7 LV remodelling in those with echo follow-up

	Mean absolute change per year / mm (se)	P value	Percentage change (se)	P value
LA	0.33 (0.09)	<0.0001	0.63 (0.19)	0.001
LVED	0.35 (0.09)	<0.0001	0.82 (0.20)	<0.0001
LVES	0.75 (0.09)	<0.0001	2.94 (0.39)	<0.0001
FS (%)	-1.19 (0.17)	<0.0001	-3.05 (0.41)	<0.0001
MAX LVWT	-0.08 (0.01)	<0.0001	-1.85 (0.30)	<0.001

In view of the gradual increase in LV size and wall thinning during follow-up, as shown above, and presented elsewhere (Thaman et al., 2004; Thaman et al., 2005) the effect of

age on annual change in echo dimensions was determined. Table 6.8 demonstrates no convincing correlation between age and change in echo dimensions in this cohort.

Table 6.8 Correlation between age and change in echo dimensions

	Absolute change		Percentage change	
	Correlation	**P value**	**Correlation**	**P value**
LA	r=0.06	p=0.21	r=0.07	p=0.20
LVED	r=-0.04	p=0.41	r=-0.04	p=0.40
LVES	r=0.05	p=0.37	r=0.05	p=0.33
FS	r=-0.09	p=0.07	r=-0.09	p=0.07
MAXLVWT	r=-0.06	p=0.25	r=-0.05	p=0.36

Due to the possible effect that Beta Blockers and ACE inhibitor (or ARB) use may have on in remodelling as seen in heart failure (Sharpe et al., 1988; Bristow et al., 1996) and hypertensive LVH (Mathew et al., 2001), change in echo parameters by Beta Blocker and ACE inhibitor (or ARB) use was also examined (Table 6.9). No convincing remodelling effect was conferred by either medication in this cohort.

Table 6.9 Change in echo parameters by Beta Blocker and ACE inhibitor (or ARB) use

LA	N	Absolute change Mean (se)	P value	Percentage change Mean (se)	P value
Beta Blockers					
No	184	0.39 (0.13)	0.002	0.76 (0.27)	0.006
Yes	196	0.27 (0.13)	0.04	0.49 (0.28)	0.08
P value		0.52		0.49	
ACE inhibitor / ARB					
No	344	0.32 (0.10)	0.001	0.60 (0.21)	0.005
Yes	37	0.50 (0.24)	0.04	1.09 (0.43)	0.02
P value		0.84		0.65	
LVED					
Beta Blockers					
No	189	0.37 (0.13)	0.004	0.90 (0.32)	0.005
Yes	196	0.31 (0.12)	0.009	0.73 (0.26)	0.005
P value		0.74		0.68	
ACE inhibitor / ARB					
No	349	0.33 (0.09)	0.0005	0.78 (0.22)	0.0004
Yes	37	0.44 (0.22)	0.05	1.00 (0.49)	0.05
P value		0.52		0.57	
LVES					
Beta Blockers					
No	188	0.69 (0.13)	<0.0001	2.86 (0.61)	<0.0001
Yes	195	0.82 (0.14)	<0.0001	3.06 (0.49)	<0.0001
P value		0.50		0.80	
ACE inhibitor / ARB					
No	347	0.73 (0.10)	<0.0001	2.87 (0.42)	<0.0001
Yes	37	0.69 (0.21)	0.003	2.56 (0.73)	0.001
P value		0.54		0.62	
FS					
Beta Blockers					
No	188	-1.06 (0.21)	<0.0001	-2.74 (0.53)	<0.0001
Yes	195	-1.33 (0.26)	<0.0001	-3.40 (0.63)	<0.0001
P value		0.43		0.43	
ACE inhibitor / ARB					
No	347	-1.14 (0.18)	<0.0001	-2.89 (0.45)	<0.0001
Yes	37	-1.01 (0.23)	0.003	-2.79 (0.67)	0.0002
P value		0.44		0.33	
MAX LVWT					
Beta Blockers					
No	186	-0.32 (0.11)	0.002	-1.46 (0.48)	0.003
Yes	197	-0.45 (0.07)	<0.0001	-2.25 (0.38)	<0.0001
P value		0.33		0.19	
ACE inhibitor / ARB					
No	348	-0.39 (0.07)	<0.0001	-1.86 (0.33)	<0.0001
Yes	36	-0.33 (0.09)	0.0005	-1.76 (0.50)	0.001
P value		0.84		0.96	

Change in echo parameters by genotype

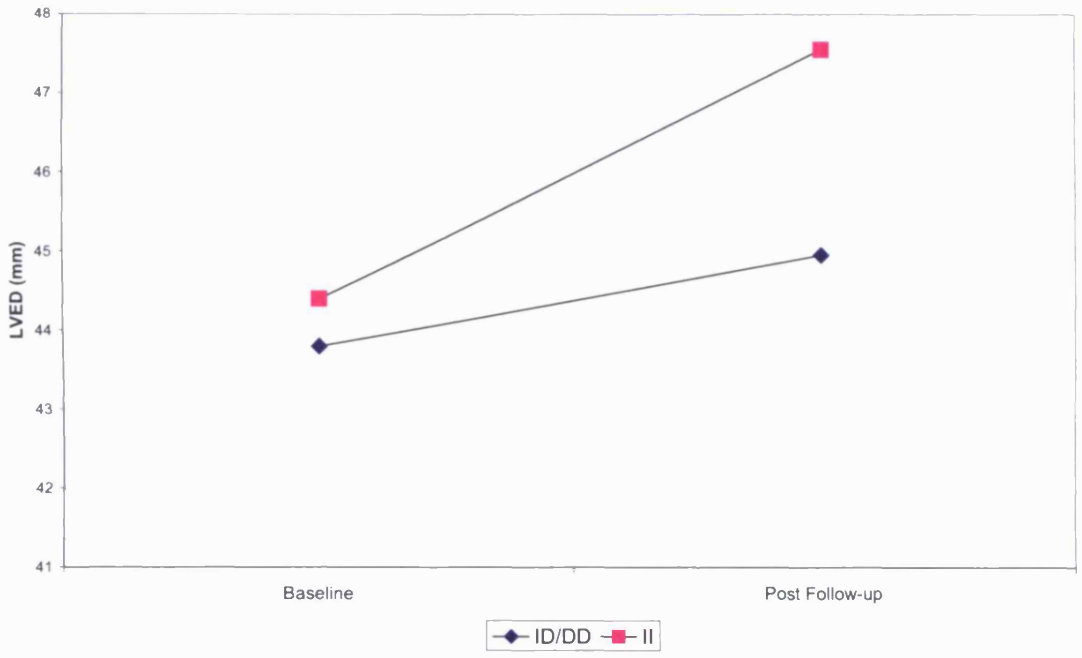
Of the 390 with echo follow-up, 362 were successfully genotyped for *ACE*. The *ACE* effect on left ventricular remodelling was therefore examined (Table 6.10).

Table 6.10 Effect of *ACE* genotype on left ventricular remodelling

	ACE	N	Absolute change Mean/mm (se)	P value	% change Mean (se)	P value
LA	DD/ID	273	0.31 (0.10)	0.004	0.56 (0.23)	0.01
	II	89	0.32 (0.21)	0.13	0.66 (0.43)	0.13
	P value		0.95		0.88	
LVED	DD/ID	273	0.23 (0.09)	0.01	0.54 (0.19)	0.006
	II	89	0.63 (0.26)	0.02	1.50 (0.64)	0.02
	P value		0.07		0.06	
LVES	DD/ID	273	0.70 (0.10)	<0.0001	2.72 (0.37)	<0.0001
	II	89	0.89 (0.26)	<0.001	3.69 (1.21)	0.003
	P value		0.43		0.35	
FS	DD/ID	273	-1.20 (0.21)	<0.0001	-3.01 (0.51)	<0.0001
	II	89	-1.18 (0.36)	0.002	-2.91 (0.86)	0.001
	P value		0.95		0.95	
MAX LVWT	DD/ID	273	-0.36 (0.07)	<0.0001	-1.70 (0.30)	<0.0001
	II	89	-0.44 (0.17)	0.01	-2.13 (0.84)	0.01
	P value		0.61		0.56	

ACE genotype was associated with a trend for change in LVED, with D allele carriers having a mean annual increase of 0.23mm vs 0.63mm in II homozygotes (p=0.07 for absolute change and p=0.06 for percentage change), as shown in Figure 6.1. No significant genotype differences were observed for the remaining echo parameters.

Figure 6.1: Effect of *ACE* genotype on change in LVED



These changes in LVED do not differ significantly between those genotyped and not genotyped as shown below (Table 6.11).

Table 6.11

ACE	Not genotyped N=28	Genotyped N=362	P value
LVED			
Absolute change (se)	0.75 (0.22)	0.32 (0.09)	0.25
Percent change (se)	1.63 (0.49)	0.76 (0.21)	0.32

Effect on change in Peak VO_2 data

Of the original 541, 461 subjects had baseline pVO_2 measurements and 252 had follow up pVO_2 data (median follow up 4.0 years with a range of 42 days to 13.9 years). Mean absolute change per year was -0.39 ml/min/kg (SE=0.23) p=0.08. Table 6.12 shows that those without follow-up were older and less likely to be on Beta Blockers at baseline. This may be consistent with tertiary referral centre practice, as described earlier.

Table 6.12 Baseline characteristics in those with and without follow-up pVO_2 measures.

	No follow-up N=208	Follow-up N=252	P value
Age	42.0 (17.4)	35.1 (15.7)	<0.0001
% male	64.9 (135))	64.3 (162)	0.89
% on Beta Blockers	36.7 (76)	52.4 (132)	<0.001
% ACEI/ARB	5.8 (12)	9.9 (25)	0.10
LA	44.8 (8.5)	44.7 (7.4)	0.90
LVED	44.1 (5.6)	43.8 (6.4)	0.64
LVES	25.4 (5.3)	24.7 (6.2)	0.19
FS (%)	42.7 (8.1)	44.2 (9.0)	0.07
MAX LVWT*	19.9 (4.7)	20.8 (5.3)	0.08
pVO_2*	20.9 (9.2)	21.3 (8.1)	0.65

*geometric mean (approximate SD)

Table 6.13 Change in pVO_2 by ACE-I and Beta Blocker use

pVO_2	N	Absolute change Mean (se)	P value
Beta Blockers			
No	130	0.23 (0.37)	0.54
Yes	134	-0.90 (0.28)	0.002
P value		P=0.01	
ACEI / ARB			
No	237	-0.35 (0.25)	0.16
Yes	27	-0.55 (0.23)	0.02
P value		P=0.62	

Of the 252 with pVO_2 follow-up, 243 were successfully genotyped for *ACE*. In this cohort there was no significant correlation between age and annual change in pVO_2 (r=-0.08 p=0.22). Beta Blocker use was adjusted for, because as demonstrated in Table 6.13, their use was associated with a significantly greater drop in pVO_2.

Table 6.14 Effect of *ACE* genotype on change in pVO_2

pVO_2	N	Absolute change Mean (se)	P value
DD/ID	184	-0.45 (0.27)	0.10
II	59	-0.02 (0.51)	0.96
P value unadj.		0.27	
P value adj*		0.31	

*adjusted for Beta Blocker use

Table 6.14 shows that there was a trend for a greater drop in pVO_2 in D allele carriers than II homozygotes.

Discussion

These results suggest the possibility that the *ACE* D allele has a mild association with favourable left ventricular dimensions and remodelling in HCM. At baseline, carriage of the D allele was non-significantly associated with a smaller LV cavity, greater max LVWT, higher FS and pVO_2. D allele carriers had a trend for a lower rise in LVED but there were no other genotype differences in remodelling. There was no effect on mortality. In terms of change in pVO_2, there was a trend for this being worse in D allele carriers than II homozygotes.

The underlying mechanism behind the possible remodelling benefit conferred by the D allele is unclear. RAS activation, mainly through AngII (but also possibly through Bradykinin inactivation), causes high levels of reactive oxygen species which drives cardiomyocyte hypertrophy (Wenzel et al., 2001; Li et al., 2002), fibrosis and pathological remodelling (Siwik et al., 2001). *ACE* D allele which is strongly associated with higher tissue and plasma ACE levels would therefore be predicted to be associated with a worse phenotype in HCM. The D-allele has already been associated with a greater LV growth response to a variety of stimuli (Estacio et al., 1999; Montgomery et al., 1997) and has been associated with greater LVH in hypertensive subjects (Iwai et al., 1994). The results here do not tie in with the evidence, albeit inconsistent, that the D allele is associated with greater LVH in HCM (Lechin et al., 1995; Tesson et al., 1997). It is interesting that ACE inhibitor (or ARB) use was associated with a trend for improvement in all-cause and cardiac mortality suggesting that RAS activation is harmful in HCM, however numbers of events in those on ACE inhibitors (or ARB) were very low.

The D allele in this cohort was associated with a higher baseline pVO_2, and pVO_2 is a marker of disease progression in HCM (Sharma et al., 2000), therefore the D allele may be of benefit through non-cardiac mechanisms e.g. skeletal muscle or vascular RAS. Indeed the D allele has been associated with greater skeletal muscle strength (Folland et al., 2000) and lower rate of decline in functional ability (in an elderly cohort) compared to II homozygotes (Kritchevsky et al., 2005). The non-significant association of the D allele with greater drop in pVO_2 however goes against this proposition, but may be consistent with the greater muscular efficiency in those with the II genotype

(Montgomery et al., 1999). The putative explanation for association between D allele carriage and baseline pVO_2 also does not directly explain the possible benefit in remodelling by the D allele carriers.

There are several limitations to this study. It was underpowered to demonstrate the trends seen at baseline and the survival effect. Despite the cohort being the largest one to date of HCM patients in the identification of modifier genes, with an adequate follow up period (of median 6.5 years in those where no event occurred), the effect of the I/D polymorphism was however still too low to be statistically significant in this sample. This was demonstrated by the power calculations in Table 6.1, in which more than 2000 patients are required to detect a statistically significant difference in baseline FS and LVES between D allele carriers and II homozygotes.

The combination of drop-outs (due to genotyping failure) and high number of subjects being lost to follow-up, led to substantial drop in numbers, which reduced the power of the study further, already with insufficient numbers and mild genetic effect. In the baseline analysis, there was a 8% drop out rate leaving only 496 subjects. In the survival analysis, there were 56 subjects lost to follow-up and in view of genotyping drop-outs there were only 446 individuals with *ACE* genotype and survival data. There were even less numbers with echo and pVO_2 follow-up data.

The follow-up of survival, echo and pVO_2 data had a very wide range, with some individuals having follow-ups of less than a year, and it is difficult to conceive of a mild genetic effect that has a measurable impact within a year. These numbers were however small e.g. for echo follow-up, only 10 subjects had follow-up of less than one year. In

terms of the echo and pVO_2 data, associations were made with respect to 'change per year' thus short periods of follow-up was not a substantial issue.

The majority of the sample were young adults (mean age 38.5 years) but there were 17 subjects who were under 16 years old with four subjects under five years old. Though it may be argued that paedeatric HCM may behave differently to adult HCM e.g. in LV growth it is reasonable to look for an underlying genetic effect on phenotype. This cohort had an annual mortality rate of approximately 1% which is consistent with other large HCM cohorts (Elliott et al., 2006). The highly significant remodelling effect in this cohort, demonstrated in others (Thaman et al., 2005) is further evidence that this sample is comparable to others in the literature.

It may well be the case that the *ACE* I/D polymorphism is not having a clinically important effect in HCM, however studies have also looked at polymorphisms in RAS as a candidate system demonstrating a cumulative influence on hypertrophy (Ortlepp et al., 2002; Perkins et al., 2005), though in the latter study this was only evident in the subgroup with no previously identified mutation. A recent study has demonstrated a cumulative effect of several polymorphisms in RAS genes – *ACE* I/D, *AGT* M235T, *CMA*-1903 A/G, *AGTR*1 1666 A/C and *CYP11B2*-344 C/T - on LVH prospectively in a group of paediatric HCM patients (Kaufman et al., 2007). In this cohort, it would therefore be useful to genotype these other polymorphisms and combine this information to evaluate this in adult HCM subjects. Further work would be to specifically identify the causative HCM mutation in these subjects, investigating whether candidate polymorphisms only modify phenotypes in those individuals with particular sarcomeric mutations for example.

In conclusion, the results presented here suggest that the D allele of the *ACE* I/D polymorphism may be associated with a small effect on LV remodelling in HCM, however replication in a larger study with evaluation of other candidate polymorphisms is essential.

Chapter 7: **Discussion**

This thesis has presented evidence demonstrating the effect of the *ACE* I/D polymorphism in influencing the risk of complex phenotypes, namely CHD and T2D, in the presence of common environmental stimuli. There is good biological plausibility for this in that (1) ACE is a key component of the RAS, (2) RAS has been found in many tissues implicated in these diseases, (3) AngII (and other components) have been shown to be disease modifying and (4) there is clinical evidence for benefit of ACE inhibitors in CHD and possibly T2D. This polymorphism may also be having a mild beneficial effect on ventricular remodelling in HCM. It is important to note it is probably not the I/D polymorphism that is causing differences in risk but a gene variant which is in strong LD with it.

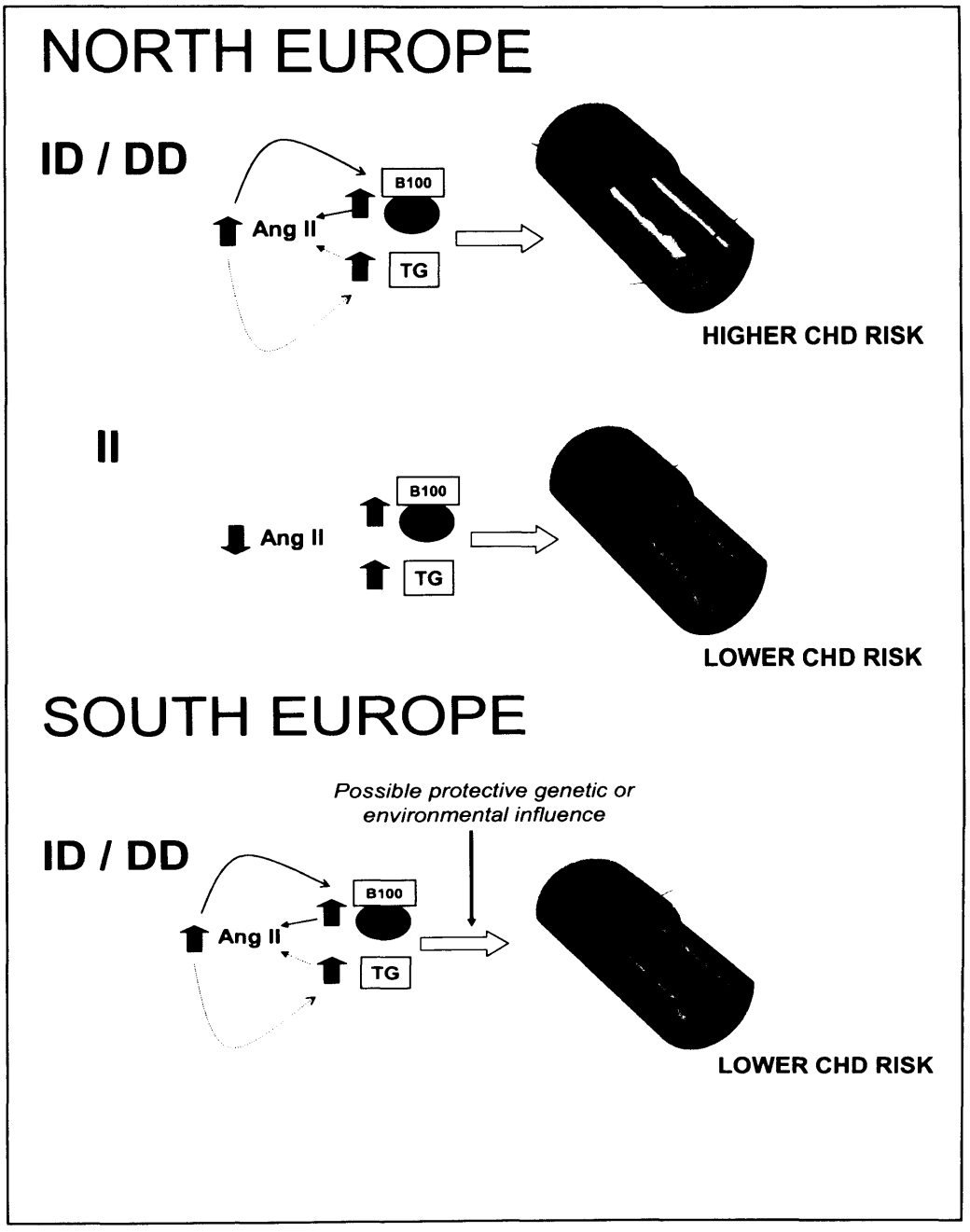
ACE-Lipid interaction on CHD risk in North Europe:

As Figure 7.1 demonstrates, there is an interaction between *ACE* genotype and lipid trait (triglycerides or apoB) in North Europe, where within North Europe, D allele carriers with higher triglycerides or apoB had a higher CHD risk compared to their II homozygote counterparts. Furthermore, D allele carriers with these elevated lipid traits had higher CHD risk than their D allele counterparts in South Europe. An interaction between *ACE* and apoB has been previously reported where D allele carriage was associated with higher CHD risk, in those with *low* apoB levels and low BMI (Cambien et al., 1992).

The mechanism for the greater risk conveyed by the D allele with higher apoB in the North may be due to a positive feedback mechanism of AngII and cholesterol with each other (Keidar et al., 2001). The triglyceride interaction may be due to an 'AngII raising' effect (Ran et al., 2004). The protective mechanism in the South is yet to be established but may be due to other genetic or environmental influences. The difference in CHD risks were more substantial for higher apoB levels than for higher triglycerides.

Figure 7.1 Mechanism of *ACE* Lipid interaction

(Dotted line between TG (triglyceride) and AngII represents less evidence available for a direct interaction)



ACE – SBP interaction on CHD risk

In Chapter 4, in a prospective study of healthy middle-aged men, D allele carriers who had higher SBP had a greater CHD risk than II homozygotes with higher SBP. The excess risk in the D allele carriers has been ascribed to the detrimental tissue effects of raised AngII (and lower Bradykinin) – including that of vascular remodelling, inducing a pro-inflammatory and pro-hypertrophic state, adhesion molecule up-regulation,– thereby worsening the already 'stressed' hypertensive tissue environment (as shown in Figure 7.2).

Figure 7.2: Mechanism of *ACE* SBP interaction (BRK Bradykinin)

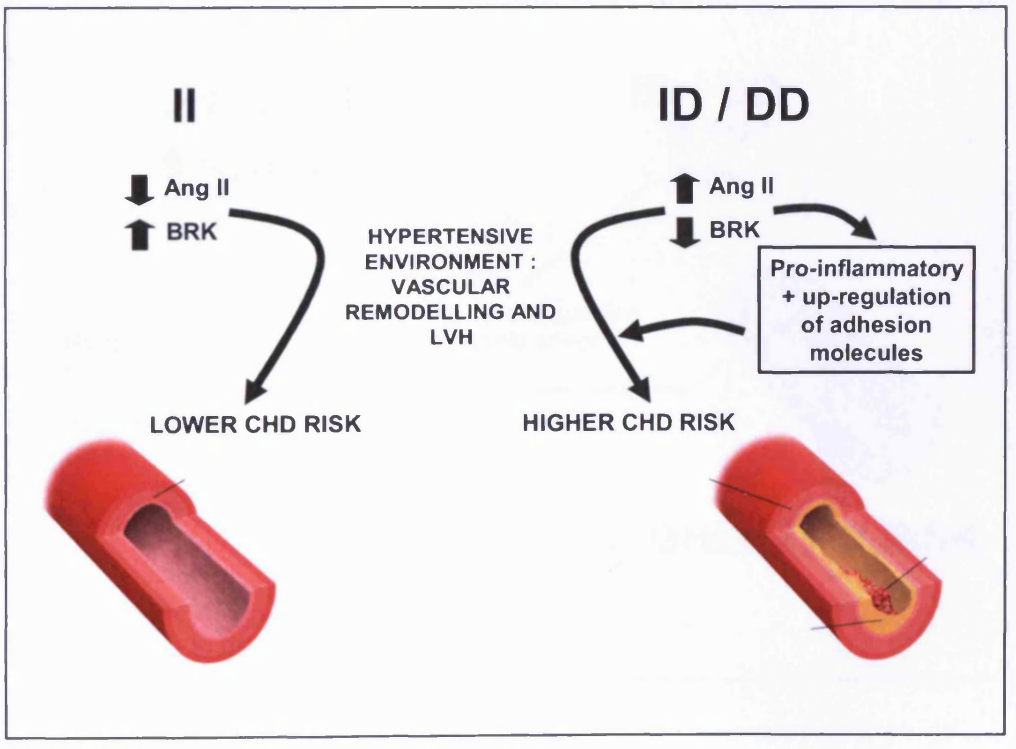
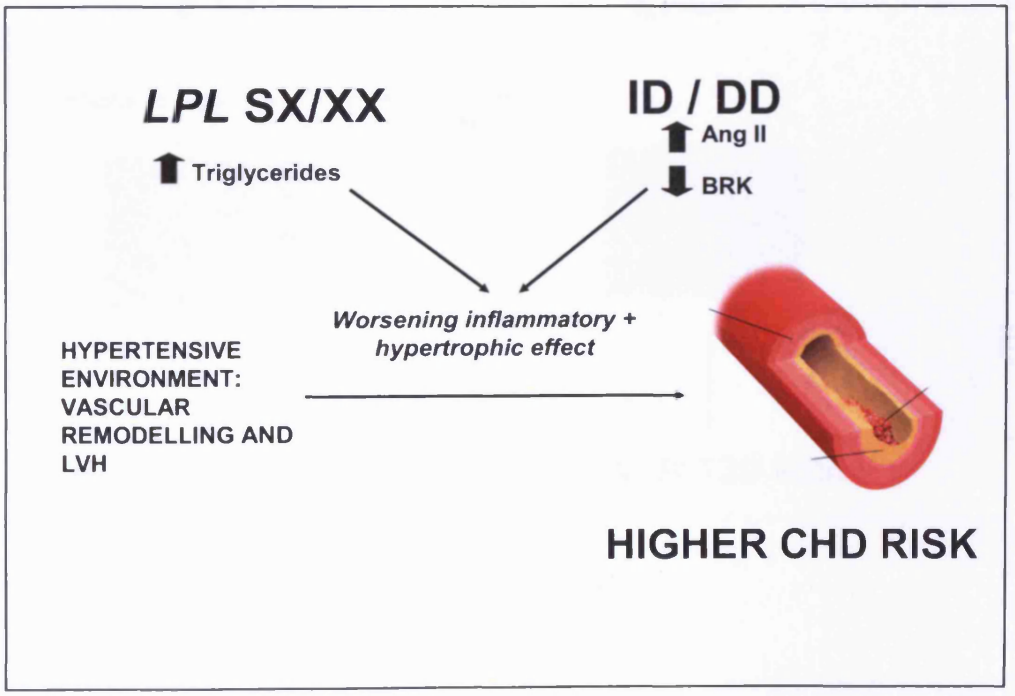


Figure 7.3 demonstrates the combined pro-inflammatory and pro-hypertrophic influences of *LPL* S447X X allele and *ACE* D allele carriers on the hypertensive state to further increase CHD risk. There is direct evidence for interaction between these two

variants with AngII also causing an increase in triglycerides (Ran et al., 2004). The putative mechanism for raised triglycerides (in *LPL447* X allele carriers) being harmful in hypertension (as opposed to normotension) is interesting. During ischaemia, in order to conserve ATP there is a switch from myocardial triglyceride to glucose metabolism (Neely et al., 1972; Allard et al., 1994), so similarly in hypertension,where there is raised tissue stress (e.g in myocardium and vascular endothelium), triglyceride metabolism may be unfavourable. Thus in hypertensive *447*X allele carriers, the raised triglyceride levels would presumably prevent shift to glucose metabolism and be energetically inefficient possibly promoting further damage (Talmud et al., 2007).

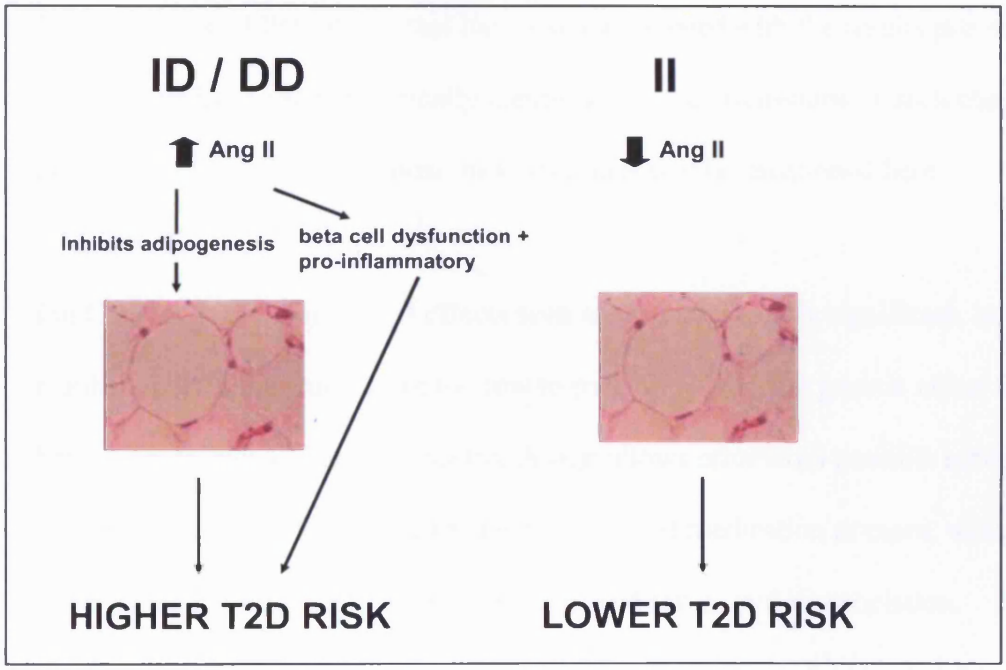
Figure 7.3 Mechanism of combined *LPL ACE* SBP interaction



ACE – Obesity interaction on T2D risk

D allele carriers in NPHS2 who are obese have been shown to have a greater T2D risk than their II counterparts. The suggested explanation for this was the multiple diabetogenic effects of the raised AngII levels on the already susceptible obese state thereby further raising the T2D risk (as demonstrated in Figure 7.4).

Figure 7.4 Mechanism of *ACE* Obesity interaction



ACE effect in HCM

The mechanism underlying the smaller left ventricular cavity size and wall thickness at baseline, and the lower increase in LVED, in D allele carriers is unclear, given that the greater ACE activity (associated with D allele) is correlated with greater cardiac

hypertrophy. Though the D allele may be associated with greater muscular strength which could explain the higher baseline pVO_2, this does not explain the association with favourable ventricular dimensions. It is important to state that all these associations were statistically non-significant so they may indeed be no effect of this polymorphism in HCM.

Limitations

There are several limitations that have been associated with the results presented in this thesis. They have been specifically mentioned in the discussions of each chapter however a summary of the most important ones will be mentioned here.

For Chapter 3, the majority of effects seen are not statistically significant, however the numbers within the study were too low to pick up the modest genetic effect that was being hypothesised. The case-control design allows error from possible survivor bias. The other main source of error relates to the use of medication in cases, which would affect blood pressure but most importantly lipid levels in this association.

The most important limitations of the NPHS2 study (Chapters 4 and 5) included the low number of cases, and the lack of data regarding details of medication. A huge strength of the study was the prospective design but because it recruited healthy men, and CHD and T2D have a low annual rate, despite 15years of follow-up, there were only a few hundred cases. In terms of lack of medication data, this is important in that effects of the *ACE* genotype may be confounded by ACE inhibitors and other anti-hypertensives

however there is as yet no consistently observed effect between I/D genotype, medication and trait.

Effects of ACE on HCM (Chapter 6) was carried out in an observational study which was also limited by numbers, approximately 500, though it is the largest cohort of HCM patients in which modifier genes have been studied reporting to date. The loss of follow up in survival data, but mainly in echo and pVO_2 measurements, further reduced the power of the study to detect an effect of this polymorphism. The causative mutation was unknown in the majority of the cohort and this may be important in identifying precise gene-gene interactions in determining phenotype. Only limited basic measurements of LV size, function and wall thickness were available. 17 of the cohort were under 16 years of age and three individuals were under three years old though it is very unlikely that the genetic effects would differ between the paedeatric and adult populations.

The absence of measurements of intermediate traits such as plasma RAS components and the use of multiple statistical testing are two limitations throughout this thesis. ACE levels would have been useful in determining the link between genotype, trait and risk, however the association between *ACE* I/D genotype and ACE levels has been consistently demonstrated. The multiple testing, including the use of interactions do increase the false positive rate, however there were *a-priori* hypotheses that genotype would only have environment-dependent effects, with biological evidence for strong prior probability, therefore the need to adjust for multiple testing is unnecessary and would also increase false negative rate.

Further studies

It is important that the key findings in this thesis are replicated in further studies with similar design and specifically targeted interactions. In view of the modest effect of the *ACE* I/D polymorphism which has a context dependent effect, very large prospective studies should be carried out where sufficient numbers of cases will occur. The cohorts should be phenotyped in a detailed manner including plasma and/or mRNA levels of RAS components and with extensive numbers of biomarkers e.g. of the inflammatory pathway. Endothelial function and LV mass can be measured as relevant intermediate traits and repeated at regular intervals. Ideally the cohort should be kept homogenous with regard to age, gender and especially to race, in that the I/D polymorphism does not associate with ACE levels in black Africans. Medications with doses and duration should be recorded.

Rodent models can be used to further define the mechanisms underlying the suggested interactions. Examples of such models in the study of atherosclerosis and hypertension include the *APOE* knockout mouse (Plump et al., 1992) which develop dyslipidaemia and atherosclerosis, and the Spontaneously Hypertensive rat (SHR) (Samani et al., 1989) which develop hypertension, LVH, RAS activation and insulin resistance. For the *ACE* lipid interaction, Keider et al (2001) demonstrated that when AngII was infused into APOE knockout mice, peritoneal macrophages increased oxidised LDL degradation compared to placebo. This effect was blocked by administration of Losartan, an ARB. Further experiments could quantify apoB levels and examine whether these mice had even more atherosclerosis than placebo. If these mice do get more atherosclerosis then they can be then randomised to a chow diet enriched with different oils (Calleja et al.,

1999) such as virgin olive oil or sunflower oil (i.e. a Mediterranean diet or Southern European environment stimulus), a normal chow diet (5% cholesterol) and an atherogenic diet ('Paigen' diet – (Paigen et al., 1985)) to help determine mechanism underlying North South Europe interaction.

For the *ACE* SBP interaction, SHR can be infused with AngII to see if they develop more atherosclerosis or LVH than SHR alone. Rodent models cannot be used to study the *ACE* Obesity interaction in T2D because AngII has the opposite effects in rodents of promoting adipocyte differentiation (Saint-Marc et al., 2001). Sharma et al has suggested further studies to address the deleterious effect of RAS in obesity through performing fat biopsies from obese humans before taking, and whilst taking ARB, to investigate whether adipocyte size was reduced, and insulin resistance improved while ARB were being taken (Sharma et al., 2002).

To examine the *ACE* effect in HCM, again a very large, ideally homogenous and well phenotyped cohort of patients are required, which would need a multi-centre, and perhaps multi-national consortium. Knowledge of the causative mutation in each individual would be helpful. Long term follow-up would enable assessment of genotype impact on survival and remodelling. Regular and detailed cardiac assessment through echocardiography or magnetic resonance imaging would be necessary for this. In large families, *ACE* and other candidate RAS genotypes could be compared between members who have the causative mutation but no phenotype and those who have the causative mutation with phenotype.

With regard to the genetic effect, a tagging SNP haplotype approach to *ACE* can be done, though whether this is better than the I/D polymorphism at marking ACE levels in Caucasians needs to be convincingly established first. To look at the combined genetic effect of the RAS, functional polymorphisms, where possible tagging SNPs in each gene can be combined, to look for gene-gene interactions that have the largest RAS effect i.e. highest AngII and lowest Bradykinin.

Angiotensin-Converting Enzyme Genotype Interacts With Systolic Blood Pressure to Determine Coronary Heart Disease Risk in Healthy Middle-Aged Men

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Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/HYPERTENSIONAHA.107.086843

Clinical Science

www.clinsci.org

online data

Clinical Science (2007) 113, 467–472 (Printed in Great Britain) doi:10.1042/CS20070158

Is the influence of variation in the *ACE* gene on the prospective risk of Type 2 diabetes in middle-aged men modified by obesity?

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