BEYOND GENOME WIDE DISCOVERY:

AN EXPLORATION OF NOVEL GENETIC VARIANTS FOR CORONARY HEART DISEASE

A Thesis Submitted for the degree of

Doctor of Medicine (MD)

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School of Medicine

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BSc (Hons), MBBS, MRCP (UK)

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For Umar and Hawaa, whose earliest memories will be of sitting in daddy's lap while he wrote his thesis

Summary of Thesis

Recent developments spurred on by the Human Genome Project have for the first time permitted genome wide association studies leading to identification of multiple novel variants for complex diseases. This thesis consists of a series of studies exploring recent genetic findings for coronary heart disease (CHD) within the broader context of the promises of the genomic era that new findings would ultimately lead to 1) Identification of new disease mechanisms 2) Permit genotype based risk prediction and 3) Promote development of novel and targeted therapies based on genotype.

We sought to address these questions, using the Emory Genebank, a collection of angiographically phenotyped subjects with stored blood samples and long-term follow up.

We first refined the phenotype for CHD to help understand underlying mechanism and demonstrated differential associations between 8 novel risk variants including 9p21, and sub-phenotypes of CHD and thereby proposed differing mechanisms of risk for these loci. With two non-CHD cohorts we then demonstrated further association between one particular risk variant at 6p24 and the intermediate phenotype of arterial elasticity and related this to a potential novel mechanism of risk.

Despite significant association with first events in population cohorts, we showed that these risk variants including 9p21 have limited value in secondary risk prediction, failing to demonstrate any association with prospective events in our cohort as single markers or when combined into a cumulative genetic risk score.

Finally in subjects carrying leukotriene pathway CHD risk variants, we administered an oral leukotriene synthesis inhibitor and after just 4 week of therapy observed significant improvement in their endothelial function.

In summary, these studies demonstrate the value of refining the phenotype to understand potential mechanisms, the complexities of genetic risk prediction and the feasibility and benefit of targeting therapy based on risk genotype.

Declaration

These studies were performed during my tenure as a visiting scientist in the Division of Cardiology at Emory University School of Medicine, Atlanta, GA, USA between July 2007 and July 2010. All work was performed under the direct supervision of Arshed A. Quyyumi MD, FRCP, FACC, Professor of Medicine and Director of the Cardiovascular Research Centre at Emory University, Atlanta, USA, with co-supervision from Julian P. Halcox MD, FRCP, Professor of Cardiology and Director of the Cardiovascular Research Group, Cardiff University, Cardiff, UK. I hereby declare that:

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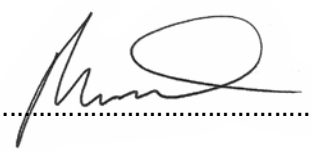

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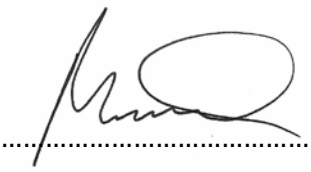

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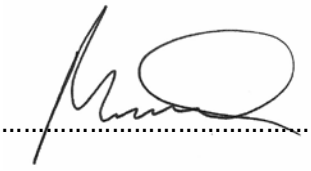

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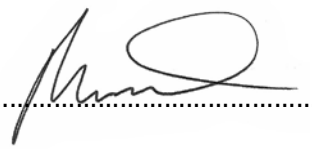

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Riyaz S. Patel, 2012

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Published Manuscripts

1. **Patel RS**, Sun YV, Hartiala J, et al. Association of a genetic risk score with prevalent and incident myocardial infarction in subjects undergoing coronary angiography. *Circ Cardiovasc Genet.* 2012;5:441-9.
2. **Patel RS**, Morris AA, Ahmed Y, et al. A genetic risk variant for myocardial infarction on chromosome 6p24 is associated with impaired central hemodynamic indexes. *Am J Hypertens.* 2012;25:797-803
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1. Neeland IJ & **Patel RS** et al. Coronary angiographic scoring systems: An evaluation of their equivalence and validity. *American Heart Journal. 2012 (in press)*
2. **Patel RS** et al. Mechanistic insights from novel myocardial infarction variants
3. Chan K & **Patel RS** et al. A collaborative meta-analysis on 9p21 and coronary artery disease

Abstracts Presented at International Meetings

1. **Patel RS,** Syed H, Blanco RR, Al Mheid I, Sher S, Eapen DJ, et al. Abstract 15501: The 5-Lipoxygenase Inhibitor Zileuton Improves Endothelial Function in Carriers of Coronary Heart Disease Risk Haplotypes in the ALOX5AP and LTA4H Leukotriene Pathway Genes. *Circulation 2011*;124(21_S):A15501
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6. **Patel RS**, Zhao J, Warren F, et al. Abstract 1437: The Chromosome 9p21 Risk Locus is Associated with a Greater Severity of Angiographically Defined Coronary Artery Disease. *Circulation* 2008; 118: S_325.
7. **Patel RS**, Al Mheid I, Jorgensen JP, et al. Abstract 3075: The Chromosome 9p21 Risk Variant rs10757278 is Associated with Increased Arterial Stiffness**.** *Circulation* 2008; 118: S_810.
8. Gender Specific Association of Depression and a Haplotype in Leukotriene A4 Hydrolase gene. Zhao J, Quyyumi A, **Patel RS**, Qureshi A, Warren F, Zafari AM, Velder E, Onufrak S, Gulcher J, Vaccarino V. *Circulation* 2008; 117: S_e244

List of Relevant Awards and Funding

1. American Heart Association Postdoctoral Fellowship Grant (2009-2011): The Genomics of Coronary Artery Disease
2. American College Cardiology (Georgia): Winner Best Poster Prize; 9p21 and CAD severity (2009)
3. Doris Duke Foundation Award (2009); Scholarship to attend workshop at the Broad Institute, led by Prof David Altshuler
4. American Heart Association (2010); Travel Award to present at AHA 2010 – only one of 4 awardees in the US
5. The Wellcome Trust: Design and Analysis of Genetic Association Studies (2011); Selected (on merit) to attend a week long intensive course at the Wellcome Genome Campus, Hinxton, Cambridge.

List of Abbreviations

5-LO:	5-Lipoxygenase
ACS:	Acute Coronary Syndrome
AIx:	Augmentation Index
AP:	Augmented Pressure
BMI:	Body Mass Index
CABG:	Coronary Artery Bypass Grafting
CAD:	Coronary Artery Disease
CHD:	Coronary Heart Disease
CNV:	Copy Number Variants
CRP:	C - Reactive Protein
CV/CVD:	Cardiovascular Disease
DBP:	Diastolic Blood Pressure
DNA:	DeoxyriboNucleic Acid
ECG/EKG:	Electrocardiogram
FLAP:	Five Lipoxygenase Activating Protein
FMD:	Flow Mediated Dilatation
GWAS:	Genome Wide Association study
HDL:	High Density Lipoprotein
LAD:	Left Anterior Descending
LCX:	Left Circumflex
LD:	Linkage Disequilibrium
LDL:	Low Density Lipoprotein
LMS:	Left Main Stem
LT:	Leukotriene
LTA4H:	Leukotriene A4 hydrolase
MAP:	Mean Arterial Pressure
MI:	Myocardial Infarction
NO:	Nitric Oxide
NSTEMI:	Non ST Elevation Myocardial Infarction
PCI:	Percutaneous Coronary Intervention
PCR:	Polymerase Chain Reaction
RCA:	Right Coronary Artery
SBP:	Systolic Blood Pressure
SNP:	Single Nucleotide Polymorphisms

STEMI:	ST Elevation Myocardial Infarction
TC:	Total Cholesterol
TG:	Triglycerides
TG:	Triglycerides
Tr/TrW:	Time to Reflected Wave

Normal Values and Units

	Mass units		Conversion Factor	SI Units	
Total Cholesterol	mg/dL	<200	x 0.0259	<5.18	mmol/L
LDL	mg/dL	<160	x 0.0259	<4.14	mmol/L
HDL	mg/dL	>40	x 0.0259	>1.03	mmol/L
Triglycerides	mg/dL	<160	x 0.0113	<1.8	mmol/L
Creatinine	mg/dL	0.6-1.2	x 88.400	53-106	µmol/L
Glucose	mg/dL	70-110	x 0.0555	3.9-6.1	mmol/L
CRP	mg/L	0.08-3.1	x 9 .524	0.76-28.5	mmol/L

Chapter 1: Background

Introduction to coronary heart disease

Coronary heart disease (CHD) is currently the most important cause of mortality accounting for 12% of all deaths worldwide.(http://www.who.int/mediacentre/factsheets/fs317/en/) It refers to a complex spectrum of disorders, mostly arising from atheroma or plaque formation within the inner lining of the coronary arteries (coronary artery disease) which leads to impaired or disrupted blood flow to the myocardium (myocardial ischaemia or infarction).

G*radual* build up of atheroma and a reduction in the luminal diameter of coronary vessels causes diminished blood flow to the myocardium which can cause ischaemia and chest pain particularly during times of increased cellular oxygen demand. This clinical syndrome was first termed “angina pectoris” by William Heberden in his classical description of the syndrome in 1768 entitled "Some Account of a Disorder of the Breast", while the association between angina and underlying disease of the coronary vessels was postulated almost 20 years later by others including Jenner, Parry and Black.(Heberden 1772; Proudfit 1983)

Sudden disruption of coronary flow however, leads to the potentially more fatal consequence of myocardial infarction with cellular necrosis. Early autopsy findings demonstrated coronary thrombosis as the cause of this condition and while the link with diseased coronary vessels was considered in the early 19th century, it was not until much later that rupture of underlying coronary atheromatous plaque was conclusively shown to be the underlying cause.(Poole-Wilson 2008)

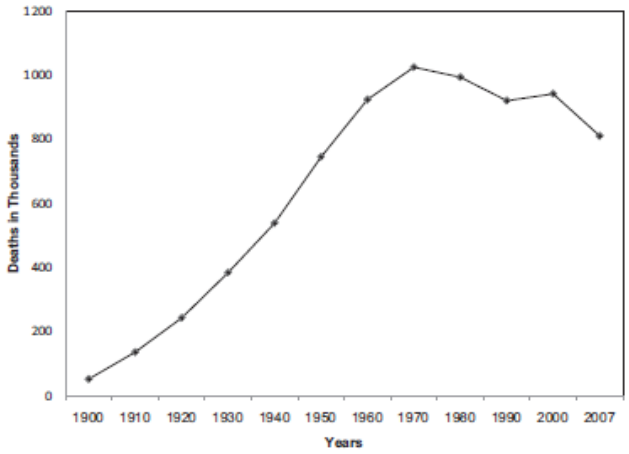
More than 200 years since these early descriptions, our understanding of the pathogenesis of coronary heart disease, atheroma formation and its sequelae of both ischaemia and infarction has progressed considerably, aided in part by the advent of invasive coronary imaging. However, despite enormous advances in elucidating the complex vascular biology of the underlying process of atherosclerosis and in development of treatments and approaches to reduce risk, CHD remains a significant health burden in both developed and developing nations worldwide.(Lusis 2000; Libby and Theroux 2005)

Extent of the problem and epidemiology

In 2010, an estimated 7.2 million deaths worldwide were due to coronary heart disease.(http://www.who.int/mediacentre/factsheets/fs317/en/) This enormous health burden has traditionally been greatest in developed or high income countries. Recent years however have seen a decline in CHD in many of these nations, mostly due to better primary prevention strategies (Figure 1). Nonetheless, many individuals continue to succumb to the disease with estimates suggesting that almost 750,000 Americans and 124,000 Britons will experience an acute coronary event this year despite aggressive attempts to manage traditional risk factors.(Roger et al. 2010; Scarborough 2010)

Figure 1: Deaths from CVD by decade in the US population (1900-2007).

Graphic illustrating a significant decline in cardiovascular (CVD) deaths in recent decades (Roger et al. 2010)



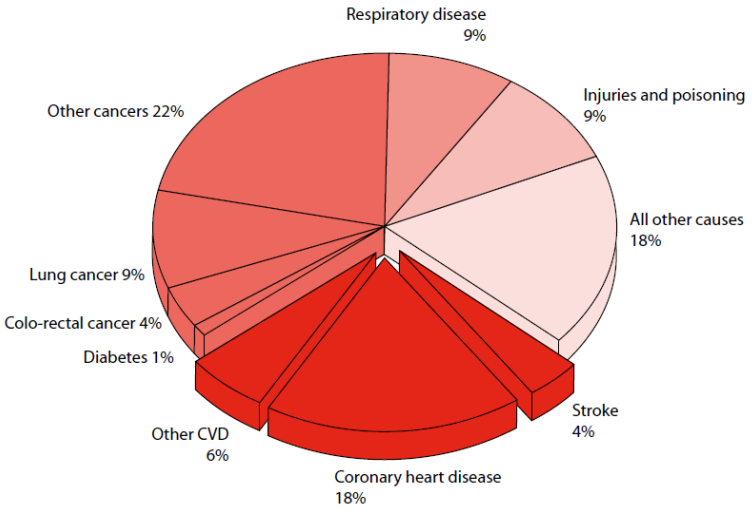
In sharp contrast however, the incidence of CHD is dramatically increasing in both middle and low income countries, due to changing diets and lifestyles and a rising burden of traditional risk factors such as diabetes and smoking. The World Health Organization (WHO) estimates that 60% of the global burden of CHD is now in developing nations and that the largest increase in deaths from the disease for example will be in South East Asia. (http://www.who.int/mediacentre/factsheets/fs317/en/) Furthermore the WHO state that "heart disease, stroke and diabetes are estimated to reduce GDP between 1 and 5% in low- and middle-income countries experiencing rapid economic growth" because of premature deaths from cardiovascular disease. (http://www.who.int/mediacentre/factsheets/fs317/en/)

Even in developed nations this burden of premature CHD is substantial. In the UK for example, there were 88,000 deaths in 2008 from CHD, of which 28,000 were

considered premature as they occurred before the age of 75 (Figure 2). Prevalence of CHD or the number of people living and being treated for angina or myocardial infarction (MI) is estimated at 2.7 million, of which 1.6 million are potentially of working age.(Scarborough 2010)

Figure 2: Mortality in men under the age of 75 by cause in the UK.

Mortality from cardiovascular disease accounts for approximately 1/3 of all premature deaths (Scarborough 2010)



In the UK, an estimated 2/3 of all those with acute MI do not reach hospital and die in the community. This form of sudden cardiac death is often the first and only presentation of CHD for many individuals. Of those that do reach hospital alive, 11% of men and 15% of women die within 30 days.(Scarborough 2010) Of the remainder who survive the initial MI, many will experience long term disability, requiring treatments for consequences such as heart failure and arrhythmias. This has substantial implications for quality of life for those individuals as well as financial consequences for the state with treatments such as implantable cardioverter-defibrillators, repeat hospitalizations and lost working days.

It is clear that beyond continued attempts to manage known risk factors on an epidemiological level, there is an urgent need to focus our efforts (1) on developing new strategies to identify individuals at risk of CHD with greater precision so that meaningful interventions can be targeted earlier in life in a cost effective manner and (2) to better understand the pathophysiology of CAD and atherosclerosis in order to develop new high impact therapies.

Overview of the stages of atherosclerosis

While coronary heart disease (CHD) can include any disease process involving the coronary arteries such as vasculitis, embolism or dissection, the most common cause of CHD is atherosclerosis, a progressive and systemic disease process affecting the entire arterial tree. The arterial wall consists of an inner monolayer, the endothelium, beyond which is a thin sub-endothelial space which contains elastin and collagen fibres along with a few smooth muscle cells (SMCs), bounded by the medial and adventitial layers of the arterial wall.

Early insults to the endothelium are believed to trigger innate immune and inflammatory responses with monocyte migration and then entrapment of lipid laden macrophages within the sub-intimal space. The formation of characteristic foam cells promotes a further inflammatory cascade resulting in modifications in extracellular matrix composition and smooth muscle cell migration and development of mature plaque with varying compositions and stability.(Libby and Theroux 2005)

While the clinical sequelae of atherosclerosis, namely luminal compromise or plaque rupture with sudden thrombosis, may present in later life, the process itself starts almost from birth as evidenced by the early changes of atherosclerosis termed "fatty streaks" identified in the aortas of teenagers and in young soldiers killed during the Korean War.(Enos et al. 1953; Strong et al. 1999) Under microscopy these streaks consist of fat laden macrophages (foam cells), T cells and smooth muscle cells.(Stary et al. 1994)

Initiation of fatty streaks, the early precursors of atherosclerosis, is widely held to occur as a "response to injury" to the endothelium, a complex monolayer of cells responsible for maintaining homeostasis in a variety of biological systems relevant for the vasculature.(Ross 1993)

Endothelial dysfunction and initiation of atherosclerosis

In health, one the most important roles of the endothelium is the maintenance of vasomotor tone.(Quyyumi 1998) Broadly, the endothelium regulates vasomotor tone by secreting both vaso-dilatory as well as vaso-constrictive factors and by maintaining a balance between the relative concentrations of these counteracting substances. One of the most important vaso-dilatory mediators in this regard is nitric oxide (NO), synthesized by nitric oxide synthase (NOS, of which three isoforms exist including eNOS, iNOS and nNOS) that converts L-arginine to NO and L-citrulline in the presence of cofactors such as tetrahydrobiopterin.(Furchgott and Zawadzki 1980); (Bonetti et al. 2003; Forstermann and Munzel 2006) NO diffuses into vascular smooth muscle cells and induces smooth muscle relaxation through cyclic GMP production. Production of NO is regulated through multiple pathways including hormones such as acetylcholine, norepinephrine, bradykinin and importantly shear stress.(Bonetti et al. 2003) Increases in shear stress enhance eNOS expression, whereas low levels of shear decrease it and this response forms the basis of clinical assessment of endothelial function termed flow mediated dilatation (FMD), described later.(Celermajer et al. 1992; Bonetti et al. 2003) In contrast, inflammatory and oxidant processes decrease eNOS expression.(Liao et al. 1995) Other vaso-dilatory factors secreted by the endothelium include the endothelium-derived hyperpolarizing factors (EDHF) and prostacyclin,(Ito et al. 1980; Feletou and Vanhoutte 1999) while vasoconstriction is achieved through prostaglandin H_2, thromboxane and 20-hydroxyeicosatetraenoic acid, expression of angiotensin-converting enzyme (ACE) and through the family of endothelins that can produce either constriction or relaxation by acting through their receptors (ET-1, -2, and -3).(Attina et al. 2005)

Other important functions of the endothelium include: (1) maintenance of a thrombo-resistant lining of the arterial wall via regulation of the balance between secreted anti- and pro- coagulant agents such as NO, prostacyclin, plasminogen activator inhibitor (PAI), tissue factors; (2) modulation of immune function through chemokine signalling and surface expression of adhesion molecules; (3) lipid transport and metabolism via low density lipoprotein receptor regulation; (4) cellular growth and vascular remodelling through synthesis of components of the extracellular matrix including collagen, elastin, glycosaminoglycans, fibronectin as well as matrix metallo-proteinases and growth factors that regulate smooth muscle cell proliferation and (5)

regulating active and passive transport of cells and macromolecules into the sub-intimal space and beyond.(Poole-Wilson 2008)

Thus, under physiological conditions, the endothelium plays a critical role in preserving vascular health by expressing a quiescent protective phenotype. However dysfunction or activation of this layer leads to a shift in the homeostatic systems to promote a more reparative or host defence response with immune and inflammatory mechanisms, that ultimately forms the basis of the atherosclerotic process.(Deanfield et al. 2007) Cardiovascular risk factors including dyslipidaemia, smoking, advanced age, hypertension and hyperglycaemia activate these various pro-inflammatory and endothelial dysfunctional mechanisms.(Kinlay and Ganz 1997)

Oxidative stress and redox signalling plays a key role in mediating risk factor-mediated endothelial activation. A variety of cellular processes lead to formation of reactive oxygen species (ROS), which in the presence of superoxide dismutase generate hydrogen peroxide (H2O2). Like NO, H2O2 can diffuse into cells and modify cysteine residues to alter transcription and activation of multiple proteins and genes.(Rhee 2006) These effects may include activation of various molecular systems ranging from increased inflammatory signalling to abnormal lipoprotein transport, all of which may promote formation of plaque.

While risk factors are often systemic, plaques generally form in certain regions of the vasculature such as bifurcations and branch points. One important factor determining lesion initiation is shear stress. Physical shear forces on the endothelium appear to alter endothelial cell morphology, such that cells in regions of laminar flow appear distinct from those in areas of low shear stress. These areas appear to show increased predilection to accumulate lipid and plaque formation.(Gimbrone 1999) Recent studies using advanced computational flow mapping have further confirmed these findings of increased plaque formation at sites of altered shear stress.(Samady et al. 2011) Changes in flow influence gene transcription and lead to altered expression of receptors such as ICAM-1 which lead to enhanced monocyte migration across the endothelium.(Nagel et al. 1994)

Thus, initiation of the atherosclerotic process is widely considered to be a "response to injury" to the vascular endothelium with associated endothelial dysfunction or activation.

Plaque biology and progression

Animal models have demonstrated that atherosclerosis involves the progressive development of plaque consisting of modified lipids, collagen, extracellular matrix, macrophages, T cells, new blood vessels and smooth muscle cells.(Libby and Theroux 2005) In the presence of endothelial activation, the key initiating event in plaque formation is the accumulation of lipid within the sub-intimal space. In normal health low levels of circulating low-density lipoprotein (LDL), which is the most atherogenic type of lipoprotein, binds to the LDL receptor (LDLR), is transported into the sub-endothelial space and is eventually extruded as high-density lipoprotein (HDL) back to the liver via the blood stream.(Poole-Wilson 2008) Antioxidant properties of the endothelium ensure the molecule remains in its reduced state in the intima. In the presence of hyperlipidaemia and other cardiovascular risk factors this antioxidant capacity however is overwhelmed such that LDL is modified to oxidized LDL (oxLDL), which is not easily cycled out of the intima and becomes trapped in the sub-endothelial space. Substantial evidence supports the role of oxLDL in development of atheroma and studies confirm its presence in atherosclerotic plaques.(Yla-Herttuala et al. 1989)

The presence of oxLDL and greater oxidant stress then promotes further endothelial dysfunction, leading to inappropriate vasoconstriction, down-regulation of NO, up-regulation of inflammation and enhanced thrombogenicity, all creating a pro-atherogenic milieu. A series of events is then triggered which promote expansion of the newly formed plaque.(Ross 1999)

(1) Monocyte chemotaxis: Local cytokine production leads to greater expression of cell adhesion molecules (I-CAM; V-CAM) on the endothelial surface, leading to increased monocyte adhesion and migration into the vessel wall, with differentiation into macrophages driven by monocyte colony stimulating factor (M-CSF).(Smith et al. 1995; Collins et al. 2000) Activated macrophages and vascular cells secrete yet more inflammatory cytokines that amplify the whole process of cell adhesion, infiltration and inflammation.
(2) Macrophage infiltration: Macrophages phagocytose oxLDL via scavenger receptors to form characteristic foam cells subsequently evolving into fatty streaks. These foam cells and the overlying endothelium express monocyte chemotactic protein 1 (MCP-1), which further enhances monocyte chemoattraction and adhesion.(Gu et al. 1998) Modified LDL also activates Toll-

like receptors (TLRs), in particular TLR4, which in turn induce pro-inflammatory gene expression leading to more macrophage infiltration.(Akira et al. 2001)

(3) Migration of SMCs: Dysfunctional endothelial cells result in decreased NO availability, which together with the direct effects of oxLDL, growth factors and cytokines secreted by the foam cells all promote smooth muscle cells (SMCs) in the vessel wall to switch into a proliferative phenotype (hyperplasia), with division and migration into the intima. These cells proliferate and elaborate a rich and complex extracellular matrix which forms the fibrous cap and causes the lesion to expand.(Lusis 2000)

(4) MMP activation: Endothelial cells, monocytes and SMCs all secrete matrix metalloproteinases (MMPs), enzymes which modulate vascular cell functions, including activation, proliferation, migration, and cell death, as well as remodelling, healing, or destruction of extracellular matrix of arteries and the myocardium.(Lusis 2000) As the plaque expands, calcification may occur through mechanisms similar to those in bone formation. In addition to proliferation of smooth muscle cells and extracellular matrix, death of lipid-laden macrophages can lead to extracellular deposition of lipid which can coalesce and form the lipid-rich "necrotic" core of the atherosclerotic plaque.

Thus through a process of early endothelial dysfunction, inflammation and lipid modifications, coronary artery disease is initiated. Established lesions then grow and expand through further accumulation of lipid, foam cells and extracellular matrix with continuous remodelling through the effects of MMPs.

With progressive inflammation, macrophage infiltration and foam cell accumulation, the plaque expands and may begin to cause clinical consequences.

Plaque rupture, arterial remodelling and the concept of the vulnerable plaque

It has long been recognized from pathological studies that superimposed thrombosis on a plaque is a crucial feature of fatal and non fatal MI. Subsequent in-vivo imaging techniques along with the success of antithrombotic and fibrinolytic therapy has firmly established the role of thrombosis in the pathogenesis of MI.(Libby and Theroux 2005) According to pathological studies, a complete rupture of the plaque's fibrous cap is often responsible for fatal MI. However, coronary thrombosis at the site of a plaque can also arise secondary to superficial erosion of the plaque, intra-plaque haemorrhage and the erosion of a calcified nodule leading to non-fatal MI.(Virmani et al. 2002)

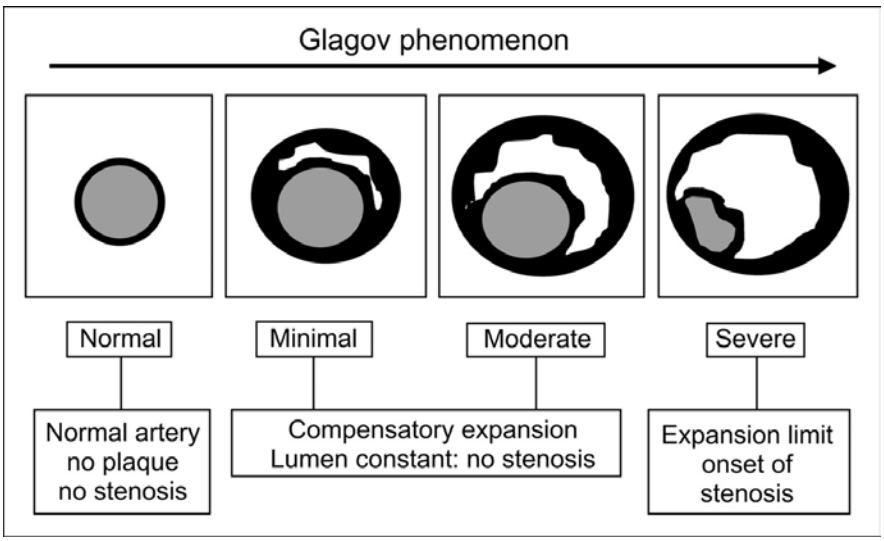
Thus, some degree of physical disruption of the atherosclerotic plaque accounts for almost all acute coronary events. Plaque disruption may occur due to sudden changes in wall stress, with vulnerable points being shoulder regions of plaques. Disrupted plaques provoke thrombosis by direct contact with collagen in the plaque's extracellular matrix, triggering platelet activation. Along with this stimulus, tissue factor secreted by activated macrophages and SMCs within the plaque further activates the coagulation cascade.(Libby and Theroux 2005) Additionally plasma levels of PAI-1, which are elevated in diabetes, obesity and other metabolic states inhibit natural fibrinolysis in these conditions.(Vaughan 2003)

The dominance of luminal imaging techniques formulated early ideas that atherosclerosis was a focal disease affecting segments of the coronary tree with lesions or plaques slowly growing into the lumen until flow was compromised or thrombosis on a tight lesion occluded flow altogether. However, it is now accepted that atherosclerosis is a widespread and progressive process which is influenced by systemic inflammation and oxidant stress.(Libby and Theroux 2005) Importantly, acute thrombosis may occur on lesions not compromising flow. Two observations have in particular altered our understanding of atherosclerosis and plaque biology.

(1) Affected coronary arteries appear to undergo a process called Glagov remodelling such that plaque may expand outwards before encroaching on the lumen (Figure 3). Visible disease in the lumen is analogous to the tip of the iceberg, with the bulk of atheroma residing in the intima.(Glagov et al. 1987) This has been confirmed by in-vivo ultrasound techniques as well as ex-vivo studies, even in healthy subjects. However, this is not a universal rule and

plaque initially growing outwards may suddenly begin to grow inwards and vice versa.

Figure 3: Glagov arterial remodelling



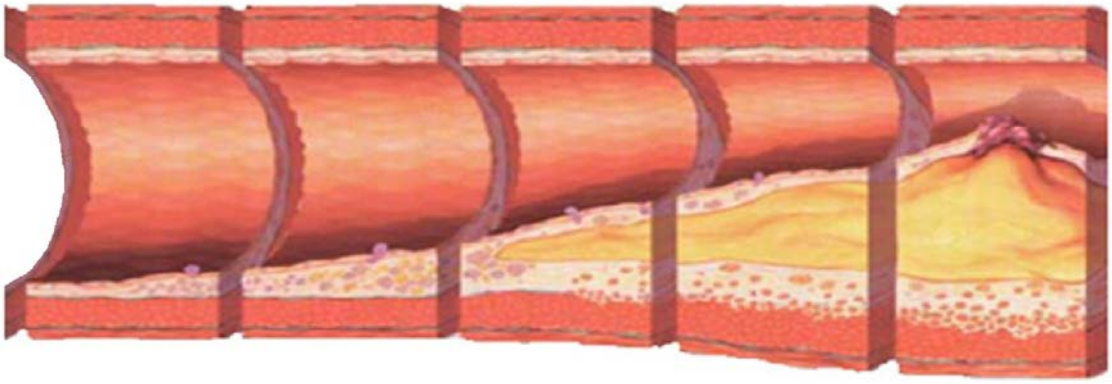
Theoretical model of Glagov remodelling postulates that plaque grows outwards before reaching a limit of expansion, after which lumen encroachment begins (Berman et al. 2006)

(2) Pathological and intravascular ultrasound (IVUS) studies have shown that plaques with moderate or <50% luminal obstruction can cause acute coronary syndromes with plaque rupture and thrombosis. It was suggested that these plaques experienced greater wall tension, which changes in direct proportion to their radii. In the coronary arteries, it was subsequently identified that plaque with a weak fibrous cap, large necrotic core and inflammatory cell infiltration at the shoulder regions were more susceptible to rupture and are considered vulnerable whereas those with greater collagen content were more stable and less prone to rupture or erode and cause infarction.(Davies 1996) This has been recently confirmed in-vivo using virtual histology ultrasound techniques.(Stone et al. 2011)

These findings have consolidated the concept of the vulnerable plaque.(Davies 1996) The amount of collagen in the fibrous cap depends on the balance between synthesis and destruction of intracellular matrix and inflammatory cell activation. Existing collagen may be degraded by macrophages that produce proteolytic enzymes and by matrix metalloproteinases (MMPs). Efforts have been made to identify such

vulnerable plaque with techniques such as virtual histology and near-infra red spectroscopy. However, further studies have also revealed that many such plaques exist and not all lead to clinical events.(Stone et al. 2011) Furthermore, it is unclear how to handle such vulnerable lesions once discovered. All of these features have shifted focus in turn towards a more holistic approach aimed at tackling atherosclerosis and inflammation systemically as opposed to a lesion centric approach. This has subsequently led to the idea of the "vulnerable patient".(Libby and Theroux 2005)

Figure 4: Progressive stages of atherosclerosis



Endothelial Dysfunction - Fatty Streak - Early atheroma – Plaque - Expanding plaque – Ruptured plaque

Schematic representation of the main stages of atherosclerosis leading to myocardial infarction: the corresponding angiographic phenotype attempting to capture each of these stages is also presented. Figure modified from the original illustration found at http://en.wikipedia.org/wiki/File: End_ dysfunction_Athero.PNG. Permission not required under the GNU free documentation license.

In summary, it is apparent that the umbrella term "CHD" encompasses a wide spectrum of disease processes and mechanisms ranging from endothelial dysfunction, early fatty streaks, advanced stable plaque through to acute coronary syndromes secondary to plaque rupture and erosion (Figure 4). Studies attempting to examine CHD as a single pathological entity or phenotype will therefore be unable to adequately explain or shed mechanistic insight into any observed associations with novel biomarkers.

Assessment and diagnosis of CHD

Given the complexity of CHD and atherosclerosis as described above, several techniques have arisen to diagnose CHD at its various levels of manifestation permitting detailed study in-vivo (Table 1). Clinical use has predominantly focused on the later stages of the disease especially for determination of myocardial infarction and coronary stenoses. Research studies in contrast have explored the earliest stages at the level of endothelial dysfunction, while population screening and demand for non invasive testing has expanded the role of imaging in the preclinical phase of atherosclerosis.

Vascular dysfunction, particularly endothelial dysfunction is considered the earliest stage of atherosclerosis and can be assessed in several ways. Early invasive studies sought to examine coronary endothelial function as dilatation in response to agents that stimulate NO release such as acetylcholine.(Quyyumi 1998) Non invasive methods, based on the assumption that endothelial dysfunction is systemic, have since evolved as surrogates for vascular risk.

(1) Brachial reactivity assesses the degree of dilatation in the brachial artery in response to shear stress induced by hyperaemia. Hyperaemia is produced from occlusion and release of a cuff placed on the forearm that is inflated to supra-systolic pressures to produce ischaemia followed by release. The degree of dilatation of the ultrasound imaged brachial artery is termed flow-mediated dilatation (FMD) and is a reflection of NO-mediated endothelium-dependent vasodilatation in response to shear stress.(Celermajer et al. 1992) Thus, a healthy endothelium will elicit a greater response to NO compared to a dysfunctional endothelium. This technique has gained widespread acceptance as a surrogate marker of risk and measure of endothelial dysfunction. Clinically, however, it is cumbersome to perform, requires a controlled environment and skilled operators to obtain reliable and reproducible readings. Nonetheless, in recent large studies, FMD has been shown to have prognostic value for incident cardiovascular events and is correlated with coronary endothelial dysfunction which too has shown prognostic value.(Halcox et al. 2002; Yeboah et al. 2009)

Table 1: Methods to assess the different stages of atherosclerosis

Vascular dysfunction	***Preclinical or early CAD***	***Plaque Presence***	***Plaque Severity***	***Plaque Burden***	***Functional Significance***	***Vulnerable plaque***	***MI (STEMI or NSTEMI)***
Biomarkers	Biomarkers	Biomarkers	Biomarkers	Biomarkers		Biomarkers	Biomarkers
FMD	CTCA/Ca	CTCA/ Ca	CTCA/ Ca	CTCA/ Ca	Perfusion CMR		ECG
PWA/PWV	CIMT	CT Calcium	CT calcium	CT Calcium	SPECT/ PET		Troponin
					Stress Echo		
					Exercise ECG		
		Angiography	Angiography	Angiography	Pressure wire		
	IVUS	IVUS	IVUS	IVUS		IVUS	
						Virtual IVUS	
						NIrS	
		OCT	OCT	OCT		OCT	

FMD: Flow Mediate Dilatation; PWA/V – pulse wave analysis/velocity; CIMT: Carotid Intima Media Thickening; CTCA: CT Coronary Angiography; CMR: Cardiac Magnetic Resonance; Electrocardiogram; IVUS: Intravascular ultrasound; OCT – Optical coherence tomography; NIrS – Near infra-red spectroscopy

(2) Arterial elasticity indices can be derived non-invasively and represent structural alterations in the vessel wall leading to arterial stiffening. Greater arterial stiffness leads to enhanced left ventricular after-load, myocardial ischaemia and stroke.(Laurent and Boutouyrie 2007) Age and risk factor-mediated changes in the collagen content of large vessels is considered to represent vascular dysfunction. Oxidative stress plays a key role in mediating this change. Pulse wave analysis (PWA) permits non-invasive derivation of central pressure augmentation indices. These indices are thought to represent wave reflection properties of the arterial tree and a composite of arterial stiffening but also vasomotor tone in the periphery.(Laurent et al. 2006) The consequence is to increase LV after-load and promote ischaemia. The gold standard assessment of arterial stiffness is currently carotid-femoral pulse wave velocity (PWV). (Laurent et al. 2006) Both arterial stiffness and central haemodynamic indices have been shown to associate with adverse prognosis in recent large meta-analyses. (Vlachopoulos et al. 2010a; Vlachopoulos et al. 2010b)

(3) Biomarkers for endothelial dysfunction are diverse, a review of which is beyond the scope of this thesis introduction. Measures of endothelial progenitor cells have particularly attracted attention as markers of reduced reparative capacity and have been correlated with endothelial dysfunction and adverse prognosis.(Hill et al. 2003)

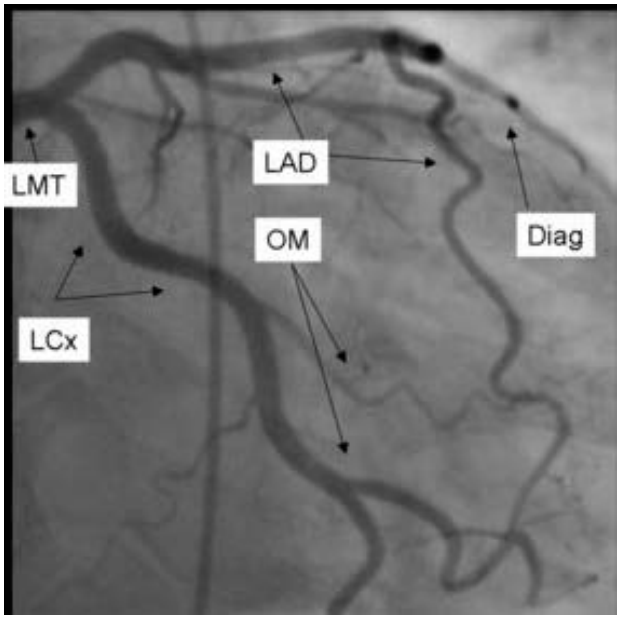
Methods also exist to identify early or preclinical CAD non-invasively using carotid B- ultrasound to identify intima media thickening, a tool which is used in large population studies as an early marker of atherosclerosis. Computed tomography is now also widely used to determine coronary calcification and to visualize coronary anatomy non-invasively.(Ibanez et al. 2009)

The current gold standard of assessment of established CAD is through invasive coronary angiography. First devised in 1954, the technique involves passing small lumen catheters retrogradely from a peripheral artery to the aortic root, with selective intubation of the coronary vessels and injection of contrast dye. X-ray fluoroscopy permits acquisition of high quality images with excellent temporal and spatial resolution (Figure 5). Lesions are identified and assessed for severity based on visual estimation in reference to the nearby lumen diameter in unaffected segments.(Poole-Wilson 2008) Researchers have attempted to quantify CAD burden and plaque size with a variety of

techniques including semi-quantitative scores as well as computerized techniques such as quantitative coronary angiography (QCA).

Despite evidence of Glagov remodelling coronary angiography remains the most widely used tool for assessing CAD for clinical and up until recently research purposes.

Figure 5: Coronary angiography



Example of coronary angiography, with injection of contrast into the left coronary system, demonstrating smooth unobstructed luminal silhouettes, suggesting absence of significant intra-luminal coronary lesions. LMT- Left Main Trunk; LCx – Left Circumflex; OM – Obtuse Marginal; LAD: Left Anterior Descending; Diag - Diagonal

More detailed invasive assessments of coronary disease are available with intravascular ultrasound (IVUS). This technology has revolutionized our appreciation of plaque burden not visible on angiography.(Nair et al. 2002) It is now considered the gold standard for assessing coronary plaque burden, but its expense and technical limitations have restricted its application to smaller scale research studies and specific clinical applications. Furthermore, using spectral analysis of radiofrequency backscatter, virtual IVUS has demonstrated in-vivo appreciation of plaque with thin fibrous caps and those with thick caps, revealing presence of multiple "vulnerable

plaque" in any one individual.(Nasu et al. 2006) Other newer techniques include optical coherence tomography and near infrared spectroscopy, which are described elsewhere.(Stamper et al. 2006; Schaar et al. 2007)

Functional significance of existing lesions is assessed using a variety of perfusion scans, based on inducing stress to determine e differences in perfusion before and after to infer presence of flow limiting lesions in coronary territories (Table 1).

Diagnosis of myocardial infarction (MI) is predominantly based on significant enzyme, specifically troponin, release from the myocardium into the blood stream as evidence of myocardial necrosis in a clinical setting consistent with myocardial ischaemia.(Thygesen et al. 2007) As such current definitions of MI emphasize a triad of clinical symptoms, ECG changes and enzyme elevation. Acute coronary syndrome is a broader definition and encompasses a spectrum of entities including (1) myocardial infarction, either transmural or sub-endocardial full thickness or transmural infarction (2) Chest pain with myocardial necrosis below the threshold for diagnosis of infarction and (3) Unstable angina, presenting with symptoms at rest or increasing in frequency without evidence of myocardial necrosis.

Despite sophisticated methods to diagnose CHD, simpler more cost effective tools are needed to screen the population at large from which many individuals will experience an event for the first time. One way to do this is to identify risk factors and estimate risk of future coronary events as a means to filter those needing further investigation or primary prevention therapy.

Traditional cardiovascular risk factors and CHD risk models:

Large scale epidemiological studies have established several key risk factors which predispose individuals to CHD risk. The INTERHEART study from 52 countries around the world identified 9 risk factors which were estimated to account for 90% of the population attributable risk for first MI. These included smoking, dyslipidaemia, hypertension, diabetes, abdominal obesity, psychosocial factors, daily consumption of fruits and vegetables, regular alcohol consumption, and regular physical activity.(Yusuf et al. 2004) The following risk factors, in addition to age and gender are widely established as promoting atherosclerosis and increasing risk of CHD.

(1) Diabetes; is characterized by insulin deficiency or resistance and results in endothelial dysfunction, decreased endothelial thrombo-resistance, and increases platelet activity. It predisposes significantly to atherosclerosis. Studies have unequivocally confirmed an increased risk of about 2-3 fold for future cardiovascular (CV) events in diabetic patients.(Almdal et al. 2004) There is also substantial evidence that glucose levels per se, even in those without diabetes predisposed to CV risk.
(2) Hypertension; causes endothelial dysfunction and increases collagen, elastin, and endothelial permeability and platelet and monocyte accumulation. Prospective studies have demonstrated a clear association with high blood pressure and CV mortality.(Miura et al. 2001)
(3) Hyperlipidaemia; a variety of lipid abnormalities are associated CHD risk including elevated LDL, triglycerides, non-HDL, apoB, Lp(a) and low HDL. Furthermore familial disorders of lipid metabolism are associated with severe premature coronary atherosclerosis, while clinical trials with drugs to lower lipid levels have demonstrated conclusive benefit in mortality reduction.(Shepherd et al. 1995)
(4) Obesity; is associated with the metabolic syndrome and itself leads to and is associated with insulin resistance, hyperlipidaemia and hypertension. However in a study of the Framingham cohort, body mass index (BMI) was found to be independently associated with CV risk.(Wilson et al. 2008)
(5) Smoking; increases platelet activity and catecholamine levels, alters prostaglandins, and decreases high-density lipoprotein (HDL) levels. It has consistently been shown to increase risk of MI and CAD and more so in women more than men.(Prescott et al. 1998)

Other risk factors known to contribute to CHD include the metabolic syndrome, lack of exercise, a diet high in saturates and cholesterol, psychosocial factors such as stress, lack of oestrogen, chronic kidney disease and inflammation assessed by hsCRP.(Yusuf et al. 2004)

Risk models:

These risk factors often act in concert and risk factor assessments using multivariate modelling is a useful means of attempting to identify those individuals without known CHD at greatest risk of future events. A variety of risk scoring algorithms have been devised for this purpose of risk stratification with the view that those in the highest risk categories would benefit most from intensive lifestyle interventions and drug therapy.(Cooney et al. 2009) Most scores currently available estimate an average risk over a 10-year period. Perhaps the most widely known algorithm is the Framingham risk score based on the Framingham population in the US, a longitudinal study of multiple generations. The first score incorporated age, gender, LDL-cholesterol, HDL-cholesterol, blood pressure (including whether the patient is treated or not), diabetes, and smoking to derive an estimated risk of developing CHD (MI, coronary death, and angina) within 10 years.(Wilson et al. 1998) Despite multiple problems with the score, including the fact that it was derived from a very specific ethnic group and that it often over- or under-estimated risk, the score has gained widespread acceptance and is used to determine the degree of risk for individuals upon which treatment decisions are based. Other scores have been devised to overcome issues with the Framingham scores and include the European SCORE, QRISK1 and QRISK2 and the Reynolds Risk Score, which take into account features such as ethnicity, parental history and other variables.(Cooney et al. 2009)

While these risk models have some value they clearly do not capture all risk and although high risk individuals will more often than not be identified, those at lower or intermediate risk, especially women, may be falsely reassured. This has led to multiple other efforts to develop novel tests to capture risk more accurately. Some of these include measures of oxidative stress, homocysteine, endothelial and vascular dysfunction, endothelial progenitor cells, hyperuricaemia, carotid IMT, coronary calcification, non-CRP markers of inflammation (eg WBC, ESR, IL6, IL18, TNFalpha, Adiponectin), infections (CMV, Chlamydia, h pylori) and coagulation factors to among others.

However, one particular risk factor that is pervasive and highly predictive has not received much attention. Genetic predisposition or family history is a substantial risk factor for CHD and offers the potential to be unique to an individual rather than an average of risk across a population. The remaining sections focus on the genetics of CHD and novel approaches to understanding this risk.

The genetic basis of coronary heart disease

As described at the start of the previous section, despite recognition and treatment of known risk factors on an epidemiological level, with a resultant fall in *population* CHD mortality,(Scarborough 2010) many *individuals* continue to succumb to this disease.(Castelli 1996) CHD encompasses a spectrum of conditions and stages of atherosclerosis each with a complex interplay of environmental, lifestyle and hereditary factors. These latter genetic factors are often proposed to account for much of the individual level risk, although this nebulous phenomenon has remained poorly understood.

It is hoped that a greater understanding of genetic predisposition to CHD will unravel clues to its aetiology and pathogenesis and allow development of novel diagnostic and therapeutic tools to permit targeted interventions to reduce this global health burden

Evidence for a genetic basis to CHD

"The chief interest in the accompanying case is in the remarkable family history volunteered by the patient…. In most instances the [angina] attacks began in the third decade, occasionally in the second…. Such a group of cases suggests some common etiological factor…. The history certainly points to a familial proclivity towards the disease." (Mills 1925)

"*Entire families sometimes show this tendency to early arteriosclerosis. A tendency which cannot be explained in any other way than that in the make-up of the machine bad material was used for the tubing"* (Osler 1892)

Familial clustering of premature disease, as in the examples above, is often considered as evidence of a shared genetic predisposition. In fact, subjects with an affected parent are at a 2 fold greater risk, which is even greater if siblings are affected.(Cremer et al. 1997; Lloyd-Jones et al. 2004; Yusuf et al. 2004; Murabito et al. 2005; Chow et al. 2011) Although such families represent only 14% of the general population they account for 72% of early CHD cases and 48% of CHD at all ages, thus underscoring the social and economic importance of genetic predisposition to CHD.(Williams et al. 2001) Statistical estimates of heritability, which represents the fraction of phenotypic variability that can be attributed to genetic variation, have been

quoted as high as 63% for premature MI.(Nora et al. 1980) Twin studies support this estimate, with an 8-fold and 4-fold increased risk for monozygotic and dizygotic males, respectively, with an affected twin.(Marenberg et al. 1994) Furthermore, adoptee studies have suggested that this risk is not just due to shared environmental influences. The landmark Danish study of a 1000 families with adopted children revealed that the death of a biological parent before the age of 50 from a vascular cause was associated with a 4.5 fold increase in mortality for the offspring from the same cause whereas the death of an adoptive parent from similar causes did not significantly increase this risk.(Sorensen et al. 1988) Finally imaging based studies have also revealed that the anatomy of coronary disease (e.g. left main stem, proximal left anterior descending disease, ectasia, and coronary calcifications) may itself be inherited, while coronary calcium scores and carotid intima-media thickness (IMT) are also enhanced in those with a family history of premature CHD.(Wang et al. 2003b; Nasir et al. 2004; Fischer et al. 2005; Fischer et al. 2007; Nasir et al. 2007)

These findings, taken with clinical experience, make it difficult to dispute the existence of at least a partially heritable basis for CHD. Within this context it is worth noting that a family history is more common and the heritability estimates greater for premature CHD than for later onset disease. This is important in interpreting genetic studies and the interplay of age and environmental factors with genetic predisposition.(Marenberg et al. 1994; Winkelmann et al. 2000) Secondly, genetic effects are often attenuated after adjustment for classic cardiovascular risk factors, suggesting that some component of this genetic risk is conferred through inherited tendencies to these conditions such as dyslipidaemia, hypertension, diabetes, smoking and obesity. Nonetheless, considerable heritability is independent of these risk factors and therefore likely to be mediated by currently *unknown* mechanisms and pathways, the pursuit of which is clearly of great interest. Finally, there will likely be multiple gene-environment and gene-gene interactions in disease causation and possibly also epigenetic factors such as genomic imprinting associated with deoxyribonucleic acid (DNA) methylation.(Wierda et al. 2010)

In a small percentage of cases, CHD is a clinical manifestation of Mendelian disorders which are relatively rare, have a predictable transmission mode such as autosomal dominant or X-linked inheritance with the underlying genetic mutations often having large effect sizes. Over the last 50 years, extensive research has led to the identification of multiple genes for most Mendelian disorders that cause CHD, for

example, familial hypercholesterolaemia is now known to be caused by mutations in the genes encoding low-density-lipoprotein receptor, apolipoprotein B100, proprotein convertase subtilisin/kexin type 9, and low density lipoprotein receptor adapter protein 1.(Soutar and Naoumova 2007) However, in the vast majority, CHD is a complex disease with multiple genetic and life style risk factors. In contrast to Mendelian disorders, complex diseases do not have a predictable pattern of inheritance and are believed to associate with many DNA variants in multiple genes each having a modest effect size. Nonetheless, over the last decade there has been considerable progress in the identification of genetic determinants for complex diseases including CHD.

Genetic linkage and association studies

The key to identifying genetic predisposition is in variation within genes, i.e. differences between individuals with and without the disease. The most common type of genetic variation consists of differences in individual base pairs, known as single nucleotide polymorphisms (SNPs). Other variations include variable copies of segments of DNA (copy number variants) as well structural variants that affect large chromosomal regions. Two approaches have traditionally been used to identify causal variants. These include linkage analysis and association studies which have been the mainstay of genetic studies attempting to unravel the heritable basis of CHD. Both approaches aim to identify disease causing genes and DNA variants which predispose to the development of CHD.

Linkage analysis:

Family based linkage studies are so called due to their reliance on the principle of linkage disequilibrium. This states that the process of meiosis at conception permits exchange of genetic material between chromatids. The closer the genes on a chromosome the less likely they are to be exchanged and thereby will be inherited together as a single unit. By using traits expressed from known genes, or DNA markers whose genomic position is known and which co-transmits with the disease in families, it is possible to map the disease causing gene in the genome, i.e. to identify the chromosome and the location on that chromosome where the disease gene resides.

This approach, has proven very successful in mapping genes for Mendelian (monogenic) disorders, but has had much less success for complex diseases. This may be

due to a number of reasons including phenotypic heterogeneity and locus heterogeneity of complex diseases and the low statistical power of this method to detect modest genetic effects. Several groups have nonetheless used linkage analysis to study complex CHD. Examining DNA microsatellite polymorphisms (short tandem repeat DNA sequences) or single nucleotide polymorphisms (SNPs) dispersed throughout the human genome, in DNA samples from collections of families with premature CHD, these studies have identified regions of a number of chromosomes that potentially harbour CHD genes (Table 2). However, these findings in general have not been cross replicated in the various listed linkage studies, which may be due to different population ethnicities, different software algorithms and varying phenotypic criteria for CHD. Furthermore, with a few exceptions, the likely disease genes in these, often extensive, chromosomal regions have also not been identified.(Wang et al. 2003a; Helgadottir et al. 2004)

For example, a potentially exciting finding was a linkage peak at chromosome 15q26, harbouring 92 genes of which variation in a gene encoding myocyte enhancement factor 2a, a transcription factor in endothelial cells (MEF2A) was identified. (Wang et al. 2003a) This seemed promising given its role in development of coronary arteries, but subsequent follow up studies failed to replicate the genetic or functional studies reported. (Weng et al. 2005; Guella et al. 2009) Meanwhile the largest of these linkage studies, the British Heart Foundation Family Heart Study, consisting of over 4000 patients with CHD from 1,900 families in the UK did not identify any statistically significant linkage peak for any of the cardiovascular phenotypes studied.(Samani et al. 2005)

Table 2: Major genome wide linkage scans for CHD

Population	*Phenotype(s)*	*Families/ Individuals*	*Locus Identified*	*Plausible Candidate Genes*	*Possible Gene Function*	*Reference*
Finnish	Premature CHD; Age <55 male, <65 female	156/364	2q21.1-22 Xq23-26	GPD2 AGTR2	Glucose-insulin axis Unknown cardiovascular function	Pajukanta(Pajukanta et al. 2000)
Mauritian	Premature CHD; Age <52yrs	99/535	16p13 10q23	SOSC1, SSTR5 CYP2C8, LIPA	Cytokine regulation. Glucagon-insulin axis Vascular tone. Tissue accumulation of lipids	Francke(Francke et al. 2001)
Australian	ACS <70 yrs	61/122	2q36-37.3	IRS1	Insulin sensitivity and metabolic syndrome	Harrap(Harrap et al. 2002)
European	Premature CHD; Age <59yrs	513/1406	14	Unknown	-	Broeckel(Broeckel et al. 2002)
American	Autosomal Dominant Pattern CHD	1/13	15q26	MEF2A*	Vascular morphogenesis	Wang(Wang et al. 2003a)
American	Premature CHD; Age <51 male, <56 female	420/1168	3q13 1q25	Unknown	Possible lipid genes CRP, P-Selectin, E-Selectin. Inflammation	Hauser(Hauser et al. 2004)
American	Premature CHD; Age <45 male, <50 female	428/1613	1p34-36	CX37	Vascular/ endothelial integrity	Wang(Wang et al. 2004)
Icelandic	MI	296/713	13q12-13	ALOX5AP*	Leukotriene modulation, inflammation	Helgadottir(Helgadottir et al. 2004)
Icelandic	MI	#	12q22	LTA4H	Leukotriene synthesis, inflammation	Helgadottir(Helgadottir et al. 2006)
British	CHD <66 yrs	1933/4175	2p12-q23.3	IL1A,B PLA2R1, OSBPL6	Interleukin 1 cluster HDL cholesterol regulators	Samani(Samani et al. 2005; Nsengimana et al. 2007)
European	CHD <66 yrs	2036/5316	17p11.2 (MI)	Unknown	-	Farrall(Farrall et al. 2006)

*Major genome wide linkage scans performed for CHD. Phenotypic definitions vary for each study with the broad definition of CHD given here to encompass the spectrum of CAD and MI phenotypes unless specifically stated phenotype. Candidate genes and functions are those proposed by the authors of the respective publications. * Mapped genes by further sequencing. # Based on work from earlier linkage scan. MI - Myocardial Infarction; CHD - Coronary Heart Disease; ACS - Acute Coronary Syndrome; HDL - High Density Lipoprotein; CRP - C-Reactive Protein*

Leukotriene pathway variants

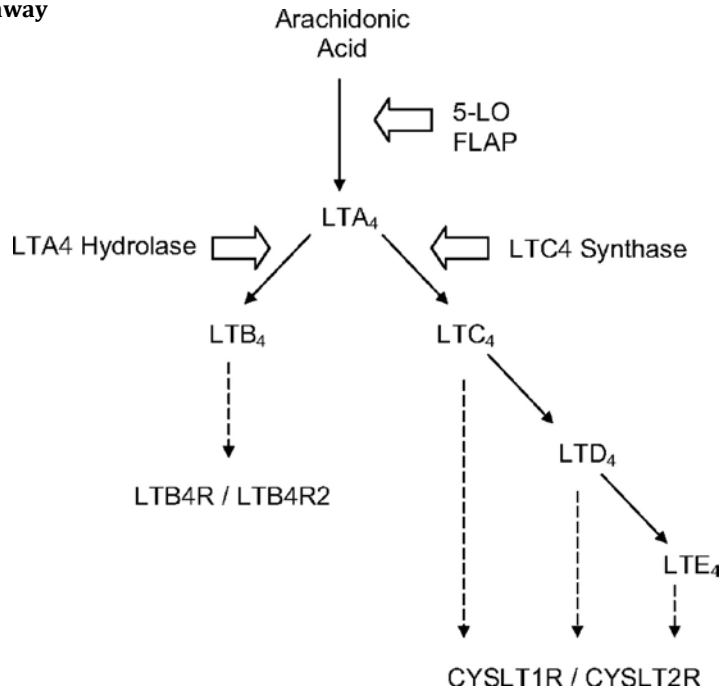
Perhaps the most promising finding using this approach was the identification of sequence variants within genes involved in the leukotriene (LT) pathway (Figure 6). Using over a thousand microsatellite marker, Helgadottir et al performed a genome wide linkage scan in 296 families (713 individuals with MI) Icelandic families with MI and identified a 4 SNP combination termed HapA in the ALOX5AP gene encoding the 5-lipoxygenase activating protein (FLAP) which was found to be associated with MI (RR 1.9) (frequency 15.8% compared to 9.5% in controls) and stroke (RR 1.8).(Helgadottir et al. 2004) A slightly different combination of SNPs in the same gene termed hapB was also identified as conferring risk of MI and stroke in a second British cohort. (Helgadottir et al. 2005) A subsequent study, again in Icelandic individuals, reported a moderately increased risk of MI (RR 1.16), for carriers of a haplotype this time in another gene but still within the LT synthesis pathway, LTA_4H (termed hapK), which encodes LTA_4 hydrolase (Figure 6). The authors replicated the association of hapK with MI and stroke in a pooled population from three North American cohorts, but interestingly found that the risk was 3-fold greater in African American subjects.(Helgadottir et al. 2006) About 28% of European American controls carried at least one copy of HapK, compared to only 6% of African American controls. Data from the HapMap Project show that HapK is rare in Africa, suggesting that the occurrence of HapK in African Americans is due primarily to admixture.

Several other groups have attempted to replicate these early findings for hapA and hapB. Crosslin et al confirmed a modest association with both hapA and hapB and MI,(Crosslin et al. 2009) while others have shown association mostly with hapB.(Girelli et al. 2007; Linsel-Nitschke et al. 2008; Crosslin et al. 2009; Tsai et al. 2009; van der Net et al. 2009) However, it is worth noting that some studies have failed to show any significant association and explanations have been proposed to account for this including phenotypic heterogeneity and ethnic variability in haplotype frequency.(Zee et al. 2006; Koch et al. 2007) Few studies have examined replication for hapK, with only

one study confirming association with MI and one failing to do so.(Crosslin et al. 2009; Hartiala et al. 2011) Despite some of these negative studies, on the whole, genetic variation in LT synthesis genes has shown significant association with risk of MI, stroke and CAD.

Prior to the landmark study by Helgadottir et al, the Los Angeles Atherosclerosis Study, evaluated the prevalence of variant genotypes in the 5-LO gene (ALOX5) promoter in 573 patients without a history of CVD, and correlated variant genotypes with severity of atherosclerosis as measured by carotid-artery intima-media thickness (IMT).(De Caterina and Zampolli 2004; Dwyer et al. 2004) These findings, together with the significant elevation of CRP in carriers of two variant alleles compared to those with none (2.6 vs. 1.3 mg/L), suggested enhanced vascular inflammation and atherogenesis related to increased 5-LO expression caused by the variant promoter. The biological mechanism by which these variants confer risk is thus speculated to be due to elevation of leukotrienes. This makes intuitive sense because LTB_4 is a potent chemotactic agent for leukocytes and may promote inflammation within blood vessels and atherosclerotic plaques, leading to accelerated atherogenesis, plaque instability or both. Indeed functional studies by Helgadottir et al revealed that males with MI who were also carriers of hapA had greater levels of neutrophil stimulated LTB4 than matched controls without MI and subjects with MI without the risk genotype.(Helgadottir et al. 2004) This data further support the concept that these variants are functional and may up-regulate LT activity.

Figure 6: Leukotriene synthesis pathway



(Bevan et al. 2009)

The initial step in leukotriene synthesis is the formation of leukotriene A_4 (LTA_4) from the oxidation of arachidonic acid (AA) by 5-LO; this transformation is dependent upon the binding of 5-LO to FLAP (Vila 2004). LTA_4, is converted to either leukotriene B_4 (LTB_4) by LTA_4-hydrolase (LTA_4-H) or to leukotriene C_4 (LTC_4) by LTC_4-synthase (LTC_4-S). LTC_4 and its downstream products leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) are collectively referred to as the peptide-LTs or cysteinyl-LTs (CysLTs). 5-LO expression occurs primarily, if not exclusively, in leukocytes, but its product, LTA_4, can be released from leukocytes and taken up by surrounding cells lacking significant 5-LO expression but containing LTA_4-H and/or LTC_4-S activity. This process, allows for the production of LTB_4 and LTC_4 in a broad range of cell types including endothelial cells, vascular smooth muscle cells, and platelets (Fabre et al. 2002; Jala and Haribabu 2004; Vila 2004).

The LTs exert their effects via binding to specific G protein-coupled seven trans-membrane receptors and subsequent kinase activation(Jala and Haribabu 2004). Receptors for LTB_4 and the CysLTs have been identified and cloned: BLT1 and BLT2 are the high- and low-affinity LTB_4 receptors respectively, while the CysLT receptors have been termed CysLT1 and CysLT2. The expression of these receptors varies by cell type, with macrophages expressing BLT1, BLT2, CysLT1 and CysLT2, endothelial cells expressing both BLT1, BLT2, and CysLT2, vascular smooth muscle cells expressing BLT1, CysLT1 and CysLT2, and neutrophils and T lymphocytes expressing only BLT1(Jala and Haribabu 2004; Heller et al. 2005). CysLT1 is also expressed in pulmonary interstitial macrophages, bronchial smooth muscle cells, and mast cells; its role in airway inflammation, edema, and smooth muscle constriction is the rationale behind the development of the CysLT1 antagonists montelukast (Singulair®) and zafirlukast (Accolate®), both of which are FDA approved for the treatment of asthma (Appendix).

This genome wide linkage finding generated considerable interest, as firstly the genes were discovered to associate with MI in an unbiased, non-hypothesis-driven way through a genome wide scanning approach. Second, genetic evidence for the leukotriene pathway in MI risk appeared to validate growing evidence from in vitro, animal and human studies of LT involvement in atherosclerosis.

Leukotrienes have long been associated with several pathologic inflammatory states, including asthma and rheumatoid arthritis. Although a link between LTs and CAD was identified as early as 1992, with elevated urinary LTs in subjects with cardiac ischaemia,(Carry et al. 1992) only recently have they been implicated in vascular pathology.(Jala and Haribabu 2004) The biological actions of LTs provide several potential pathophysiologic mechanisms in atherogenesis. While the primary effects of CysLTs (LTC_4,D_4,E_4) are to mediate smooth-muscle contraction and vascular

permeability, LTB_4 plays an important role in chemotaxis and adhesion of leukocytes (neutrophils, monocytes, and T lymphocytes) to endothelial cells, migration across the endothelium, and activation of both neutrophils and macrophages, with generation of reactive oxygen species and formation of foam cells. (Yokomizo et al. 2001; Friedrich et al. 2003) (Aiello et al. 2002)

In addition, genetic mouse models revealed that mutations in the 5-LO gene conferred resistance to atherosclerosis in hyperlipidaemic animals.(Mehrabian et al. 2001; Welch et al. 2001; Mehrabian et al. 2002; Kuhn et al. 2003) In $apoE^{-/-}/BLT1^{-/-}$ mice, aortic atherosclerotic lesion area is reduced by up to 55% compared to $apoE^{-/-}$ controls (Subbarao et al. 2004; Heller et al. 2005) and is accompanied by a significant reduction in atheroma smooth muscle cells, (Heller et al. 2005) suggesting a significant role for LTB_4 in atherogenesis**.**

These findings were also corroborated by human studies demonstrating that the 5-LO/LT pathway participates in atherosclerotic processes. Studies showed that 5-LO, FLAP, and LTA_4 hydrolase (LTA4H) are abundantly expressed in arterial walls of CVD patients and that 5-LO has markedly greater expression in advanced lesions and localizes to inflammatory cells, such as macrophages, dendritic cells, and neutrophilic granulocytes.(Spanbroek et al. 2003) Additionally, over expression of 5-LO in carotid plaques appeared to identify those subjects who were symptomatic and had events compared to those that weren't.(Cipollone et al. 2005) BLT1 receptors have also been identified in human carotid artery plaques and shown to be functional through patch clamp techniques, with evidence that their activation promotes migration and proliferation of smooth muscle cells in vitro while antagonism leads to reduced hyperplasia after injury.(Back et al. 2005)

Dwyer et al also reported that persons carrying variant alleles of a 5-LO promoter polymorphism consisting of tandem Sp1-binding sites have significantly greater carotid atherosclerosis.(De Caterina and Zampolli 2004) An interesting extension of these analyses was that high dietary arachidonic acid (AA), the primary 5-LO substrate that leads to greater levels of pro-inflammatory LTs, exacerbated the atherogenic effect of the variant alleles, whereas high dietary n–3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), that are also substrates for 5-LO but that generate non-inflammatory LTs, blunted this effect. (De Caterina and Zampolli 2004; Dwyer et al. 2004)

Thus, genetic evidence along with in vitro, mouse and human studies have demonstrated a strong role for LTs in CHD development. Given these findings, there is an immediate opportunity to devise therapy based on LT synthesis inhibition or receptor blockade to ameliorate cardiovascular risk. LT synthesis and receptor blockers have been licensed for use in asthma but have not yet been utilized in treating vascular disease.

Thus two distinct genes in the LT metabolic pathway, with a plausible mechanistic basis were associated with CHD using the genome wide linkage approach. Whether these findings can lead to therapeutic benefit remains to be determined.

Association studies:

In contrast, association studies involve direct comparison of the frequency of the genetic variant between those with a clinical condition and those without in unrelated individuals and with greater power than linkage studies.

Previously, almost all genetic association studies were focused on variation in genes related to known pathological pathways or risk factors such as hyperlipidaemia or inflammation. This so called "candidate gene" approach starts with the hypothesis that, because the gene to be studied encodes a protein known to be involved in the disease pathogenesis, variation in that gene may alter its expression or the function of the encoded protein, consequently leading to disease. Lists of published CHD related candidate gene variants are available online (http://hugenavigator.net/ HuGENavigator/home.do; http://www.bioguo.org/CADgene/).(Liu et al. 2010a) Surprisingly, despite over 5000 publications, very few of these findings have been replicated.(Mayer et al. 2007) Non-replication can be due to false positive findings in the original studies or false negative results in subsequent studies, all of which may arise for a variety of reasons, the most common reason being inadequate sample sizes which increases the rates of type I and type II errors. Some of the most widely studied candidate genes in relation to CHD include for example *ACE; APOE; NOS3; MTHFR; PON1; IL6* (http://hugenavigator.net/HuGENavigator/home.do) with lipid genes often showing the most successful replication.

The candidate gene association approach, however, has two major limitations. First, since the functions of most of the 20k-25k genes in the human genome and the exact pathogenesis of complex diseases such as CHD are not fully elucidated, candidacy will likely be biased. Second, such studies usually focus on the exons, introns and immediate flanking regions of the candidate genes. However, these regions represent only a small fraction of the genome, while the inter-genic regions contain many DNA elements (e.g. enhancers, silencers) that regulate gene expression. Although sequence variations in some of such DNA elements can potentially confer susceptibility to complex diseases, they are not covered in candidate gene studies.(Grant et al. 2006)

These limitations were largely overcome by the genome-wide association (GWA) approach which has only recently become feasible, thanks to advances in our knowledge of the human genome and in genotyping technologies.

The genomic era and GWAS

The Human Genome Project and HapMap

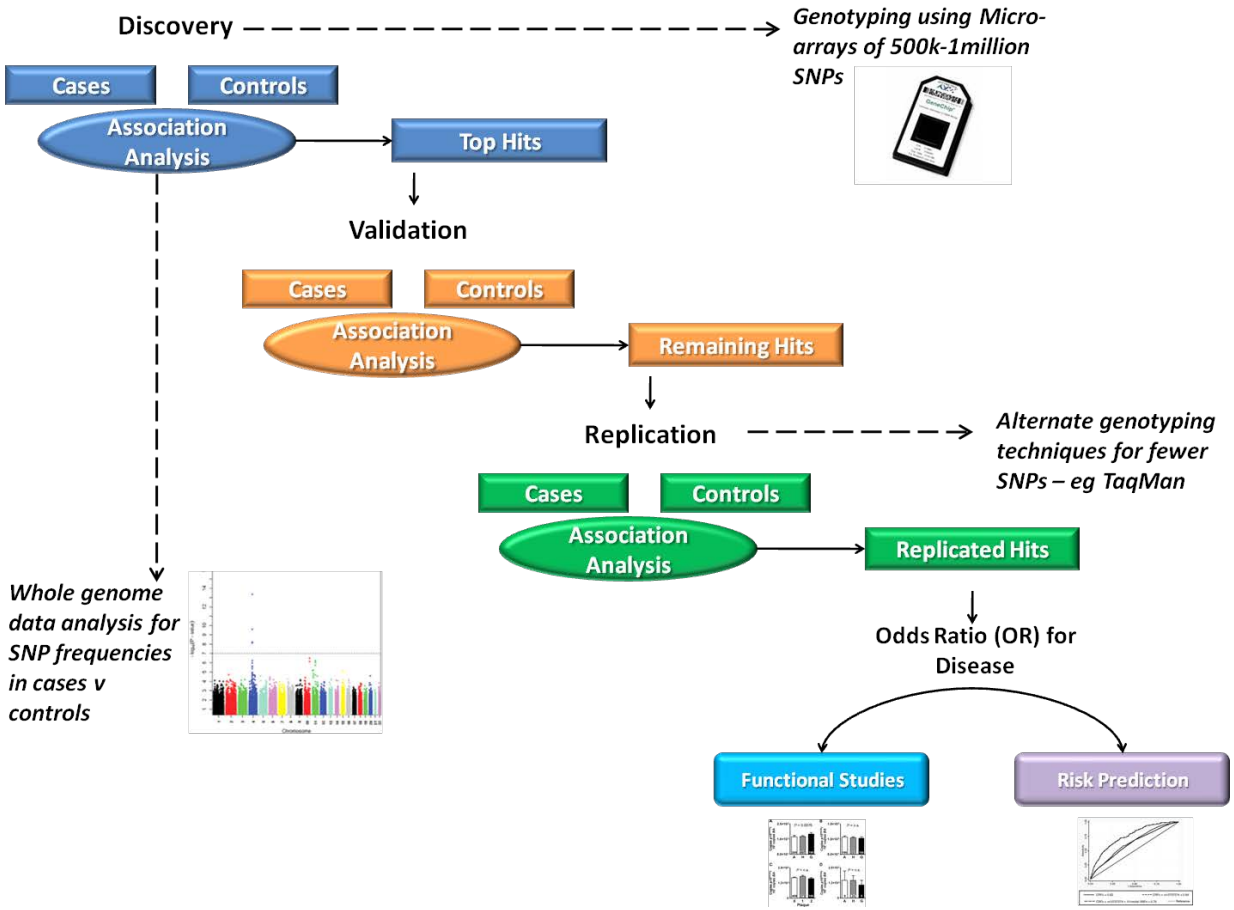
The Human Genome Project was initiated in 1990 as a joint venture between the department of energy and the NIH and after just 11 years the first draft sequence of the human genome was published, with the final version released in 2003 just 11 days short of the 50th anniversary of Watson and Crick's discovery of the double helix. It revealed the sequence of the 3 billion base pairs of the human genome and the positions of approximately 20,000-25,000 genes, far fewer than anticipated. With the exception of identical twins, every person is genetically unique, such that the genomes of any two individuals are only roughly 99.9% identical. It is however the 0.1% difference which is the key to identifying disease causative genes and mapping this variation was the remit of the international HapMap projects, which sequenced genomes of multiple individuals from different ethnic groups across the world.

As a result of these efforts, The Human Genome Project, the SNP Consortium and the International HapMap Project have now collectively identified approximately 10 million common DNA variants in the human genome, the majority of which are SNPs.("The International HapMap Project" 2003; A haplotype map of the human genome" 2005; Altshuler et al. 2010) Because DNA variants that are near each other tend to be inherited together, they can be in linkage-disequilibrium (LD), e.g. if a SNP which has a cytosine (C) in one allele and a thymine (T) in another allele is in LD with a SNP which has an adenine (A) in one allele and a guanine (G) in another allele, people who have the C allele for the first SNP will tend to have the A allele for the second SNP while people who have the T allele for the first SNP will tend to have the G allele for the second SNP. The HapMap project has now provided comprehensive knowledge of LD among SNPs throughout the human genome.(Altshuler et al. 2010) Taking advantage of this knowledge, it is possible to capture information on many SNPs across the genome by examining only a much smaller set of "tag" SNPs, therefore reducing the costs. In parallel to these scientific breakthroughs, technologies have developed rapidly allowing simultaneous typing of up to approximately 1 million common SNPs providing over 90% coverage of all common SNPs in the genome.(de Bakker et al. 2005) These scientific and technological advances have enabled genome wide association studies (Figure 7), which have led to the identification of hundreds of novel genomic loci that influence various complex diseases in the last few years. (Hirschhorn and Daly 2005; Chanock et al. 2007; Donnelly 2008)

GWAS principles and methodology

Combining the advantages of linkage and candidate gene association studies, genome wide association is essentially a method for interrogating the 12 million variation points across the human genome with a phenotype of interest. Using a case-control design and making no assumptions about aetiology, GWAS permits an agnostic or hypothesis free approach to identifying causal variants for disease. DNA from cases and controls is genotyped using micro-array chips which can examine up to a million SNPs per sample. Specific GWA analysis algorithms can then identify variants with statistically different frequencies in cases and controls. Critically, since most known common variants that influence disease have modest effects, large sample sizes are required to minimize the false negative rate while a stringent threshold of p values of less than $5x10^{-8}$, is used to limit the false positive rate due to multiple testing. Staged designs balance statistical power and efficiency,(Satagopan et al. 2002; Thomas et al. 2004; Skol et al. 2006) with top "hits" from discovery stages taken forward for replication in independent case-control sets. Once discovered, the disease loci are then subject to further study to identify causal variants through sequencing of the region and further functional studies to identify causal mechanisms (Figure 7).

Figure 7: Methodology for genome wide association studies (GWAS)



Genome Wide Association Studies are case-control studies, permitting an agnostic or hypothesis free approach to identifying causal variants for disease. DNA from cases and controls are genotyped using micro-array chips which can examine up to a million SNPs per sample. Specific GWA analysis algorithms can then identify variants with statistically different frequencies in cases and controls. Given the multiple testing involved, a stringent threshold of p values of less than $5x10^{-8}$, is used to limit the false positive rate. Large sample sizes are required to minimize the converse false negative rate. Top "hits" are taken forward for replication in independent case-control sets. Once discovered, the disease loci are then subject to further study to identify causal variants through sequencing of the region and further functional studies to identify causal mechanisms.

Genome wide association studies for CHD:

Ozaki et al first piloted the GWAS approach for CAD/MI approach by examining 65k SNPS in 1133 cases with MI and 1878 controls and identified 2 SNPS in the lymphotoxin-alpha (LTA) gene as being significantly associated with MI. Functional studies suggested a role for this protein in vascular inflammation. Subsequent studies by the same group identified LGALS2 polymorphisms, encoding galectin-2, as also associated with MI and involved in the LTA pathway. Unfortunately, attempts at replicating this finding in the ISIS cohort failed to show any significant association for 7 variants in the LTA gene. (Clarke 2006)

In 2007, using more advanced microarrays, 3 groups almost simultaneously reported findings for a novel locus associating with CHD using this new approach. In a large Icelandic cohort, Helgadottir and colleagues performed GWAS on 1670 cases (MI patients from the several Icelandic cohorts) and 6728 controls (derived from various genetic registries). After ensuring appropriate quality control on the SNPs on a 300K chip, their association analysis led to the identification of 3 SNPs (rs1333040, rs2383207, rs101162077) all within a 190kb LD block on chromosome 9p21, that were significantly associated with MI. Replication in a further 665 Icelandic patients and 3 cohorts from the US, confirmed the finding with rs2383207 remaining the most significant. Refinement of the SNPs in this region showed the strongest association was with rs10757278. This SNP, the risk allele being G, demonstrated an OR of 1.64 (1.47-1.82) for MI for homozygotes and an OR of 2.02 (1.72-2.36) in those with early MI (MI <50). The group further associated the same SNP with prevalence of MI in 2 US cohorts, showing in turn an allelic OR of 1.28. The risk allele in these Caucasian populations is particularly prevalent with 21% of the population being homozygous for this risk variant. The authors quoted for this variant a PAR of 21% for MI and 31% for early MI cases.(Helgadottir et al. 2007)

Meanwhile, in the same issue of the journal, McPherson et al. described a three stage GWAS of Caucasian subjects with CHD, using a 100K SNP microarray and identified two SNPs (rs10757274 and rs2383206) in the same narrow 58kb region of chromosome 9p21 which significantly associated with CHD (definition included MI and elective revascularization cases). They validated their findings in five other mostly Caucasian cohorts from Denmark and the United States (23000 participants in total). This study revealed a 15-20% increase in risk for heterozygotes and a 30-40% increase in risk for homozygotes.(McPherson et al. 2007)

Finally at about the same time, a third independent group, the Wellcome Trust Case Control Consortium (WTCCC), performed GWA for CAD in 1,926 cases and 3,000 controls from a British population and also demonstrated significant association with MI and CAD with another SNP in the 9p21 locus, rs1333049 showing an OR 1.90 (1.6-2.24) for subjects homozygous for the risk variant.(WTCCC 2007) This finding was then successfully replicated in a German cohort of 875 MI patients and 1644 controls with a pooled OR estimate of 1.36 (1.27-1.46) per risk allele (C).(Samani et al. 2007)

Thus despite using different cohorts in different countries, the three studies independently identified the same small region of chromosome 9p21 as associating with CHD.

This association was also independent of traditional risk factors including family history. Multiple studies have since successfully replicated these findings in a variety of populations including other European, South Asian and East Asian groups.(Hinohara et al. 2008; Hiura et al. 2008; Shen et al. 2008; Zhou et al. 2008; Ding et al. 2009; Saleheen et al. 2010) However, replication of the 9p21 risk variant in African or African-American subjects has not yet been demonstrated, although available data from multi-ethnic cohorts suggests a lack of association in this group, presumably due to smaller linkage blocks. Meta-analyses have further consolidated the significance of these findings.(Schunkert et al. 2008) One such study incorporating 36,000 cases and 96,000 controls revealed that compared to subjects with one 9p21 risk allele, those with two alleles had an OR of 1.25 and those with 0 alleles an OR of 0.8 for CHD.(Palomaki et al. 2010) The study also demonstrated clearly that the effect size diminished with advancing age.(Palomaki et al. 2010) Even considering publication biases, these findings clearly demonstrate that 9p21 is the most robust and replicated genetic variant associated with CHD to date.

Despite the significant association of this locus with CHD, the mechanism of action remains uncertain as it does not reside within any known genetic pathway. Part of the problem has been that the various studies have correlated this variant with the phenotype of CHD, using a mixture of patients with acute and chronic CAD, MI, revascularisation history etc. At the most primary level it was initially unclear if the locus influenced an atherosclerotic pathway or a plaque rupture/thrombosis mechanism.

Interestingly, the 9p21 locus was additionally associated with multiple other potentially related phenotypes. These include abdominal aortic aneurysm, intracranial aneurysms, ischaemic stroke, peripheral arterial disease, sudden death, heart failure, human longevity, prognosis after bypass surgery, platelet reactivity and arterial stiffness.(Helgadottir et al. 2008; Cluett et al. 2009; Newton-Cheh et al. 2009; Wahlstrand et al. 2009; Yamagishi et al. 2009; Emanuele et al. 2010; Liu et al. 2010b; Musunuru et al. 2010a) No association has been noted for cardiac structure, endothelial progenitor cells, carotid IMT, flow mediated dilatation and in-stent restenosis phenotypes.(Samani et al. 2008b; Farzaneh-Far et al. 2009; Hoppmann et al. 2009; Ye et al. 2010)

Importantly, a significant association was later demonstrated with carotid atherosclerosis suggesting an atherosclerotic mechanism. However, direct association with coronary atherosclerosis was lacking and the few studies that examined the association of severity of coronary artery disease with genotype were inadequately powered to detect any association.

Thus, a direct test of association between the 9p21 variant and coronary artery disease burden would help determine if the locus influenced atherosclerosis mechanisms or a more abrupt thrombotic/plaque rupture phenotype.

In many ways, 9p21 was the low hanging fruit and subsequent GWAS studies identified multiple other genetic variants associating with CHD. Those identified by mid 2009 are listed in Table 3. Worthy of specific mention is the MIGEN consortium study by Kathiresan et al. In a discovery sample of 5000 and an effective replication sample size of 19000 subjects, they identified 9 variants associating with premature MI of which 3 were novel (21q22, 6p24, 2q33) and 6 which had been discovered previously (9p21, 1q41, 1p13, 10q11, 19p13, 1p32).(Kathiresan et al. 2009a) Other individual GWAS also identified variants associating with CHD and MI phenotypes at loci including 3q22 and 12q24.(Erdmann et al. 2009; Gudbjartsson et al. 2009)

Table 3: Major GWAS loci for CHD identified by mid 2009

Locus	*SNP (rs)*	*Risk Allele & Freq*	*OR (95% CI)*	*Phenotype*	*N control/ case*	*Discovery Studies*	*Positive Replication*	*Negative replication*
9p21	1333049	C 0.50	1.24 (1.18-1.30)	MI/CAD	12k/11k	WTCC/ Samani	Samani (2)	
	4977574	G 0.56	1.29 (1.25-1.34)	MI	12k/12k	Kathiresan		
	10757278	G 0.50	1.28 (1.22-1.35)	MI/CAD	13k/4.5k	Helgadottir		
1p13	646776	T 0.81	1.19 (1.13-1.26)	MI	12k/12k	Kathiresan		
	599839	A 0.80	1.11 (1.05-1.18)	MI/CAD	12k/11k	WTCC/Samani	Samani (2)	
	599839	A 0.78	1.20 (1.10-1.31)	CAD/MI	12k/2k	Willer		
21q22	9982601	T 0.13	1.20 (1.14-1.27)	MI	12k/12k	Kathiresan		
10q11	1746048	C 0.84	1.17 (1.11-1.24)	MI	12k/12k	Kathiresan		
	501120	T 0.88	1.15 (1.07-1.24)	MI/CAD	12k/11k	WTCC/Samani	Samani (2)	
2q33	6725887	C 0.14	1.17 (1.11-1.23)	MI	12k/12k	Kathiresan		
19p13	1122608	G 0.75	1.15 (1.10-1.20)	MI	12k/12k	Kathiresan		
1p32	11206510	T 0.81	1.15 (1.10-1.21)	MI	12k/12k	Kathiresan		
	11206510	T 0.81	1.13 (1.03-1.23)	CAD/MI	12k/2k	Willer		
1q41*	17465637	C 0.72	1.14 (1.10-1.19)	MI	12k/12k	Kathiresan	Erdmann	
1q41*	3008621	G 0.88	1.09 (1.01-1.17)	MI/CAD	12k/11k	WTCC/Samani	Samani (2)	
3q22.3	9818870	T 0.17	1.15 (1.11-1.19)	CAD	21k/20k	Erdmann		

12q24	3184504	T 0.47	1.13 (1.08-1.18)	MI/Eos	40k/7k	Gudbjarsson	
	653178	G 0.45	1.12 (1.07-1.17)				
6p24	12526453	C 0.65	1.12 (1.08-1.17)	MI	12k/12k	Kathiresan	
12q24.31	2259816	A 0.36	1.08 (1.05-1.11)	CAD	21k/20k	Erdmann	
	Non replicated						
2q36	2943634	C 0.66	1.03 (0.98-1.09)	MI	12k/11k	Samani	Samani (2)
		C 0.66	0.95 (0.91-0.98)	MI	12k/12k		Kathiresan
6q25	6922269	A 0.26	1.08 (1.03-1.14)	MI	12k/11k	Samani	Samani (2)
	6922269	A 0.26	1.09 (1.05-1.14)	MI			Kathiresan
	3798220	G 0.04	~3	CAD	1.3k/1.8k	Luke	
15q22	17228212	C 0.27	1.02 (0.98-1.07)	MI	12k/11k	Samani	Samani (2)
	17228212	T 0.73	1.05 (1.01-1.09)	MI	12k/12k		Kathiresan

**Not in linkage disequilbrium*

(Helgadottir et al. 2007; Samani et al. 2007; 2007; Willer et al. 2008; Erdmann et al. 2009; Gudbjartsson et al. 2009; Kathiresan et al. 2009a; Samani et al. 2009)

GWAS for CHD risk factors:

GWAS has also been performed for key risk factors known to predispose to CHD. In total, approximately 40 loci are currently known for type 2-diabetes. The most robust is a variant within the TCF7L2 gene, which has subsequently been shown to be associated with islet cell function.(McCarthy 2010) Similarly, there are over 30 variants for BMI and obesity, of which FTO is the most widely replicated.(McCarthy 2010) Furthermore, there are currently over 90 variants known to associate with lipid phenotypes while approximately 20 are known for hypertension.(Teslovich et al. 2010; Wang and Snieder 2011) A GWAS has even been performed for smoking tendency and has identified a variant on chromosome 15 associated with greater smoking behaviour. Thus, cumulative genomic risk is likely to be complex when all metabolic and risk pathways for CHD are integrated, even before gene-gene interactions and epigenetic effects are considered.

Copy Number Variants

Common genetic variation in humans is composed not only of SNPS but also copy number variations (CNVs). These are deletions or duplications of chunks of DNA and appear to be quite common, affecting more than 10 per cent of the genome. Several CNVs have been associated with a variety of human conditions.

Kathiresan et al in the MIGEN consortium study tested 554 common copy number polymorphisms (>1% allele frequency) and 8,065 rare CNVs but none, in cases compared to controls, in genes compared to the genome as a whole, or at any individual locus were found to significantly associate with risk of MI.(Kathiresan et al. 2009a) Similarly, the Wellcome Trust Case-Control Consortia also examined 3000 healthy volunteers with 16000 subjects with disease (2k each of 8 conditions including coronary disease) and found no significant CNV associating with coronary artery disease.(Craddock et al. 2010) It is currently felt that CNVs are unlikely to account for a significant proportion of the heritability of complex diseases.

Summary of genetic studies to date

A general summary of key findings from genetic studies in coronary heart disease over the last 50 years can thus be proposed as follows:

1. Candidate gene studies have been uniformly disappointing in identifying genetic susceptibility for coronary artery disease.
2. Family based genome wide linkage scans have also failed to show any convincing genetic susceptibility. Perhaps the most promising findings from these studies is that of leukotriene pathway polymorphisms.
3. GWA has revealed that, (at the time of these studies), there are at least a dozen common variants associating with coronary heart disease and these have been reliably and robustly replicated. Of these, the variants at the 9p21 locus are the strongest and most widely validated genomic finding for coronary disease to date
4. The mechanisms for most variants remains unknown, with only a few potentially related to lipid metabolism.
5. Despite their high prevalence these common variants each confer very small risk on their own, with 9p21 for example prevalent in 50% of the population conferring risk of 1.25. Combinations of alleles may provide greater estimates of risk as a measure of genetic burden

Beyond genome wide discovery

On June 26th, 2000, President Clinton announced, "Today, we are learning the language that allowed God to create life." Mapping the human genome would lead to "the diagnosis, prevention and treatment of most, if not all, human diseases". Francis Collins, director of the genome agency at the National Institutes of Health, concurred, saying at a news conference that the genome project would lead to a "complete transformation in therapeutic medicine" but ten years later, have these promises been realized? More immediately, where do we go next armed with these GWAS findings? One way to address this question is to look back at what genetics and genomics promised to deliver and to see if these goals are being realized.

"Genetic findings will lead to the identification of novel disease mechanisms and provide new therapeutic targets"

Candidate gene association studies were unable to identify new mechanisms of disease simply because they were necessarily restricted to pathways that were known such as inflammation or lipid metabolism.

Genome wide linkage and association studies however offered a hypothesis free approach unrestricted to known mechanisms, thereby opening up the possibility of identifying novel mechanisms. Overall, genome wide linkage scans have delivered few prominent findings. Of these the most significant is that of polymorphisms in the leukotriene synthesis pathway. While technically not a novel mechanistic pathway, given this is an inflammatory mediator, its role in coronary artery disease is novel. Prior to this discovery, studies had begun to demonstrate the effect of leukotrienes in atherosclerosis. Genetic association with disease added weight to this finding and promoted the role of leukotrienes in CAD. Significantly, this pathway is already well established and treatments exist to block it for asthmatics. In one ongoing study, treatment of 200 MI patients with at-risk variants of the FLAP or LTA_4-H genes with the FLAP inhibitor DG-031 was found to reduce LTB_4 production and plasma MPO and CRP levels.(Hakonarson et al. 2005)

Leukotriene antagonists have not yet been shown to improve CHD risk. There is clear need to demonstrate that blockade of this pathway can improve CHD risk, especially in carriers of the leukotriene risk variants/haplotypes.

Perhaps the greatest hope for discovering novel biology was laid on GWAS. Of the GWAS variants identified for CAD, only 3 are known to influence known lipid gene mechanisms, with the remaining variants not directly involved in any known pathways. Many variants reside in intronic regions or gene deserts with nearby genes many kilobases away. In many respects the GWAS revolution was driven by technological and bioinformatics advances. The science itself is only now slowly catching up and the challenge lies in making sense of this wealth of new data. For example, of the 12 CHD loci identified so far, only a few contain genes that are known to influence traditional risk factors. As a result, functional studies exploring these variants are gathering pace and the possibility of uncovering novel disease mechanisms remains very real.

One key problem hindering our understanding of GWAS variants is the inaccuracy of the phenotype. As described in the previous sections CHD is a broad phenotype encompassing a wide range of pathophysiology from endothelial dysfunction all the way through to plaque rupture and thrombosis. Thus, it is unclear whether these variants for example influence elements of atherosclerosis or thrombosis or both. The first step is therefore to refine these phenotypes to identify where in the CHD spectrum these variants act. This can then inform more detailed functional studies.

As an example, the 1p13 locus was first identified as a CHD risk variant associating with MI and clinical CAD. Through association studies using related phenotypes it was found to associate significantly with lipid levels.(Samani et al. 2008a) Following this discovery, mechanistic studies focused on lipid metabolism. Musunuru and colleagues subsequently demonstrated that in the liver, three genes at the 1p13 locus were expressed at reduced levels in carriers of the risk allele.(Musunuru et al. 2010b) By fine-mapping they then identified that the causal variant resided within a possible binding site for the CCAAT/enhancer binding protein (C/EBP), a transcription factor. Binding of this transcription factor to the small region of DNA was disrupted in carriers of the variant. Their experiments suggested a series of events linking the risk allele of SNP rs12740374 to reduced expression levels of genes on chromosome 1p13. The gene most affected in expression was SORT1, encoding Sortillin 1 protein. Knockout of this gene in a mouse model led to greater LDL-cholesterol levels. Further work has shown that Sortillin is an intracellular sorting apparatus for particles with apolipoprotein B and in its absence particles show more apolipoprotein B and less endocytosis of LDL particles.(Kjolby et al. 2010) Thus, again while the exact mechanism

is still unclear this work shows exciting potential for novel LDL-cholesterol lowering therapies.

Critics of genomic studies comment that GWAS findings are likely to be irrelevant, given they have such small effect sizes. However, this ignores the fact that the effects on a phenotype can be much greater either if (1) they result from as yet unidentified rarer mutations at these loci that have greater impact on gene expression/function, or (2) from drug therapy targeted to the gene product or related pathway. This is best highlighted by the discovery of variants in the HMGCR gene associating with modest lipid levels in large GWAS.(Kathiresan et al. 2008; Willer et al. 2008) Had the HMG-CoA reductase pathway not been known, it is not inconceivable that functional studies following this finding could have led to identification of this pathway and development of statins, one of the most widely prescribed drug in the world. Similarly, multiple variants such as this are under intense investigation and the promise of discovering new biology and new treatments remains very much alive, even if it may not be imminent.

"Genetics and genomics will establish risk prediction and personalized medicine"

It was also hoped that the genomic discoveries could lead to personalized medicine, whereby healthcare interventions would be guided based on an individual's genomic make up. To some extent this personalization already occurs in various guises. Children are tested at birth for PKU so their diets can be modified, relatives of breast cancer patients can be tested with BRCA1 mutations and then screened and managed appropriately. With coronary disease the emphasis has been on identifying individuals at risk so that early interventions and lifestyle modifications can be initiated.

Genotyping risk variants aims to capture the genetic burden of risk over an individual's lifespan. Efforts are ongoing to use currently available genetic data as biomarkers to improve risk prediction beyond what is currently available in the hope that they may offer more precise estimates of individual risk. Genotype based risk prediction has a number of advantages over conventional biomarkers as genotypes are (1) fixed from birth, allowing early risk prediction; (2) less susceptible to biological variation over life, such as during inter-current illness, etc; and (3) are easy to obtain with minimal measurement error.

Previously a number of studies attempted to utilize risk scores from individual candidate genes in the hope of improving predictive value, but failed to demonstrate clinical utility.(Morrison et al. 2007) With the recent advances in genomic research, hopes are again raised of utilizing genetic data to improve risk prediction.

There is therefore particular interest in exploring the risk prediction value of common CHD variants both, on their own and in combination as a genetic risk score.

Summary

Despite the successes witnessed over the last few years, much remains to be learned about the genetics of CHD. As we contemplate the findings of the first wave of GWAS, the focus has shifted towards attempting to define their mechanisms of action. Furthermore, it remains to be seen whether their use as genetic biomarkers will provide added prognostic value to current risk prediction models and whether new therapies will arise from this new genomic era.

The overall aim of this thesis is to advance our understanding of recently discovered GWAS variants. We have attempted to (a) refine the CHD phenotype to guide functional inquiry, (b) explore their predictive value for determining risk of future events and (c) target drug therapy based on genomic heterogeneity in the leukotriene pathway.

Aims and objectives

The overall aim of this thesis, composed of a series of studies, is to explore findings from recent GWAS of CHD in greater depth and within the broader context of the promises of the genome era that new findings would lead to:

1) Identification of new disease mechanisms

2) Permit genotype based risk prediction and

3) Promote development of novel and targeted therapies based on genotype.

Specific Aim 1: GWAS variants and refined CHD phenotypes

In a population of ~2500 patients undergoing coronary angiography for investigation and/or treatment of CAD we sought:

Aim 1a: To refine the phenotypic associations with the 9p21 genotype, the first major GWAS finding for CHD, by testing for its association with the presence, severity and progression of angiographically documented CAD.

Aim 1b: To test association between the 8 novel risk loci, without known patho-physiological mechanism, and CHD phenotypes including presence and severity of CAD, progression of CAD, and occurrence of MI.

Specific Aim 2: GWAS variants and intermediate CHD phenotypes

In a group of 400 subjects without known CHD, we sought:

Aim 2a: To examine association between the 8 CHD risk variants, without known mechanisms of risk, with quantitative intermediate phenotypes representative of traditional risk factors

Aim 2b: To examine association between these variants with non-invasively derived indices of central pressure augmentation to determine if these variants may confer risk by influencing arterial stiffness and abnormalities in pressure wave reflection

Specific Aim 3: GWAS variants and genetic risk prediction

In a population of ~2500 patients undergoing coronary angiography for investigation and/or treatment of CAD, we sought:

Aim 3a: To examine the prognostic value of 9p21 by associating genotype with adverse outcomes over 2 years of follow-up.

Aim 3b: To examine the effect of a cumulative risk score using the 11 established variants on prevalent and incident MI risk.

Specific Aim 4: Genomic discovery and novel therapy for CHD

In a subset of 11 subjects, from the coronary angiography population, we sought:

Aim 4: To determine if treatment with a leukotriene synthesis inhibitor in patients carrying the leukotriene MI risk haplotypes would improve endothelial function as a surrogate marker of cardiovascular risk.

Chapter 2: Methods

Study design and overview of specific aims

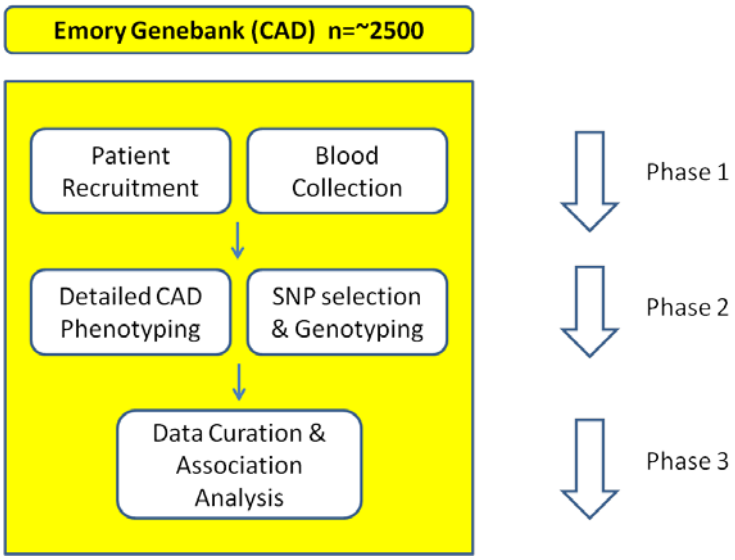
The studies described in the following sections are based predominantly on genotype-phenotype associations using genome wide discovered variants with a series of related atherosclerosis phenotypes.

Aim 1 (Chapter 3):

Aim 1a: To refine the phenotypic associations with the 9p21 genotype, the first major GWAS finding for CHD, by testing for its association with the presence, severity and progression of angiographically documented CAD.

Aim 1b: To test association between the 8 novel risk loci, without known patho-physiological mechanism, and CHD phenotypes including presence and severity of CAD, progression of CAD, and occurrence of MI.

For Aims 1a and 1b we used a cohort of subjects undergoing investigation for CAD with coronary angiography (Genebank). These subjects were extensively phenotyped using medical questionnaires, chart abstraction and by scoring their coronary angiogram data for presence and severity of CAD. Blood samples were collected at the time of enrolment and stored. Samples were catalogued and aliquoted prior to genotyping. Thereafter statistical analyses were performed to correlate genotypes with phenotypes.

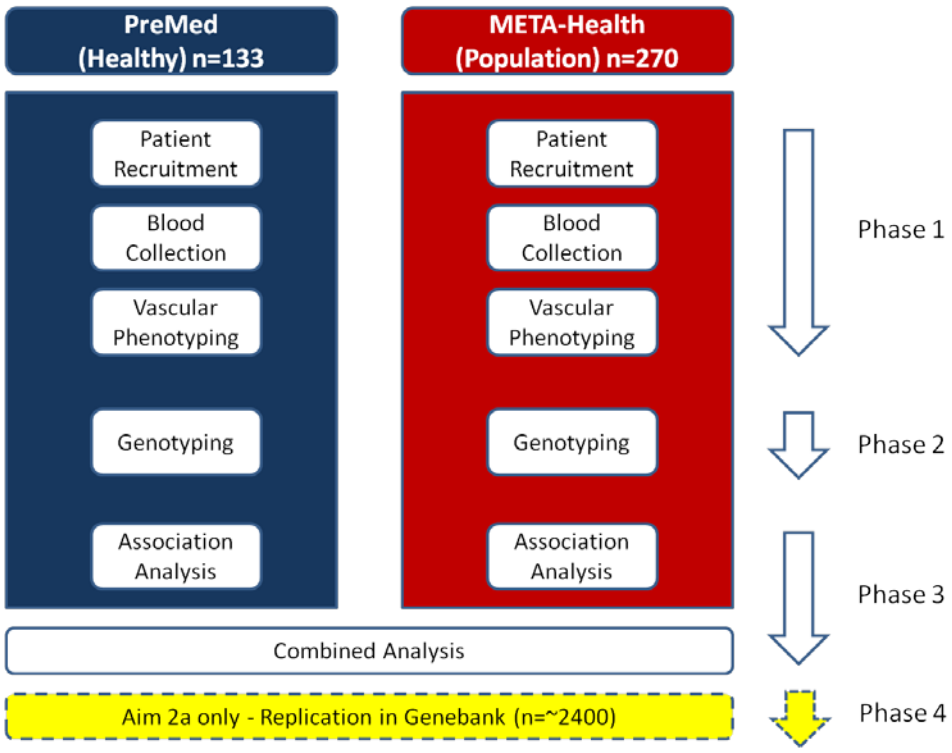


Aim 2 (Chapter 4):

Aim 2a: To examine association between the 8 CHD risk variants, without known mechanisms of risk, with quantitative intermediate phenotypes representative of traditional risk factors

Aim 2b: To examine association between these variants with non-invasively derived indices of central pressure augmentation to determine if these variants may confer risk by influencing arterial stiffness and abnormalities in pressure wave reflection

In order to investigate associations with phenotypes upstream of atherosclerosis, we chose to examine non-CAD cohorts for this aim. This included two groups (screened healthy (PreMed) and population (META-Health) subjects free of overt CAD) recruited through advertisement under separate study protocols within the same department. Vascular phenotyping was performed in both groups in the same laboratory, by the same technicians using standardized protocols. Blood samples were collected and stored with subsequent genotyping. Statistical analyses were then performed to associate genotypes with phenotypes in each group separately and then combined.

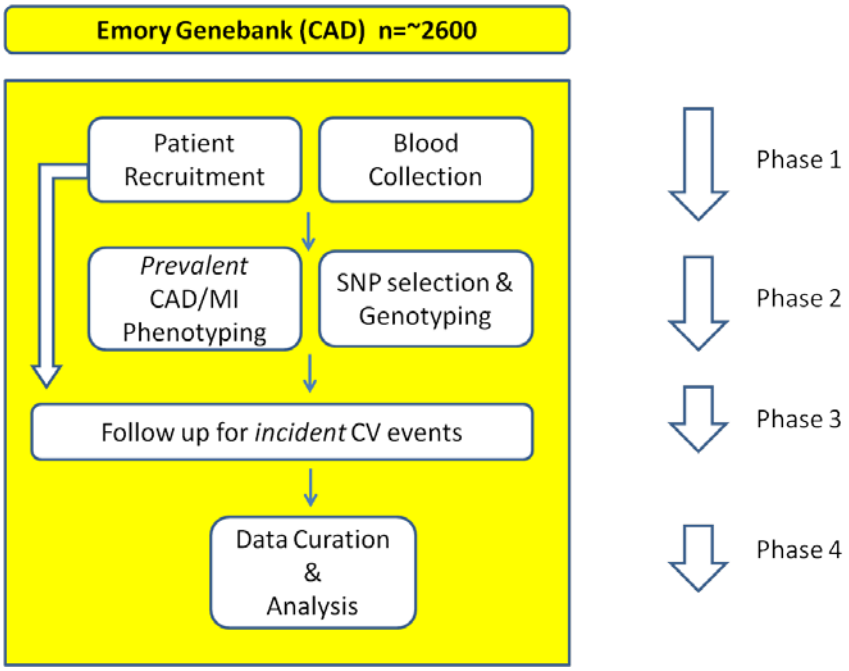


Aim 3 (Chapter 5):

Aim 3a: To examine the prognostic value of 9p21 by associating genotype with adverse outcomes over 2 years of follow-up.

Aim 3b: To examine the effect of a cumulative risk score using the 11 established variants on prevalent and incident MI risk.

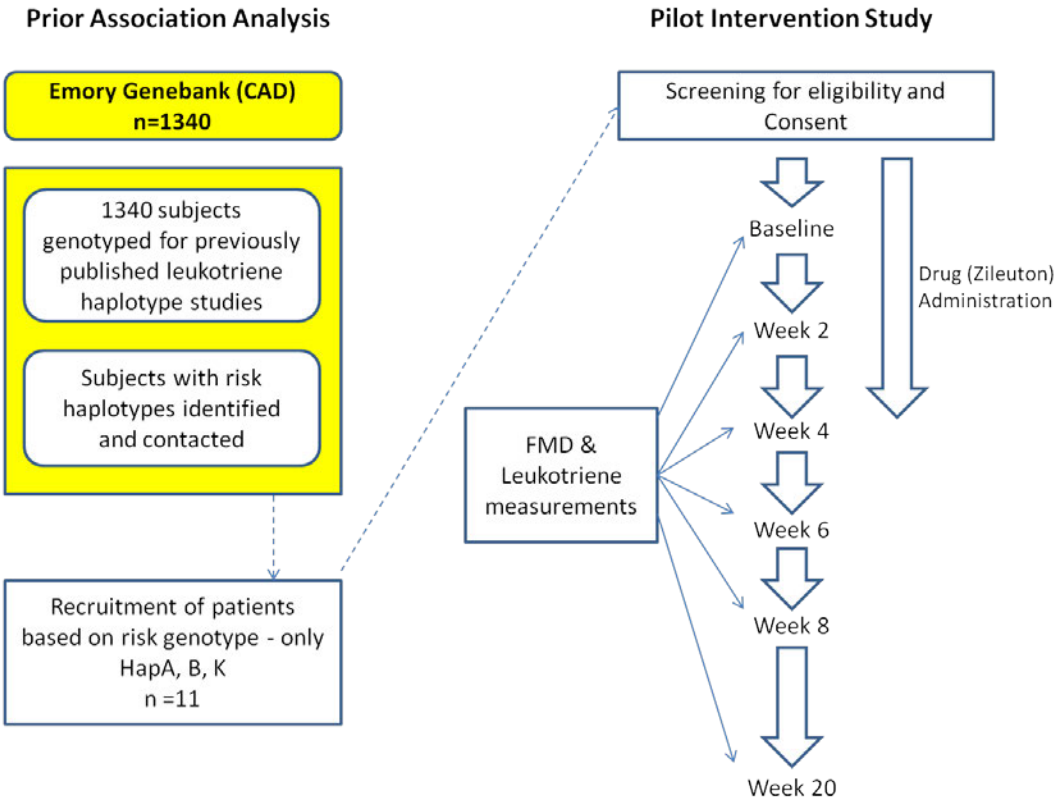
Prospective follow up of the CAD (Genebank) cohort was performed through search of state death records, telephone interview and chart abstraction by trained research staff after a minimum of 12 months. All cardiovascular events were validated by an adjudicating committee. We performed survival analysis using the single SNP for 9p21 and with a genetic risk score created from the variants genotyped for Aim 1.



Aim 4 (Chapter 6):

Aim 4: To determine if treatment with a leukotriene synthesis inhibitor in patients carrying the leukotriene MI risk haplotypes would improve endothelial function as a surrogate marker of cardiovascular risk.

Association analysis for leukotriene haplotypes and myocardial infarction has been published previously using the Emory Genebank cohort.(Helgadottir et al. 2004; Helgadottir et al. 2006) We identified patients carrying the risk haplotypes and recruited 10 subjects irrespective of CAD status for the pilot intervention study. The study drug was administered for 4 weeks with flow mediated dilatation (FMD) and leukotriene level measurements every 2 weeks for 8 weeks and a final visit at 20 weeks.



Contribution:

With the cohorts and resources available in Professor Quyyumi's department, I formulated these studies, their aims, hypotheses and analysis plans. I was responsible for organizing and collating the data, phenotyping and performing all genetic and statistical analyses. For aim 4, an association analysis had already been published by Dr Quyyumi's group but the study design for the pilot intervention was performed by me.

Study populations:

Three study cohorts were examined for the purposes of this collection of genetic studies. All of these studies, under the same PI (AQ) were separately funded with IRB approvals and had dedicated research coordinators collecting and recruiting subjects. Full unrestricted access was provided to data from all three studies.

CAD cohort ("Genebank")

The Emory Genebank is an ongoing bio-repository/database recruiting patients undergoing cardiac investigations and treatments. Although its scope is broad and encompasses all of cardiology, it is predominantly a cardiac catheterization laboratory (cath lab) based coronary artery disease database, consisting of patients undergoing coronary angiography +/- percutaneous intervention (PCI). Patients have a range of disease severity ranging from normal to extensive CAD. At Emory and its affiliated hospitals, over 7,000 cardiac catheterization procedures are performed annually. Recruitment for the biobank commenced in 2004 and had reached approximately 2000 subjects at the start of these research studies and was close to 4500 by their end, with an ultimate aim of recruiting 12000 patients.

Study participants were enrolled prior to undergoing elective or emergent cardiac catheterization. Patients, aged 20-90, undergoing assessment and consent for cardiac catheterization were considered and approached for participation in the bio-bank by either 1) research coordinators; 2) cardiology clinical fellows; 3) cardiology research fellows; 4) trained nurses. All patients were recruited sequentially without reference to gender or race. Patients were excluded if they were unable to give consent, had a recent blood transfusion (within 4 months) or had severe anaemia precluding 50cc of phlebotomy for the study or had been previously enrolled. Race was self-reported as 1) Caucasian 2) African American (AA) 3) Asian 4) South Asian/Pacific Islander 5) Hispanic 6) Other.

Consented patients were then interviewed by research coordinators to collect information on demographic characteristics, medical history and behavioural (lifestyle) habits using a detailed questionnaire (Appendix). Risk factor prevalence was determined by physician diagnosis and/or treatment for hypertension, hyperlipidaemia and diabetes. Smoking was classified as non-smoker or "ever smoked" if there was a lifetime history of smoking at least 100 cigarettes. A history of CAD, previous

revascularization, and MI along with ages at diagnosis was collected. Electronic medical records (Power-Chart, Cerner Millennium) were then reviewed to confirm self-reported medical history of all major conditions as well as to document previous angiographic findings and prior coronary revascularization details (Appendix). Medication data at the time of enrolment was also abstracted and documented. All data was subsequently entered by research coordinators into a secure SQL database, with rigorous data management and quality control strategies.

All subjects were fasting for their cardiac procedure. At the time of catheterization, blood samples were drawn from the arterial sheath prior to heparin or drug administration. Pre-labelled blood tubes were filled and samples sent immediately to the on-site processing laboratory (Atlanta Clinical Translational Institute, Emory University Hospital) where they were processed for plasma, serum, RNA and whole blood storage at -80C conditions and for other parallel studies protocol whenever relevant. (Appendix)

All sample and clinical data was de-identified and a unique study ID assigned to each patient. A key linking patient details and study ID was maintained in a secure password protected encrypted database by the PI (AQ). The bio-banking protocol was approved by the Institutional Review Board at Emory University, Atlanta, GA. All subjects provided written informed consent at the time of enrolment.

Contribution:

The Emory Genebank was established in 2004 and had been running for 3 years prior to my involvement and initiation of these studies. As the lead fellow for this biobank, I coordinated and streamlined patient recruitment and managed all aspects from ethics to sample collection, data entry and analysis. Subject recruitment, sample collection, storage and questionnaire data entry was performed by trained coordinators and cardiology staff under my supervision. I was responsible for ensuring and performing accurate phenotyping for the entire cohort for CVD traits and for organizing and managing data and samples for all analyses.

Healthy cohort ("PreMed")

The Predictive Medicine (PreMed) study was designed as part of an effort to establish normal range values for novel cardiovascular risk markers including among many others, arterial elasticity markers. A total of 300 healthy non-smoking volunteers, aged 20-70 years with approximately equal numbers of males and females were recruited through public advertising and after careful screening for absence of hypertension (SBP<130 or DBP<90 mmHg on three occasions), hyperlipidaemia (total cholesterol level <200 mg/dL, LDL <120 mg/dL), impaired fasting glucose or diabetes (fasting glucose <100 mg/dL), and obesity (BMI <=25). Other exclusion criteria included history of any CVD or valvular heart disease, pregnancy, any acute or chronic illnesses, taking prescription medications or any vitamin supplements for 6 weeks. Specific dietary intake patterns were not documented but all blood samples and measurements were taken after an overnight fast. Race was self reported. Subjects satisfying inclusion criteria were then invited back for further testing.

Participants underwent full medical history and examination with multiple tests including screening blood tests, blood pressure assessment, and measurement of BMI, waist circumference, body fat composition (as % of body weight by bioelectrical impedance or BIA). Subsequent testing after confirmation of eligibility included multiple novel markers of vascular risk, with the most prominent being pulse wave analysis, through non invasive arterial tonometry (See Methods).

All blood testing was performed after an overnight fast in a quiet room with the subject at rest. Blood was collected for screening FBC, complete metabolic profile (Electrolytes, blood urea nitrogen, creatinine, liver function tests, fasting glucose) and fasting lipid profile. Further samples were collected for biomarker analysis, with storage of plasma, serum. Whole blood was also collected and stored specifically for subsequent DNA extraction and analysis. All samples were managed and stored using standardized cross study protocols by the on-site processing laboratory (Atlanta Clinical Translational Institute).

The study was approved by the Emory University Institutional Review Committee. All data was stored in a secure encrypted database. Informed consent was obtained from all subjects.

Population cohort ("META-Health")

The Morehouse and Emory Team up to Eliminate Health Disparities (META-Health) Study was designed as a two-stage cross-sectional study of both traditional and psychosocial risk factors for CVD. The first stage was a random digit dialling telephone survey of African American and White residents of metropolitan Atlanta, ages 30 to 65 years (n=3391); the second stage included a subset of participants (n=753) who agreed to come to either Emory or Morehouse Schools of Medicine for a detailed study visit. For the purposes of this work, the latter group was of primary interest. Race was self reported.

Subjects agreeing to the study visit were screened and consented for the study. Pregnant women and those with acute illnesses were excluded. Detailed information on demographics and anthropometrics was collected at the time of the visit. All subjects answered a detailed medical history survey including medication usage. Race was defined by participant self report. A history of coronary artery disease or MI was elicited. History of diabetes and hypertension were defined by participant self report or use of anti-diabetic or anti-hypertensive medications. Smoking history, obtained using standardized questionnaires, was defined as current, or never/former (no cigarettes within the past 30 days).

All subjects then underwent blood pressure assessment, measurement of BMI, waist circumference, body fat composition (as % of body weight by bioelectrical impedance or BIA) and vascular testing including arterial tonometry assessment with PWA .

All blood testing was performed after an overnight fast in a quiet room with the subject at rest. Blood was collected for complete metabolic profile (Electrolytes, BUN, creatinine, LFTs, fasting glucose) and fasting lipid profile. Further samples were collected for biomarker analysis, with storage of plasma and serum. Biomarker assays were run in batches to minimize measurement variation. Whole blood was also collected and stored specifically for subsequent DNA extraction and analysis. All samples were managed and stored using standardized cross study protocols by the on-site processing laboratory (Atlanta Clinical Translational Institute)

The study was approved by the Emory University and Morehouse University Institutional Review Committees. All data was stored in a secure encrypted database. Informed consent was obtained from all participants.

Contribution:

The PreMed and META-Health studies are NIH funded studies led by Professor Quyyumi at Emory University. As senior fellow, my contribution was 1) overall supervision of research staff and protocols 2) sample management and genotyping 2) supervision and assistance with phenotyping for arterial compliance measures 3) data cleaning and management of data for study analysis.

Cardiovascular disease definitions

These definitions were applied consistently across all studies

- Coronary Heart Disease: For the purposes of this study all patients (Genebank and META-Health cohorts) were classified as having CHD if they had either a self reported and chart confirmed history of 1) myocardial infarction or 2) revascularization (angioplasty, PCI, CABG) or 3) angiographic confirmation of coronary disease>50%. Diagnosis of any of these at the time of enrolment or after the index coronary catheterization was also considered for this purpose of defining presence of CHD. The age at first diagnosis was also documented.
- Myocardial Infarction: Subjects were determined to have had an MI based on medical record confirmation of an event, based on a firm physician diagnosis using standard WHO criteria. This was based on a combination of 1) history; 2) ECG and 3) enzyme changes. Patients in the Genebank cohort were recorded as having MI if they had a prior MI or had been admitted and were undergoing cardiac catheterization for an index event diagnosed as MI. The age at which the first MI had occurred was documented to the nearest year. The definition of MI included any acute coronary syndrome with a troponin T rise 3x ULN.

  Cardiovascular risk factors were defined as follows

- Diabetes: Prior physician diagnosis and/or treatment; Fasting blood glucose at enrolment of >110mg/dL
- Hypertension: Prior physician diagnosis and/or treatment; At least three readings of >140mmHg systolic pressures
- Hyperlipidaemia: Prior physician diagnosis and/or treatment; total cholesterol of >200mg/dL
- Smoking: "Former" if lifetime history of at least 100 cigarettes; "Current"; "Never"
- Family History: At least one first degree relative (parents/siblings) with documented CHD <60yrs

Prospective follow up and event ascertainment

All patients enrolled in the Genebank were prospectively followed for a minimum of one year, with a further planned follow up at 5 years. Consent for future contact was obtained at enrolment. Follow up was performed by telephone calling each individual. Prior to the phone call, each patient's alive/dead status was checked against state vital records. Subsequently a detailed interview was conducted by trained coordinators using a standard proforma (Appendix), with documentation of major events outlined below. After telephone interview chart abstraction was performed to verify reported events. If hospitals outside the system were visited, patient permission was sought to obtain these records. Up to 5 attempts were made to contact patients at different times and over different days before classification as lost to follow up. These subjects were then checked against the hospital records system to determine hospital visits or events.

All events were verified using hospital medical records and state vital records. If patients had died, cause of death was documented through Georgia state records and post mortem documentation if available. Age and date of death/diagnosis were documented in all cases. An adjudicating committee (2 cardiologists) reviewed all events, with group consensus in cases of uncertainty.

- All cause death – this included death from any cause
- Cardiovascular death - was defined as sudden, immediate (<24 hrs) or late (>24 hrs) cardiac death, stroke (haemorrhagic or ischemic) or acute vascular

catastrophe. A subcategory of cardiac death included only those with a cardiac cause.

- MI – this included any myocardial infarction (defined as previously) with admission to hospital. All incident MIs were documented including first or recurrent MI. Chest pain or ACS admissions were vetted and classified as MI if they fulfilled additional enzyme criteria. All NSTEMI and STEMI were considered as MI. Enzyme positive hospital admissions were not considered an MI if the treating physician did not diagnose it as an ACS.
- Revascularization – Any elective or urgent revascularization procedure including PCI and CABG. If the procedure was urgent following and MI, the latter was documented as the first event.
- Stroke – Any ischemic or haemorrhagic stroke, confirmed by CT and physician diagnosis with symptoms lasting >24 hrs

Contribution:

I was responsible for coordinating and supervising a team of 4 individuals (Dee Anderson, Joy Hartsfield, Nancy Murrah, Ronak Patel) to contact participants and collect death and mortality data from all available sources. I helped define the outcome measures and ensured data was accurate and reliable through a variety of strategies, including organizing outcomes adjudication committees and data filtering.

Examination methods

Blood Pressure – Brachial systolic and diastolic pressure measurements were taken at rest using an oscillometric technique with semi-automated sphygmomanometers (Dinamap or Omron diagnostics) with appropriate cuff sizing. Resting pre procedure blood pressures, measured for clinical purposes, were documented for the Genebank cohort. For PreMed and META-Health subjects, after a 20 minute rest, three measures were taken each 5 minutes apart and averaged. Mean arterial pressure was calculated as 2/3 diastolic + 1/3 systolic. Pulse pressure was calculated as systolic - diastolic pressures.

BMI – Weight and height were estimated in the usual manner with calibrated height bars and electronic scales. Units other than kg or m were converted prior to entry into the database. BMI was calculated as kg/m^2.

Laboratory methods

Commonly used clinical blood tests were performed for all subjects including plasma glucose, complete blood count and basic electrolytes. A lipid panel was also performed including total cholesterol, triglycerides, HDL (high density lipoprotein) and LDL (low density lipoprotein) which was calculated using the Friedewald equation. For the Genebank cohort these tests were performed for clinical indications at the Emory laboratories using commercially available systems. For PreMed and META-Health these tests were run as part of the research protocol through Quest Diagnostics.

Biomarker analysis was only actively performed for the PreMed and META-Health cohorts. Batch analysis was performed to minimize inter assay variability and samples performed in duplicate. High-sensitivity CRP was measured using a double-antibody sandwich ELISA with rabbit anti-human CRP and peroxidase-conjugated rabbit anti-human CRP (Roche Diagnostics Corporation Indianapolis, IN, U.S.A., Catalog No 1972855). The assay is a particle enhanced immunoturbidimetric assay. Anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. A Hitachi 917 analyzer was used. The assay was linear up to 5 mg/l and logarithmic thereafter. Serum leptin was measured using an enzyme-linked immunosorbent assay (Linco Research, St Charles, MO; and Alpco Diagnostics, Salem, NH) with intra-assay variations of 1.09–4.98% and inter-assay variations of 3.9–5.3%.

Measurement units for laboratory tests are presented according to US standards (conventional). Reference ranges for common tests and their conversion factors for SI units are given under the section “Normal Values and Units” on page 17.

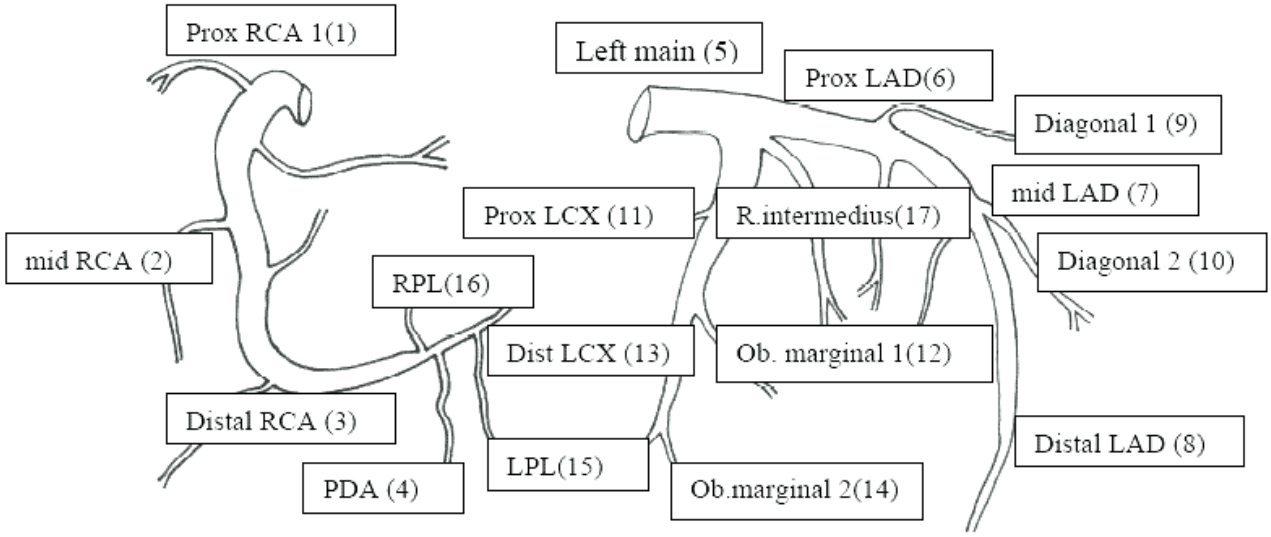
Clinical phenotyping

Coronary angiography definitions and scoring

Coronary angiography/angioplasty was performed in the cardiac catheterization laboratories at Emory Hospital sites. All patients had been referred by their cardiologist based on symptoms or other accepted clinical indications. All angiography was performed by the femoral route as per department experience. Standard diagnostic catheters and guides were used depending on individual anatomy. Research staff did not influence any aspect of the procedure or peri-procedural care of the patient.

On completion of the procedure, two operators including at least one senior cardiologist, evaluated all coronary angiograms by visual estimation of luminal narrowing in multiple segments based on a modified form of the AHA/ACC classification of the coronary tree (Figure 8).(Austen et al. 1975) This data was entered into a custom built database and exported onto a spreadsheet for further analysis.

Figure 8: Coronary tree segmentation for reporting angiograms



Modified AHA classification of the coronary arterial tree assuming a left dominant system, with a second version (not shown) for right dominant systems

Using this data, coronary angiography phenotypes were estimated by myself and a colleague (Dr Ian Neeland) including, any CAD >50%, number of epicardial vessels with >50% disease, left main and proximal vessel disease. Finally using the extracted data from the database, formulae were applied to estimate semi-quantitative angiographic scores including the Gensini and Sullivan Extent systems.(Gensini 1975; Sullivan et al. 1990) All coronary angiography evaluations were performed prior to genotyping and without knowledge of genotype status if re-reviewed.

The two primary angiographic scores were selected for use based on an extensive literature review and in-house validation through inter-score correlations and comparison with IVUS plaque burden in 50 patients.(Neeland et al. 2010) This work revealed that the ten most widely used/cited scores were highly correlated with each other and most had good correlation with IVUS plaque burden in a small cohort of subjects with both IVUS and angiographic data. The Gensini score appeared to be the most robust with inter-score correlation of approximately 0.88-0.94 (spearman rank correlation with other angiographic scores) and importantly a good correlation with IVUS plaque burden ($r=0.76$, $p<0.001$).(Neeland et al. 2010) This score was thus selected as an angiographic assessment of CAD burden. We also chose to use a second score which although had slightly weaker correlation with IVUS plaque burden (0.69), represented a different metric of coronary artery disease. The Sullivan Extent score attempts to indicate the percentage area of the coronary circulation affected by plaque, irrespective of lesion severity or luminal compromise, thereby indicating the extent of coronary atherosclerotic involvement.

The Gensini score quantifies severity of CAD by a non linear points system for degree of luminal narrowing along with a multiplier for specific coronary tree locations, thereby weighting each lesion score for prognostic significance. The total of the lesion scores is summed to give a final Gensini score. Thus, multiple severe proximal lesions gain the highest score (Figure 9).(Gensini 1975)(Appendix)

Figure 9: Gensini scoring algorithm

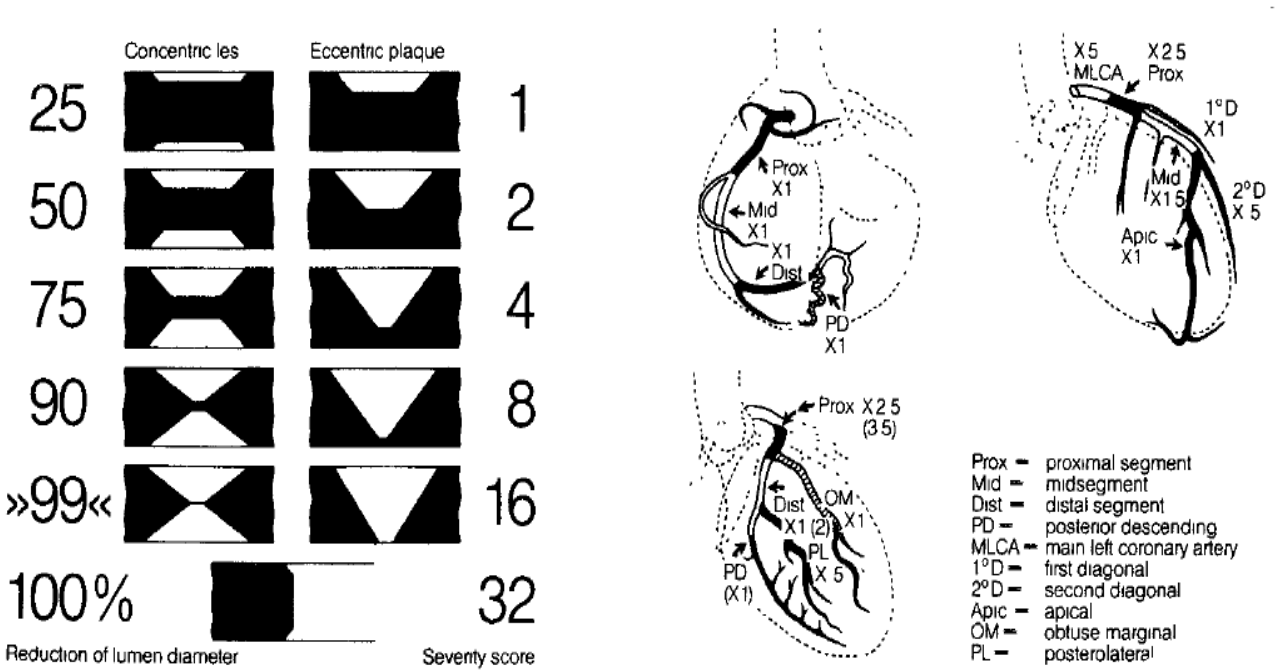


FIGURE 1. Left panel, roentgenographic appearance of concentric lesions and eccentric plaques resulting in, respectively, 25, 50, 75, 90, and 99% obstruction as well as complete occlusion (100%). The right column in this panel indicates the relative severity of these lesions using a score of 1 for 25% obstruction and doubling that number as the severity of the obstructions progresses according to the indicated reduction of lumen diameter (left column). **Right panel,** the principal vascular segments of (from left to right) the right coronary artery, the left anterior descending, and the circumflex. Each segment is followed by a multiplying factor such as X1, X2.5, and so on, depending on the functional significance of the area supplied by that segment.

Illustration by Gensini, demonstrating his method for scoring coronary angiography to estimate the Gensini score.(Gensini 1975)

The Sullivan Extent score quantifies the percentage of the coronary intimal surface area affected by atheroma, without specific weighting for the degree of luminal narrowing. The percentage involvement of each vessel is estimated and multiplied by a factor representative of the surface area of that vessel in relation to the entire coronary tree. We used a modified version based on segments of each vessel with reported disease to derive percentage involvement. RCA – four segments, each contributing 25%; LAD - three segments, each contributing 33% with the proximal segment further subdivided into two; LCx – divided into three segments each contributing 33%.(Sullivan et al. 1990)

To determine the intra-class correlation coefficient, 25 patient angiograms were randomly chosen and examined independently by myself and a colleague (Dr Ian Neeland). Lesions were visually estimated and recorded by coronary artery tree segments and then used to calculate Gensini and Sullivan Extent scores. The intra-class correlation coefficients were estimated at 0.88 (0.74-0.95) and 0.90 (0.77-0.96) for Gensini and Sullivan Extent scores respectively, which indicates good inter-observer agreement.

A subset of 308 patients who had undergone two or more coronary angiograms at least 6 months apart, were identified and the two angiograms furthest apart in time were quantified using the Gensini and Sullivan Extent scores described above. Given the variation in times between angiographies, the net change in angiographic score was divided by number of years between angiographies to give Gensini and Sulivan Extent "rates" as proxies for progression. Subjects were also arbitrarily categorized as "progressors" and "non-progressors" based on a Gensini rate of change of >1 or ≤ 0.5 points/year respectively (as a guide, one point is equivalent to a 25% lesion in the RCA). Similarly for the Sullivan Extent score, progression and non progression was defined simply as >1% and ≤0.5% change/year respectively.

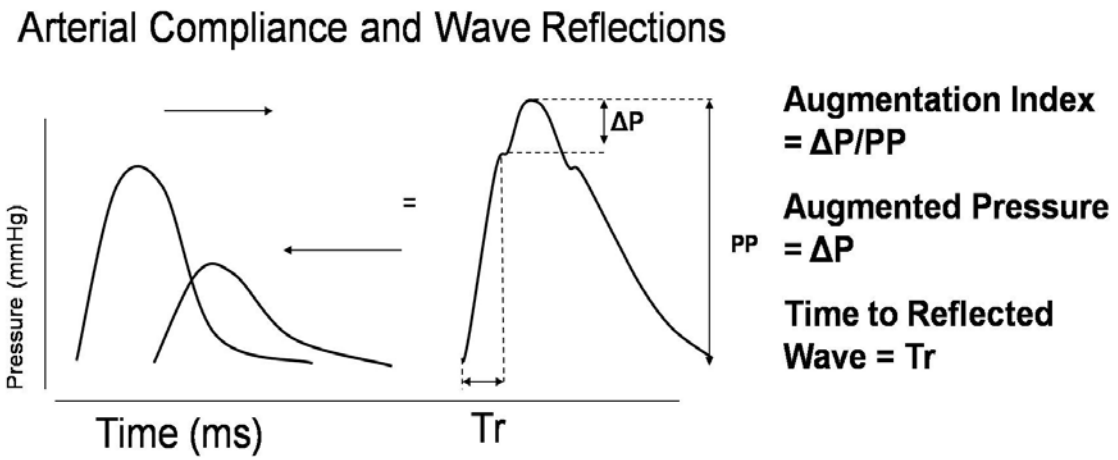
Contribution:

My primary contribution to the Genebank was to phenotype it for CAD using coronary angiography data. I coordinated retrieval of data from hospital record systems and also reviewed stored angiograms to score them individually. I reviewed all normal angiograms to ensure they were appropriately reported. I was assisted by a colleague (Dr Ian Neeland) in validating, formatting and storing this acquired data into an excel file which had advanced formulae to calculate the angiographic scores.

Pulse wave analysis

Pulse wave analysis was performed using the SphygmoCor® Pulse Wave Analysis system (PWA Medical, NSW, Australia). In brief, peripheral pressure waveforms were recorded from the radial artery at the wrist, using applanation tonometry with a high-fidelity micro-manometer. After 20 sequential waveforms were acquired, a validated generalized transfer function generated the corresponding central aortic pressure waveform. Augmentation index (AIx) and augmented pressure (AP) were derived from this with the technique of pulse wave analysis. This involves estimating the merging point of the incident and the reflected wave (the inflection point) on the generated aortic pressure waveform. AP is the maximum systolic pressure minus pressure at the inflection point. The AIx is defined as the AP divided by pulse pressure and expressed as a percentage. Larger values of AIx indicate increased wave reflection from the periphery or earlier return of the reflected wave as a result of increased pulse wave velocity (attributable to increased arterial stiffness) and vice versa. In addition, because AIx is influenced by heart rate, an index normalized for heart rate of 75 bpm (AIx@75) was also generated and used in accordance with Wilkinson et al. Time to return of the reflected wave (Tr) is the time from the beginning of the derived aortic systolic pressure waveform to the inflection point and can be used as a substitute for pulse wave velocity (a higher pulse wave velocity will lead to a shorter Tr) (Figure 10).

Figure 10: Concept of arterial pressure wave reflection



A schematic representation of pressure wave reflection and pressure augmentation in the proximal aorta which occurs secondary to the merging of the outgoing and reflected waves

All pulse wave analyses were taken in the supine position in a quiet room after a brief period (at least 5 minutes) of rest in the study room. Brachial blood pressure measurements, required to calibrate the system, were performed as described above with a validated, semi-automated blood pressure monitor, with the radial artery kept at heart level during measurement. Quality control indices were evaluated at the time of study and non acceptable readings discarded and repeated. Reproducibility studies in our laboratory on 9 subjects on consecutive days have demonstrated a coefficient of variation of 3.8%, and 20.3% for PWV and AIx respectively.

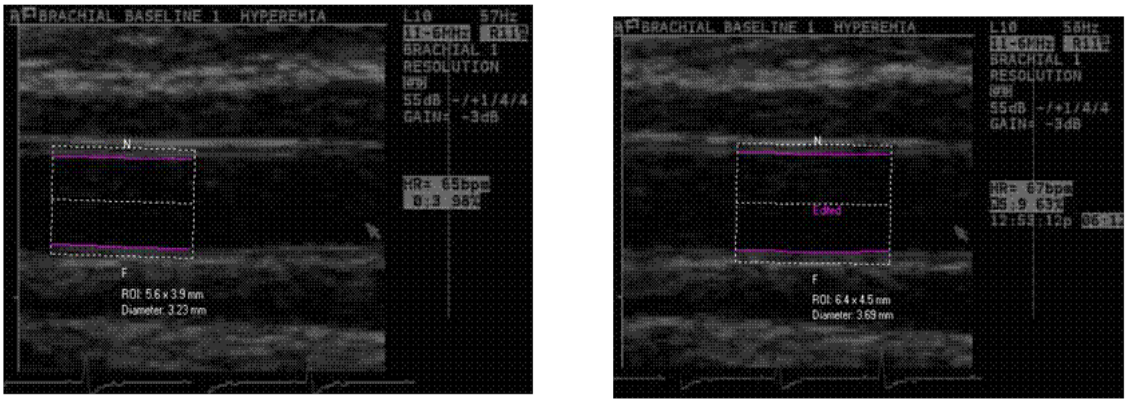
Brachial artery flow mediated dilatation

Brachial artery flow mediated dilatation (FMD) in response to shear stress was estimated as a measure endothelial function. This was performed by acquiring two-dimensional ultrasound images of the brachial artery according to established and validated methodologies. Images were obtained with an Acuson 10 mHz linear array transducer and an Acuson Aspen ultrasound system (Figure 11).(Bonetti et al. 2003) We performed imaging with the subject resting supine for at least 10 minutes on a hospital bed in a quiet setting. For each subject, optimal brachial artery images were obtained between 2 and 10 cm above the ante-cubital crease. This location was marked, and all subsequent images were obtained at the same location. After baseline measurements a blood pressure cuff was inflated to 200 mm Hg over the proximal portion of the right arm for 5 minutes (below-elbow technique). Endothelial dependent function was determined during the first two minutes of release of the cuff as previous studies have shown that maximal dilatation occurs 1 minute after cuff deflation. The flow dependent response was then be allowed to return to baseline over a period of five minutes. Endothelial independent responsiveness was evaluated with 0.4 mg of GTN administered sublingually. Brachial artery images were then obtained three minutes after GTN administration. Four triggered events (defined as the end of the T wave on the ECG) for each intervention were recorded and downloaded to an analysis system that allows offline automatic edge detection of the M-line that defines the intima-media interface for both the near wall and the far wall of the artery using a customized software (Figure 11) (Medical Imaging Applications Inc, Iowa).

Analyses were performed by individuals blinded to clinical and laboratory status of the subjects. A linear portion of the vessel was chosen for analysis. Measurements from the twelve frames were averaged for every intervention. The end point of measurement was the percent change in diameter in response to reactive hyperaemia or

to nitroglycerin. The risks involved with this procedure involved transient headache or hypotension with the administration of nitroglycerin. These subjects were supervised by a staff physician at all times during the study.

Figure 11: Tracking software to estimate FMD



Example of tracking software application to determine FMD from the brachial artery ultrasound images; for serial measures, anatomical landmarks were used to ensure the same point was measured at each visit.

Reproducibility of FMD measurements in the Quyyumi laboratory: The mean difference in FMD (%) between 2 consecutive assessments performed in 11 subjects an average of 8 days apart was 1.26(±0.76)%, with r=0.75. The mean difference in the FMD (%) between 2 readings of the same 11 measurements was 0.82 (±0.48)%, with r=0.97.

Contribution:

I trained in both of these techniques (PWA/FMD) and am familiar with their limitations and methodologies. I supervised research staff collecting this data to ensure quality control and also coordinated collection of reproducibility data in the Quyyumi lab. The majority of measurements were acquired by trained research staff (Yusuf Ahmed, Ibhar Al-Mheid, Salman Sher and Irena Uphoff)

DNA extraction and genotyping

DNA extraction and genotyping was performed both at the Emory biomarkers centre and at decode Genetics, Iceland. The Genebank samples were predominantly processed at decode while the PreMed and META-Health samples were processed at Emory.

DNA extraction

DeCode: DNA was extracted from 5-10ml of whole blood collected in EDTA tubes and stored at -80C. After thawing, samples were processed using an in-house semi-automated high throughput protocol based on DNA purification technology from Qiagen and/or the Chemagic DNA Blood Kit special from Chemagen (Germany). Typical yield for all samples was 50-300ug of genomic DNA. DNA quality reports were provided including an A260/280 ratio and DNA concentration measured by OD.

Emory: Aliquots of 200ul of whole blood were thawed and processed using the Qiagen Biorobot M48. Samples were tracked via their barcode and placed into the Biorobot, where all liquid handling, incubation, washing and heating takes place using a Silica-coated magnetic bead technology. With this, DNA adheres to the silica coating in the presence of a chaotropic salt. The magnetic beads with the immobilized DNA are collected by applying a magnetic force and the solution with the unbound components are removed and discarded. The magnetic beads are re-suspended in washing solution for purification and ultimately released from the beads into elution buffer. The Biorobot places the extracted DNA into a 2.0ml vial, labelled with the appropriate barcode, at the conclusion of the procedure.

Genotyping

DeCode: Single SNP genotyping for all samples was carried out at deCODE genetics in Reykjavik, Iceland applying the same platform to all populations studied. SNP genotyping was carried out by the Centaurus (Nanogen) platform. The quality of each Centaurus SNP assay was evaluated by genotyping each assay in the CEU and/or YRI HapMap samples and comparing the results with the HapMap data. Assays with >1.5% mismatch rate were not used and a linkage disequilibrium (LD) test was used for markers known to be in LD. Key markers we re-genotyped on more than 10% of

samples and a mismatch was observed in less than 0.5% of samples.(Kutyavin et al. 2006)

Emory: Genotyping for all SNPs was performed using either TaqMan technology or the SNPstream multiplex (Beckman Coulter) platform at Emory University, Atlanta, GA. (Bell et al. 2002; Kutyavin et al. 2006)

For Single SNP genotyping we used TaqMan technology, detailed descriptions of which are available elsewhere.(McGuigan and Ralston 2002) For each subject sample, 20 ng of DNA and the same volume of positive and no template (water) controls were dried down in 384-well plates, with 6 positive and 3 NTC controls per plate. PCR amplification was performed with primers designed by Applied Biosystems to amplify the region that contained the SNP of interest, using GeneAmp PCR System 9700 thermocyclers from Applied Biosystems. Subsequent fluorescence readings were performed using the 7900HT Fast Real-Time PCR System also from Applied Biosystems.

Multiple SNP genotyping was performed using a 48SNP multiplex. The Beckman Coulter SNPstream (Beckman Coulter, Inc., Fullerton, CA) can process up to 48 SNPs per well of a 384-well plate and features single-based primer extension technology operating at over 99% accuracy. For primer design, sequence information (SNP rs#, or flanking sequences for those SNPs without rs#) was entered into www.autoprimer.com. Three primers, two for PCR and one for single-base extension were designed for each SNP, and grouped into 48-plexed panels. Using 2-10ng of genomic DNA, a 48-plex PCR was carried out in 384-well format to amplify a ~100 bp region flanking each SNP. The PCR reaction was treated with ExoSAP reagent to remove any left-over PCR primers and dNTPs. The 48-plex extension primer pool was then added to the same PCR plate. A single-base extension reaction was performed to incorporate a differentially labelled fluorescent dNTP to the SNP position. The extension reaction was transferred to a Tag Array and spatially resolved to distinguish the xx SNPs. All reagents are pre-formulated and included in the GenomeLab SNPware Reagent kit. The GenomeLab SNPstream Genotyping System Software Suite v2.3 (Beckman Coulter, Inc., Fullerton, CA) was used for array imaging and genotype calling. The genotype data was be exported into an Excel spreadsheet for further analysis. To ensure genotyping accuracy and reproducibility two internal quality control samples were included each run in triplicate, on each of the 384-well arrays.

Contribution:

I was responsible for sample management and shipping of samples to genetics laboratories at Emory and in Iceland. I personally aliquoted all samples for genotyping at Emory to ensure appropriate volumes of DNA were plated. Although I was not responsible for actual wet lab genotyping (Mark Bouzyk, Weining Tang, Freedom David) I worked closely with the technicians at each site to identify and select SNPs for genotyping and for troubleshooting. I reviewed all genotype data for consistency before admission into the database.

General statistics

Data management:

All data for the Genebank, PreMed and META-Health studies was managed centrally by a single data manager, using advanced SQL database systems. Quality control was performed at data entry level and with regular checks of the database to identify data errors (duplications, DOB errors, identifier mismatches etc). Data from the SQL database was extracted for analysis using Microsoft Excel and SPSS compatible spreadsheets.

Software:

PLINK statistical software was used for the majority of genetic analysis. This included all basic association testing (quantitative and qualitative), Hardy Weinberg equilibrium assessment; frequency and missingness analysis and genotype score estimation. The software is open access and was downloaded onto a windows PC and run in an ms-dos environment. Required files were constructed according to PLINK format as .ped and .map files. (http://pngu.mgh.harvard.edu/purcell/plink/)(Purcell et al. 2007) Example analysis codes are available in the Appendix. SPSS v17 (Illinois, USA) was used for follow up linear and logistic regression as well as all non-genetic statistical analyses. Charts were constructed using original data with SPSS v17 (Illinois, USA) or MS Excel.

General statistics:

Unless otherwise stated, continuous variables are presented as means (SD) and categorical variables as proportions (%) with one way ANOVA and chi-squared tests

used to determine differences by category or genotype. Variables were tested for normality with Kolmogorov-Smirnov statistics and (+1 log10) transformed for purposes of parametric analyses. Reverse log transformation was applied to obtain clinically interpretable values. P values were universally deemed statistically significant at $p<0.05$.

Specific statistics:

Risk alleles for tested variants were identified from published GWAS data. Minor allele frequencies were estimated in the population under study and compared to published data. Hardy Weinberg statistics were calculated for each SNP.

Association analyses were performed using the [–assoc] feature of PLINK. This is analogous to a Cochrane-Mantzel test of association. Additive models were assumed in all analyses. Where phenotypic traits were continuous a linear regression model was constructed using the [–qassoc] feature. Beta coefficients were estimated and reported along with p values. Logistic and linear regression models were constructed to test the additive effect of the SNPs on phenotypes with adjustment for specific covariates. Odds ratios (OR) are presented where appropriate along with 95% confidence intervals in parentheses.

Outcome analyses were performed using the Kaplan Meier function in SPSS with estimation of log-rank p values and construction of survival charts. Data was categorized based on variable features. Cox regression techniques were used to model variables and adjust for covariates.

Differences between visits of measured variables, or within subjects differences was tested using paired t tests and with repeated measures general linear models.

Contribution:

For these studies, I personally performed all genetic and general statistics. All analyses were checked by both a general (Dr Emir Veledar) and genetic statistician (Dr Shaoyong Su) at Emory.

Chapter 3: Results, GWAS Variants and Refined CHD Phenotypes

The results for specific aims 1a and 1b are presented in this chapter. These studies are focused on refining the CHD phenotype to determine if the true association is with atherosclerosis or plaque rupture and myocardial infarction. Study 1 describes the association between the first GWAS discovered risk marker at 9p21 with the refined CHD phenotype of angiographic CAD using semi quantitative CAD severity scores. Study 2 follows with association between all 8 CHD risk loci, identified subsequently up until 2009, without known mechanisms of risk with both angiographic CAD and MI phenotypes.

Contribution:

I conceived the ideas for both of these studies and performed all relevant analyses. My contribution to the methodology is described in chapter 2, but relevant to these studies included phenotyping for CAD, primarily for the angiographic coronary data and calculation of angiographic scores. I personally reviewed angiograms as well as medical data on all subjects to ensure validity and accuracy of phenotyping. In addition I managed and coordinated samples for DNA extraction and genotyping. I performed all statistical and genetic analyses, wrote the now published manuscript and created the relevant figures.

Study 1: The chromosome 9p21 risk locus is associated with angiographic severity and progression of coronary artery disease

Summary:

The 9p21 locus was the first variant to be identified through genome wide association, using a mixture of MI and CAD binary phenotypes. Its mechanism was and remains unknown but it was suspected to promote atherosclerotic burden rather than a thrombotic phenotype. In this study we sought to examine this hypothesis by exploring association with detailed semi-quantitative coronary angiographic phenotypes. We demonstrate a near linear relationship between 9p21 risk allele carriage and CAD severity as well as CAD progression, thereby refining the phenotype for this risk locus and developing the evidence base for its association with atherosclerosis as the underlying mechanism of risk.

Introduction:

Unbiased genome wide approaches have led to the identification of the 9p21.3 locus as a risk marker for myocardial infarction (MI) and prevalent CAD in predominantly Caucasian cohorts.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; WTCCC 2007) This association has since been replicated in several studies and in non Caucasian populations, and confirmed by meta-analyses, making this one of the most robust genetic findings for coronary heart disease to date.(Schunkert et al. 2008; Palomaki et al. 2010)

Its mechanism of risk however remains unknown. A large prospective study demonstrated that 9p21 status is predictive of first revascularization in subjects with medically treated MI suggesting a tendency to atherosclerosis.(Lina et al. 2008) In addition, recent functional studies have demonstrated enhanced expression of the non coding RNA, ANRIL, in 9p21 carriers(Jarinova et al. 2009) and this transcript has in turn been associated with greater atherosclerosis.(Holdt et al. 2010) Despite these and other studies suggesting greater atherosclerotic activity as a potential mechanism, a positive and direct association between 9p21 carrier status and CAD severity, extent or progression has yet to be convincingly demonstrated in humans.(Visel et al. 2010) This

may in part be due to inadequacy of phenotyping methods employed thus far to assess CAD severity, particularly as the effect size of common variants is often small.

Despite its many limitations, coronary angiography remains the gold standard for documenting extent and severity of CAD. We sought to test the hypothesis that the 9p21 locus promotes atherosclerosis by examining its association with angiographically defined CAD severity and extent as well as CAD progression by refining the phenotype using two validated semi-quantitative coronary scoring systems.

Methods:

Study Population

Study participants were recruited as part of the Emory Cardiology Genebank, consisting of consecutive patients enrolled prior to undergoing elective or emergent cardiac catheterization across three Emory Healthcare sites. Medical records were reviewed to confirm self reported history of MI and other conditions as well as to document previous angiographic findings and prior coronary revascularization. Full details and definitions for this population are provided in chapter 2. After excluding self reported non-Caucasian ancestry, heart transplantation, missing or incomplete angiographic data and missing DNA/blood samples, 2334 subjects were deemed eligible for this analysis. The study was approved by the Institutional Review Board at Emory University, Atlanta, GA and all subjects provided written informed consent.

Coronary Angiography Definitions and Scoring

Two operators, evaluated all coronary angiograms by visual estimation of luminal narrowing in multiple segments based on a modified form of the AHA/ACC classification of the coronary tree.(Austen et al. 1975) Using this data, coronary angiography phenotypes were estimated by myself and a colleague (Dr Ian Neeland) including, any CAD >50%, number of epicardial vessels with >50% disease, left main and proximal vessel disease and finally, quantitative angiographic scores using the Gensini and Sullivan Extent systems.(Gensini 1975; Sullivan et al. 1990) All coronary angiography evaluations were performed without knowledge of genotype status. Full

details and descriptions of angiographic scoring and methods used are available in chapter 2.

In addition a subset of 308 patients who had undergone two or more coronary angiographies at least 6 months apart, were identified and the two angiograms furthest apart in time were quantified using the Gensini and Sullivan Extent scores. The net change in angiographic score was divided by number of years between angiographies to give Gensini and Sulivan Extent "rates" as proxies for progression. Full details are again available in chapter 2.

Genotyping

Genotyping for all samples was carried out at deCODE genetics in Reykjavik, Iceland, as part of ongoing collaborative studies, with rs10757278 chosen as the representative SNP for the 9p21 region based on our group's prior work.(Helgadottir et al. 2007) All single SNP (rs10757278) genotyping was carried out using the Centaurus (Nanogen) platform as described in chapter 2.(Kutyavin et al. 2006)

Statistical Analysis

Logistic and linear regression models were constructed to test the additive effect of the SNPs on CAD phenotypes including severity and extent, with the SNP coded as 0, 1 or 2 based on number of risk (G) alleles. Analyses were repeated after adjusting for age, gender, BMI, diabetes, hypertension, hyperlipidaemia, smoking, statin use and history of MI. Analyses were also repeated after excluding subjects with normal coronary arteries (smooth or <10% luminal irregularities) to ensure any observed effect on graded severity was not being driven by those without any CAD in whom risk allele frequency is expected to be significantly lower. Interaction terms were tested for association between the 9p21 SNP and significant determinants of CAD severity, followed by stratified analysis to evaluate significant interactions.

CAD progression was tested as both a continuous variable (change in angiographic score/year) and as a categorical variable (progression vs. non progression) with regression coefficients and ORs calculated accordingly. Analyses

were adjusted for age, gender, diabetes, statin use, smoking and baseline angiographic score at first cath. A 2-tailed *P* value <0.05 was considered significant. All statistical analyses were performed using SPSS 17.0 (Chicago, IL).

Results:

A total of 2334 self-reported Caucasians were genotyped for the rs10757278 SNP and included in this study. The observed genotypic frequencies were consistent with Hardy Weinberg equilibrium (p=0.11) with a risk allele frequency of 0.50 (G allele). Patient characteristics at baseline by rs10757278 genotype are shown in Table 4. The mean age (SD) was 63.9 years (11.1), with a range of 24 to 90 years. No significant differences in patient characteristics were observed between rs10757278 genotypes for traditional risk factors, laboratory parameters or medication usage.

As described previously, we noted a significant association in prevalence of prior MI, with increasing copies of the risk allele (p=0.04, Table 4), equating to an allelic OR of 1.18 (95%CI 1.04-1.34).(Helgadottir et al. 2007) Similarly, there were significant associations with prior PCI (OR 1.17 (1.04-1.32)), CABG (OR 1.17(1.02-1.34)) and angiographically significant CAD defined as at least one vessel with 50% disease compared to normal coronary artery patients (OR 1.25 (1.08-1.45)). When further classified as normal, single and multi-vessel disease, there was a significant association with greater risk allele frequency with increasing CAD severity (p=0.003). Angiographic traits considered to be especially heritable,(Fischer et al. 2005) were also more common in carriers of the risk allele: Left Main (OR=1.36 (1.10-1.68)) and Proximal Disease (OR 1.32 (1.13-1.54)).

Table 4: Patient characteristics by 9p21 genotype for Genebank (aim 1a)

	rs10757278 genotype			
n = 2334	*AA (557)*	*AG (1206)*	*GG (571)*	p
Age, yrs	64.4 (11)	63.6 (11.1)	64.2 (11.3)	0.26
Male, %	67.9	67.4	66.0	0.78
Body Mass Index	29.1 (6.1)	29.5 (6.2)	29.1 (5.6)	0.24
Diabetes, %	26.8	30.0	27.4	0.30
Glucose, mg/dL	120.6 (41.9)	122.6 (45.9)	119.3 (38.5)	0.34
Hypertension, %	65.2	68.6	67.9	0.36
Systolic BP, mmHg	142 (23.5)	138 (22.6)	141.4 (22.7)	0.13
Hyperlipidaemia, %	67.0	70.5	71.0	0.25
Total Cholesterol, mg/dL	168.5 (38.9)	169.4 (43.4)	166.1 (45.1)	0.40
LDL, mg/dL	96.6 (33.9)	96.8 (35.0)	92.7 (36.1)	0.12
HDL, mg/dL	41.1 (12.8)	40.9 (12.4)	40.7 (12.5)	0.89
Ever Smoked, %	58.7	60.5	61.7	0.59
Statin Use, %	72.5	76.5	77.9	0.19
Beta-Blocker, %	61.5	64.1	66.3	0.28
Serum Creatinine, mg/dL	1.10 (0.56)	1.08 (0.55)	1.06 (0.53)	0.42
Prior Myocardial Infarction, %	29.4	33.5	36.7	0.04
Acute Coronary Syndrome, %	12.3	9.6	10.8	0.24
Prior CABG , %	20.1	23.2	25.6	0.09
Prior PCI, %	38.2	43.2	45.8	0.03
Ejection Fraction, %	54.3 (10.8)	53.7 (11.2)	53.4 (11.9)	0.48
Angiographic CAD (>50%), %	72.4	79.0	80.1	0.004
Coronary Disease Burden				0.003
Normal, %	27.6	21.0	19.9	
Single Vessel, %	22.9	21.5	19.5	
Multi-Vessel, %	49.5	57.5	60.5	
Left Main, %	5.6	8.5	10.2	0.017
Proximal Disease, %	60.8	72.1	72.7	<0.001
Gensini Score, Median (IQR)	10 (0 - 49)	17 (3 - 64)	19 (3 - 76)	0.001
Sullivan Extent, Median (IQR)	15 (3 - 32)	19.9 (5 - 36)	21.5 (7 - 37)	<0.001

Mean (SD) or % unless indicated. CAD – Coronary Artery Disease; CABG – Coronary Artery Bypass Grafting; PCI – Percutaneous Coronary Intervention; IQR – Inter-Quartile Range

9p21 association with semi quantitative CAD severity and extent scores

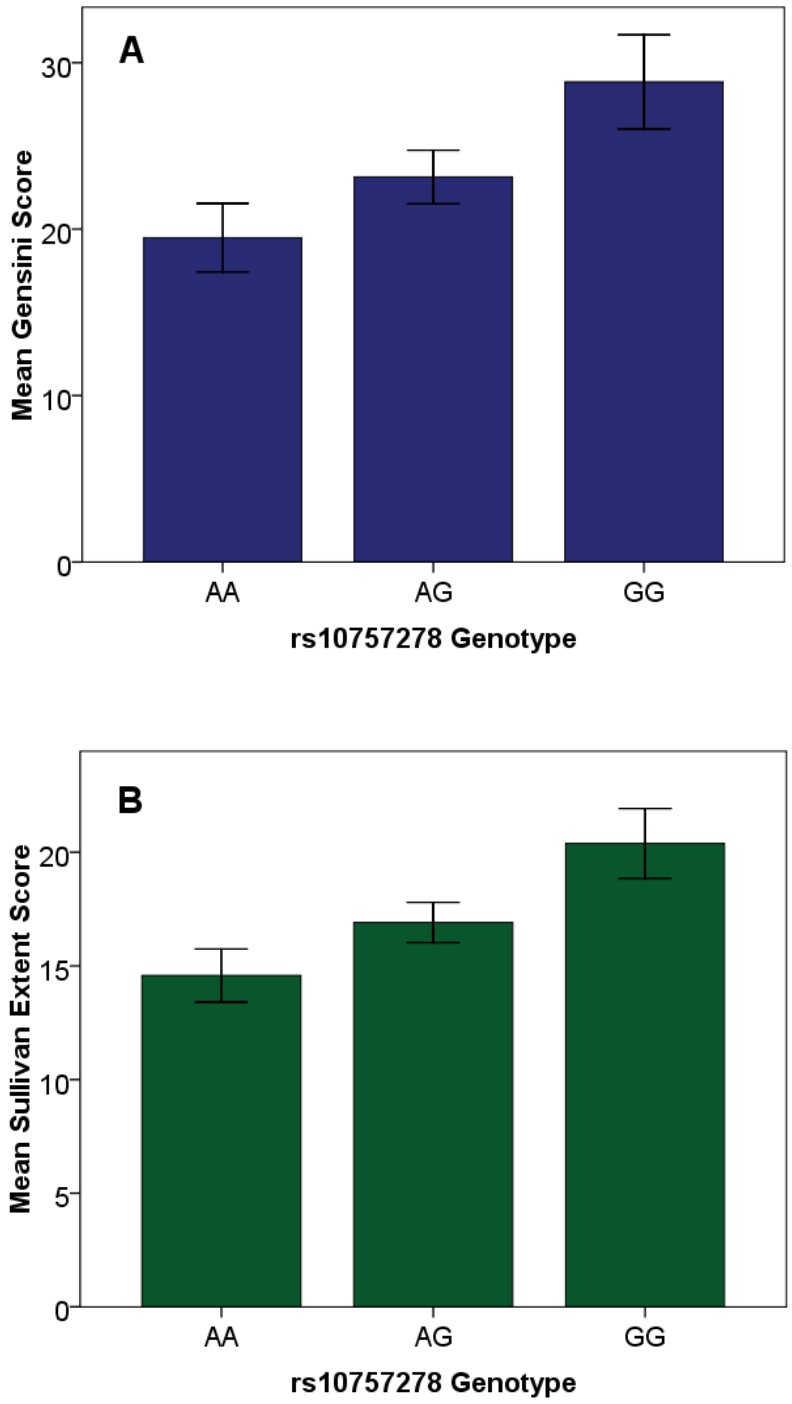
Table 4 shows the association between the rs10757278 genotype and CAD severity/extent with respect to median Gensini and Sullivan Extent scores. There was a significant additive effect of the G allele on each measure of CAD. After adjusting for age, gender, BMI, traditional risk factors, statin use and history of MI, the associations between rs10757278 and both scores remained significant (Gensini p=0.016, Sullivan p=0.005)(Table 5). Thus, possessing one copy of the risk variant equates to greater angiographic scores, which correspond to, for example, a 50% lesion in the proximal LAD, or 15% of the entire LAD intima area. (Figure 12)

Table 5: Multivariate predictors of CAD severity and extent

	Log Gensini (CAD Severity)		***Log Sullivan (CAD Extent)***	
	B (SE)	P value	B (SE)	P value
Age, yrs	0.03 (<0.01)	<0.001	0.03 (<0.01)	<0.001
Gender, m	0.88 (0.07)	<0.001	0.70 (0.06)	<0.001
BMI	-0.01 (0.01)	0.028	-0.01 (0.01)	0.07
Diabetes	0.36 (0.08)	<0.001	0.34 (0.06)	<0.001
Hyperlipidaemia	0.30 (0.08)	<0.001	0.22 (0.06)	<0.001
Hypertension	0.08 (0.08)	0.32	0.11 (0.06)	0.06
Smoking	0.14 (0.07)	0.05	0.19 (0.05)	<0.001
Statin Use	0.83 (0.08)	<0.001	0.72 (0.06)	<0.001
History of MI	1.30 (0.07)	<0.001	0.89 (0.06)	<0.001
rs10757278	0.12 (0.05)	0.016	0.10 (0.04)	0.005

Multivariate linear regression model includes age (year), gender (male=1), body mass index (BMI), diabetes, hypertension, hyperlipidaemia, smoking, statin use, history of myocardial infarction (MI) and rs10757278 (per risk allele). Model R^2 for log Gensini = 0.356 with R^2 change for rs10757278 = 0.004; Model R^2 for log Sullivan = 0.343, R^2 change for rs10757278 = 0.004. B(SE) - Unstandardized regression coefficient with standard error.

Figure 12: CAD severity and extent by 9p21 genotype



CAD severity is illustrated here as the mean Gensini score (Panel A, p for trend <0.001) and the mean Sullivan Extent score (Panel B, p for trend<0.001), all adjusted for age, gender, BMI, risk factors, statin use, MI and presented after reverse log transformation. Standard Error Bars shown; The risk allele is G.

Analyses were repeated after excluding subjects with normal coronary arteries in order to ensure that the observed effect was not being driven primarily by the absence of disease in one group. In this smaller group (n=1849), the association with both Gensini and Sullivan Extent scores remained significant and independent of covariates (adjusted p=0.03 for both).

Sensitivity analysis did not reveal any significant interactions with age, gender, or presence of diabetes, hypertension, hyperlipidaemia and statin use or smoking (data not shown). However, we did observe an interaction with history of MI (p=0.03). Stratified analysis revealed no association between rs10757278 and CAD scores in subjects with MI (n=751, Gensini p=0.91, Sullivan p= 0.74), while those with no history of MI (n=1583, Gensini and Sullivan p<0.001) maintained a significant association with both scores.

9p21 association with CAD progression

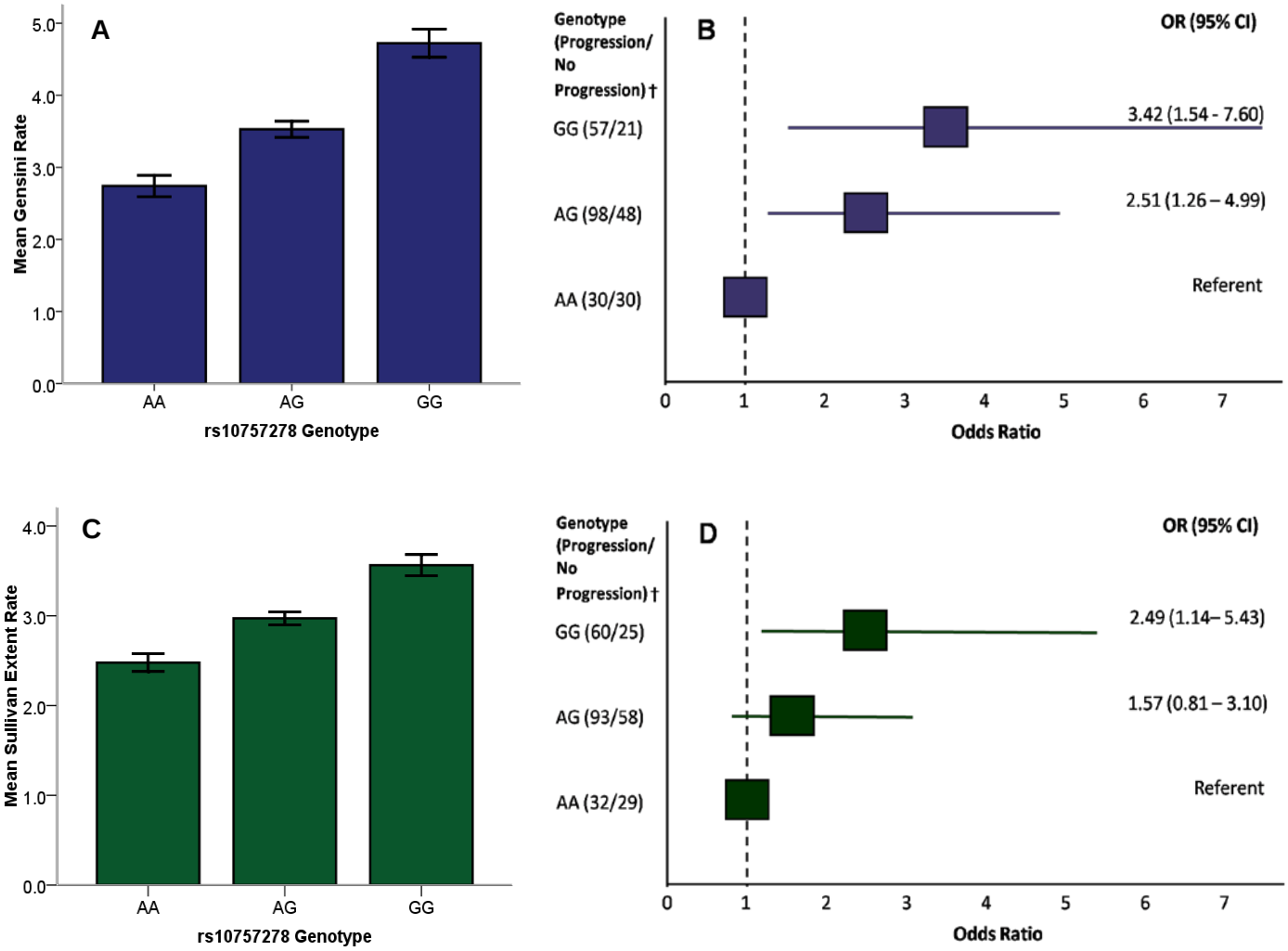
Of the 2334 patients, 308 were identified as having had repeat angiograms. These patients did not differ by genotype for basic characteristics and were similar to the main cohort (Table 6). The median length of time between angiographies was 4.5 years (IQR 2.5-7yrs). There was a significant additive effect of the G allele on risk of progression when the net change in Gensini score per year was used to quantify progression (p=0.023) with homozygotes for the risk allele progressing at a mean covariate adjusted rate of 5 Gensini points/year compared to the referent group progressing at under 3 points/year. (Figure 13a) Furthermore when treated as a binary variable, heterozygotes for the risk allele were more than twice as likely to be progressors (Methods) (OR 2.51 (1.26 - 4.99)) as compared to non-carriers, while homozygotes were greater than three times more likely (OR 3.42 (1.54 - 7.60)) after adjustment for age, gender, diabetes, statin use, smoking and baseline CAD Gensini score. (Figure 13b) A similar trend was observed when the Sullivan Extent score was used to assess progression in this manner. After adjustment for the same covariates we observed a significant association between rs10757278 and the net change in Sullivan Extent score/year (p=0.003) as well as with a binary categorization of progression (Methods) (Heterozygote OR 1.57 (0.80-3.10); Homozygote OR 2.49 (1.10-5.40) (Figures 13c & d).

Table 6: Patient characteristics by 9p21 genotype for those with serial angiograms

	rs10757278 genotype*			
n = 308	*AA (63)*	*AG (158)*	*GG (87)*	p value
Age, yrs	64.4 (11.2)	64.8 (10.3)	66.0 (11.6)	0.61
Male, %	71.4	67.1	64.4	0.66
BMI, kg/m2	30.2 (7.2)	29.8 (6.2)	29.3 (5.9)	0.66
Diabetes, %	31.7	35.4	31.1	0.75
Hypertension, %	74.6	77.0	64.4	0.09
Hyperlipidaemia, %	76.2	75.2	74.3	0.92
Ever Smoked, %	52.5	62.1	64.0	0.32
Statin Use, %	77.6	72.6	81.9	0.27
Baseline Gensini Score, Median (IQR)	7.5 (1 - 14)	10 (3 - 24)	12 (6 - 24)	0.07
Baseline Sullivan Extent Score, Median (IQR)	11.6 (5 - 23)	15 (5 -27)	17 (8 -27)	0.11
Gensini Rate, Median (IQR)	0.8 (0 - 6)	2.0 (0 - 5)	2.7 (1 - 8)	0.04
Sullivan Extent Rate, Median (IQR)	1.1 (0 - 4)	1.8 (0 - 5)	3.0 (0 - 5)	0.08

*Mean (SD) or % unless indicated. IQR – Inter-Quartile Range. *Hardy-Weinberg Equilibrium test: p=0.6*

Figure 13: CAD progression by 9p21 genotype



CAD progression by genotype using angiographic scores is illustrated here as a continuous parameter showing the mean Gensini (Panel A) and Sullivan Extent rate (Panel C), defined as net change in score/years between procedures, by genotype.

Odds Ratios and 95% confidence intervals for progression v non progression is also illustrated for Gensini (Panel B) and Sullivan Extent scores (Panel D).

All values are adjusted for age, gender, diabetes, statin use, smoking and baseline CAD angiographic score and presented after reverse log transformation. † Patients with intermediate rates of change were omitted for Gensini (n=24) and Sullivan Extent (n=11) definitions of progression/non progression (Methods). The risk allele is G.

Discussion:

Using detailed angiographic data and thereby refining the phenotype, we demonstrated a positive association between the rs10757278 SNP and the Gensini and Sullivan Extent scores that define the severity and extent of angiographic CAD. Furthermore, we demonstrated that each copy of the risk allele leads to a higher risk of CAD progression over time. Our findings add significantly to the existing clinical and functional studies linking the 9p21 risk locus to atherosclerosis, by demonstrating an independent association with a quantitative CAD phenotype, and importantly with CAD progression.

A quantitative measure of CAD is preferable to a binary phenotype as it (1) avoids misclassification bias due to the time sensitive nature of coronary disease, (2) gives a better indication of lifelong cumulative burden of disease, and (3) may be more sensitive to the small effect size of common variants. We therefore chose to use two validated semi-quantitative angiographic scores which can be easily applied as a means to estimate severity and extent of CAD. While moderately correlated with each other ($r=0.7$, $p<0.01$), each score represents a slightly different aspect of CAD.

Our results demonstrate a significant association with rs10757278 for both scores using an additive genetic model. As an example, each copy of the risk allele contributes approximately one 50% lesion in the LAD. Even after excluding subjects with normal coronary arteries, whose inclusion may potentially be driving the effect given they were shown to have a lower frequency of the risk allele, the additive trend persisted. Interestingly, while we confirmed an absence of any significant interactions between 9p21 and common risk factors, we did observe an interaction with MI, with a non-significant relationship in those who had a previous MI. This likely represents a skewed distribution of disease, as MI patients tend to have a greater degree of CAD burden at the upper range of Gensini and Sullivan Extent scores, and thus a smaller range of disease in which to identify a trend.

The positive association between the 9p21 risk genotype and graded severity of CAD is a novel finding, not previously shown in angiographic cohorts. Initial studies demonstrated association with presence/absence of CAD, either defined clinically or by 50% disease criteria on angiography.(Samani et al. 2007; Muendlein et al. 2009) Early studies failed to demonstrate an association between 9p21 risk genotype and CAD severity by number of vessels affected in Asian populations. This may have been due to

their low power to detect small effects along with a relatively insensitive estimate of severity.(Hinohara et al. 2008; Chen et al. 2009b) Another study, by Anderson and colleagues, demonstrated that CAD presence was correlated with 9p21 in 2100 Caucasian subjects but not with extent as assessed by a vessel score and the Duke CAD index.(Anderson et al. 2008) Both scores may be insensitive to the changes expected, given the complexity of the disease and the small effect size of this SNP. The Duke CAD index is a validated hierarchical prognostic score, including only vessels with >50% disease and is less suited to quantifying multiple lesions. For example, a left main lesion with 95% luminal stenosis would score a maximum of 100 for the Duke CAD Index with no room to quantify further disease that may exist in other vessels, unlike the Gensini score. Population stratification or differences in clinical selection criteria for coronary invasive investigation may also account for the divergent results.

Importantly, our study adds additional information by demonstrating association with CAD progression over time. In subjects with repeat angiograms, we observed an additive effect of the risk allele on rate of change of Gensini score per year. When classified as “progressors” and “non progressors”, homozygotes for the risk allele were 3 times more likely to be progressors compared to non carriers, even when subjects without baseline coronary disease were excluded (data not shown). Similar findings were observed with use of the Sullivan Extent score to document progression. In contrast one study, based on a quantitative angiography analysis of subjects enrolled in a statin trial (treated 147, placebo 141), reported no evidence of progression over 2 years in relation to 9p21 genotype, despite post hoc calculations to suggest adequate power.(Chen et al. 2009a) This difference may be a consequence of strict patient selection or shorter follow up time, compared to our study. On the other hand, supportive evidence comes from studies reporting progression of subclinical atherosclerosis with carotid intima media thickening(Ye et al. 2008) and greater revascularization outcomes in carriers of the 9p21 risk allele.(Lina et al. 2008)

Our findings on the whole thus support the notion proposed by clinical and functional studies that cell proliferation and atherosclerosis are mediated by this locus.(Visel et al. 2010) In addition to studies associating this locus with coronary calcium scores (Assimes et al. 2008) and peripheral vascular disease,(Helgadottir et al. 2008) others have also reported association with intracranial aneurysms and arterial stiffness suggesting that the locus may also act outside of the traditional atherosclerotic

pathway, perhaps by influencing vascular structure.(Bilguvar et al. 2008; Patel et al. 2008)

Strengths and Limitations

Some strengths of our study include: (1) a large sample size, with a broad range of disease from normal to severe multi-vessel involvement enabling accurate assessment of severity; (2) use of detailed coronary angiography phenotyping, moving beyond simple vessel scoring to carefully quantify disease burden and (3) evaluation of progression of disease. There are also some important limitations to our study. First, the use of coronary angiography to visually quantify atherosclerosis is limited as remodeling may obscure substantial disease burden in arterial walls that can be detected by intravascular ultrasound,(Glagov et al. 1987; Nissen 2001) but relatively small and limited numbers of genetic ultrasound registries are available to date. Also, subjects undergoing first or repeat catheterization are a select group who are symptomatic or otherwise at high risk and thus may not be representative of the general population. Furthermore, variations in healthcare systems and referral patterns for angiography could also be a source of selection bias. Finally, we only ascertained the effect of one SNP in this region. However, this SNP was chosen as the marker of this region based on robust prior data and is in tight linkage disequilibrium with many other commonly used 9p21 markers (for example rs1333049, $r^2 = 1$) and genotyping these would thus add little incremental value.

Conclusions

In conclusion, we have shown that the rs10757278 SNP at the 9p21 risk locus is associated with severity, extent and progression of CAD in a population undergoing coronary angiography, suggesting a role for this locus in influencing atherosclerosis and its progression.

Study 2: Refining the phenotype for novel coronary heart disease risk variants discovered through genome wide association

Summary:

By mid 2009, several additional CHD risk variants had been discovered through comprehensive GWAS and large scale replication. Of the 12 discovered in total, 8 had no definite mechanism by which they could confer risk. This study examined the association of each of these 8 variants (including 9p21) with 4 specific angiographic sub-phenotypes: 1) CAD presence or absence; 2) CAD burden; 3) CAD progression and 4) MI in the presence of CAD. We found that three variants at 9p21, 6p24 and 1q41 were associated with CAD phenotypes while two at 21q22 and 2q33 were associated with risk of MI in the presence of CAD. This study further highlights the importance of refining the CHD phenotype to potentially aid mechanistic understanding.

Introduction:

Unbiased genome wide approaches have now led to the identification of multiple novel risk loci for CHD in predominantly Caucasian cohorts.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; 2007; Clarke et al. 2009; Erdmann et al. 2009; Gudbjartsson et al. 2009; Kathiresan et al. 2009a) Only a few appear to have a mechanistically relevant basis through lipid metabolism pathways (1p13, 1p32, 6q26, 19p13).(Samani et al. 2008a; Clarke et al. 2009; Kathiresan et al. 2009b) The remaining loci span genomic regions which as yet have no clearly defined mechanism to explain coronary risk.

The diagnosis of CHD encompasses a broad range of pathophysiological entities and as a composite phenotype may be hindering attempts to understand the mechanistic basis of these variants. Refining the phenotype for these novel loci may thus prove to be a useful means to help direct functional studies.(McCarthy and Hirschhorn 2008) As an example, the 1p13 locus was initially identified through association with MI, and then followed up with association to lipid levels which in turn has led functional studies to identify a role for this locus in lipid metabolism through sortillin 1 (SORT1) with potential for therapeutic developments.(Linsel-Nitschke et al. 2009; Musunuru et al. 2010)

Myocardial infarction is the end result of complex processes including atherosclerosis, plaque destabilization, rupture and thrombosis.(Libby et al. 2009) In this context, Reilly et al recently suggested that in the presence of CAD, none of these SNPs confer additional risk of MI precipitation and thus may all be related to atherosclerosis.(Reilly et al. 2011) Atherosclerosis though is itself a complex phenotype and associated variants could be related to upstream risk factors, lesion initiation, disease extent, or rapid progression (Figure 4, chapter 1). Coronary angiography allows identification of specific CAD and MI phenotypes. For example, it can be used to define subjects as true controls having smooth unobstructed coronary arteries, those who have aggressive or widespread CAD, those who develop rapid progression of disease and those who develop CAD but do not experience plaque rupture or erosion events.

We thus sought to examine the association of 8 GWAS identified CHD risk variants, whose mechanisms of action are unclear, with detailed angiographic CAD and MI phenotypes, hypothesizing that some or all of these loci confer risk by promoting (1) atherosclerosis development (initiation), (2) greater atherosclerotic involvement of the coronary arteries (severity/burden), (3) enhancing progression of existing disease, or (4) promoting the final precipitation of MI or plaque rupture (Figure 4, chapter 1).

Methods

Study Population

Study participants were recruited as part of the Emory Cardiology Genebank, consisting of consecutive patients enrolled prior to undergoing elective or emergent cardiac catheterization across three Emory Healthcare sites. Medical records were reviewed to confirm self reported history of MI and other conditions as well as to document previous angiographic findings and prior coronary revascularization. Full details and definitions for this population are provided in chapter 2. After excluding self reported non-Caucasian ancestry (as these variants have not yet been validated in other ethnicities), heart transplantation, missing or incomplete angiographic data and missing DNA/blood samples, 2730 subjects were deemed eligible for this analysis. The study was approved by the Institutional Review Board at Emory University, Atlanta, GA and all subjects provided written informed consent.

Myocardial Infarction (MI) Phenotypes

MI cases were classified by prior history of or admission with MI defined by standard universal criteria, as reported in chapter 2.(Thygesen et al. 2007) Given that comparing subjects with MI to controls without CAD (by angiography) may also be testing the hypothesis of CAD v no CAD we defined two sets of controls determined as 1) subjects without MI and no angiographic CAD, and 2) those without MI in patients *with* angiographic CAD. To mitigate case misclassification bias with the second group of controls, given that CAD cases may eventually develop MI we specified a-priori sub-group analysis to also examine this association in cases <60 years of age and controls >70 years old.

Coronary Artery Disease (CAD) Phenotypes

Coronary artery disease phenotyping was performed using coronary angiographic data as described in chapter 2. CAD cases were defined as subjects with angiographic CAD of >50% diameter stenosis in any major epicardial vessel while controls were those without CAD or with minimal luminal irregularities only (<10% luminal obstruction). Subjects with intermediate disease, >10% and <50% diameter stenosis, were excluded (n=137) for binary analysis. CAD severity and progression were documented using the Gensini scoring system in the whole cohort.(Gensini 1975) Full details on angiographic scoring of CAD severity in the Emory Genebank have been published previously and are also described in chapter 2.(Patel et al. 2010) Analyses were repeated after excluding subjects with MI, in an attempt to remove the influence of this related phenotype.

As described in the previous study, a subset of 310 patients who had undergone two or more coronary angiographies at least 6 months apart, were identified and the two angiograms furthest apart in time were quantified using the Gensini score. The net change in Gensini score was divided by number of years between angiographies to give a "Gensini Rate", which was used as a measure of CAD progression. Full details are provided in chapter 2 and have also been published recently.(Patel et al. 2010)

SNP Selection and Genotyping

A review of published genome wide association studies identified 16 loci associating with CAD and/or MI through June 2009 at a GWAS threshold of $5x10^{-8}$ (for 1 million tests of association). These are listed in Table 3 in Chapter 1. Of these, 3 did not replicate in larger follow up studies (2q36, 15q22, 6q25).(Samani et al. 2009) Another 4 loci have potential functional relevance to lipid metabolism (1p13 –SORT1; 19p13 – LDLR; 1p32 – PCSK9; 6q26- LpA) and were therefore not included in this study, the purpose of which was to examine those loci without a defined mechanism. (Samani et al. 2008a; Clarke et al. 2009; Kathiresan et al. 2009b) One SNP at 12q24.3 was not included due to its very small effect size in the discovery study.(Erdmann et al. 2009) Lead single nucleotide polymorphisms (SNPs) at the remaining 8 loci of interest, which are independent of traditional risk factors, were thus genotyped and are listed in Table 8.

Genotyping was performed using the Centaurus (Nanogen) platform at deCODE genetics, Reykjavik, Iceland or with the SNPstream (Beckman Coulter) platform at Emory University, Atlanta, GA. (Bell et al. 2002; Kutyavin et al. 2006) Full genotyping details and quality control measures are provided in chapter 2. Genotyping rate for all 8 SNPs was >98%.

Statistical Analysis

Continuous variables are presented as means (SD) and categorical variables as proportions (%). PLINK software was used to compute Hardy-Weinberg equilibrium and minor/risk allele frequencies.

Case control analyses were performed using logistic regression under an additive model. Linear regression models were constructed to test the additive effect of the SNPs on the quantitative trait of CAD severity and extent (log+1 transformed Gensini score), with the SNP coded as 0, 1 or 2 based on number of risk alleles. Analyses were repeated after adjusting for age, gender, body mass index (BMI), diabetes, hypertension, hyperlipidaemia and smoking. A 2-tailed P value <0.05 was considered significant. Statistical analyses were performed using SPSS 17.0 (Chicago, IL).

Power calculations were performed using Quanto software (http://hydra.usc.edu/gxe/). Minor allele frequencies were varied from 0.05 to 0.5

under a log additive model. For case-control analyses we estimated prevalence of CAD and MI from our population. For Gensini scores and Gensini rate, we estimated the smallest effect size per risk allele that could be determined at 80% power with the given number of patients. Results of the power calculations are presented at the end of this chapter in Table 13.

Results

A total of 2730 self-reported Caucasians were included in this study. Patient characteristics at baseline are shown in Table 7. The mean age (SD) was 63.8 years (11.3), with a range of 24 to 90 years and 67.6% were male. The prevalence of risk factors and other characteristics were representative of typical cardiac catheterization laboratory populations in the US. The majority of subjects had obstructive coronary disease although 19% demonstrated normal coronary anatomy (smooth or minor irregularity <10%). Further patient characteristics by specific case-control status for CAD and MI are available in Tables 11 & 12 at the end of the chapter.

The eight variants representing the loci of interest along with their published effect sizes for association with MI are listed in Table 8 and were genotyped in all patients. The observed genotypic frequencies were consistent with Hardy Weinberg equilibrium with risk allele frequencies similar to those reported in published studies (Table 8).

Table 7: Patient characteristics for Genebank participants (aim 1b)

Patient characteristics	***n = 2730***
Age, yrs	63.8 (11.3)
Male, %	67.6
Body Mass Index, kg/m^2	29.3 (6.0)
Diabetes, %	28.8
Hypertension, %	68.1
Systolic BP, mmHg	138.9 (22.6)
Hyperlipidaemia, %	69.8
Total Cholesterol, mg/dL	167.7 (42.8)
Ever Smoked, %	60.8
Acute Coronary Syndrome, %	10.6
Ever Myocardial Infarction	40.7
Coronary Artery Disease	
Normal (<10%), %	19
Non obstructive (>10%,<50%), %	5
Obstructive (>50%), %	76
Gensini Score, Median (IQR)	14 (1-58.6)

Mean (SD) or % unless indicated. CAD – Coronary Artery Disease; CABG – Coronary Artery Bypass Grafting; PCI – Percutaneous Coronary Intervention; IQR – Inter-Quartile Range

Table 8: Details of CHD risk loci without known mechanism of risk

Chromosomal Locus	*Genotyped SNP*	*Nearest Gene*	*Risk allele*	*Published risk allele frequency*	*Observed risk allele frequency*	*HWE p value*	*Reported allelic CHD risk (OR)*	*References*
1q41	17465637	MIA3	C	0.74	0.73	0.84	1.10-1.14	(Kathiresan et al. 2009a; Samani et al. 2009)
2q33	6725887	WDR12	C	0.14	0.13	0.23	1.17	(Kathiresan et al. 2009a)
3q22.3	9818870	MRAS	T	0.15	0.16	0.77	1.15	(Erdmann et al. 2009)
6p24	12526453	PHACTR1	C	0.65	0.67	0.09	1.12	(Kathiresan et al. 2009a)
9p21	10757278	CDKN2A/B	G	0.50	0.50	0.36	1.20-1.33	(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; Kathiresan et al. 2009a; Samani et al. 2009; Reilly et al. 2011)
10q11	1746048	CXCL12	C	0.84	0.87	0.65	1.11-1.17	(Kathiresan et al. 2009a; Samani et al. 2009)
12q24	3184504	SH2B3	T	0.47	0.49	0.30	1.13	(Gudbjartsson et al. 2009)
21q22	9982601	SLC5A3-MRPS6-KCNE2	T	0.13	0.13	0.17	1.20	(Kathiresan et al. 2009a)

SNP – Single Nucleotide Polymorphisms; HWE – Hardy Weinberg Equilibrium

Association with CAD (case-control)

We first examined association between the eight variants and presence or absence of CAD using angiographic definitions described above. Significant associations were observed for 4 out of the 8 SNPs (at loci 9p21 ($p=5.8x10^{-3}$), 6p24 ($p=1.7x10^{-4}$), 1q41 ($p=4x10^{-3}$) and 21q22 (p=0.04)) (Table 9). When subjects without MI were examined, to exclude the effect of a parallel association with MI, only three SNPS remained significantly associated with CAD (at 9p21 (p=0.03), 1q41 (p=0.03) and 6p24 ($p=3.3x10^{-4}$). Adjustment for age, gender and risk factors attenuated the significance of the association to borderline for the 1q41 variant only (Table 9).

Association with CAD severity (quantitative)

Next we tested genotype associations with CAD severity as a quantitative trait assessed by the Gensini score in all 2730 subjects. The three SNPs that demonstrated significant association with the binary CAD phenotype were also associated with the Gensini score before and after excluding subjects with MI (at 1q41 (p=0.013), 6p24 (p=0.019), 9p21 ($p=1.4x10^{-3}$)). However, after adjusting for covariates, only two of the three SNPs (at 9p21 and 6p24) demonstrated additive association with the Gensini score (p=0.001, p=0.024 respectively) whereby each risk allele contributed incrementally to CAD severity/extent (Table 9).

Association with CAD progression (quantitative)

Using a consecutive series of 310 patients who underwent repeat angiography, we examined associations between these 9 SNPs and CAD progression. Here, only the 9p21 variant ($p=2x10^{-3}$) associated significantly with the "Gensini rate", which was determined as the net change in Gensini score/year. This remained significant after adjusting for age, gender, risk factors and baseline CAD severity by Gensini (Table 9).

Table 9: Association between CHD risk variants and CAD phenotypes

CAD:	***Initiation***		***Initiation***			***Extent***			***Progression***		
Locus	CAD versus no CAD (all) N = 2075 v 518		CAD versus no CAD (MI-) N = 965 v 518			CAD Severity/Extent (MI-) N = 1620			CAD Progression (all) N = 310		
	OR (95% CI)	P value	OR (95% CI)	P value	P – adj*	Beta (SE)	P	P–adj*	Beta (SE)	P	P- adj†
1q41	1.25 (1.07-1.45)	$4.0x10^{-3}$	1.20 (1.02-1.42)	0.03	0.05	0.17 (0.07)	0.01	0.16	-0.10 (0.09)	0.29	-
2q33	1.15 (0.94 -1.42)	0.17	1.05 (0.83-1.32)	0.67	-	-0.01 (0.10)	0.93	-	-0.18 (0.12)	0.13	-
3q22.3	0.99 (0.82-1.20)	0.96	0.98 (0.80-1.21)	0.86	-	-0.05 (0.10)	0.60	-	-0.13 (0.10)	0.22	-
6p24	1.32 (1.14-1.52)	$1.7x10^{-4}$	1.34 (1.14-1.57)	$3.3x10^{-4}$	$1.0 x10^{-3}$	0.16 (0.07)	0.02	0.02	0.13 (0.09)	0.12	-
9p21	1.22 (1.06-1.40)	$5.8x10^{-3}$	1.19 (1.02-1.39)	0.03	0.02	0.21 (0.07)	$1.4x10^{-3}$	$1.0x10^{-3}$	0.19 (0.08)	$2.0x10^{-3}$	$3.0x10^{-3}$
10q11	1.14 (0.93-1.40)	0.21	1.15 (0.92-1.45)	0.22	-	0.07 (0.10)	0.45	-	-0.12 (0.13)	0.45	-
12q24	0.96 (0.83-1.11)	0.60	0.95 (0.81-1.11)	0.51	-	-0.01 (0.07)	0.83	-	0.06 (0.08)	0.45	-
21q22	1.26 (1.02-1.56)	0.04	1.14 (0.90-1.44)	0.29	-	0.04 (0.10)	0.68	-	0.17 (0.12)	0.16	-

**Adjusted for age, gender, BMI, diabetes, hypertension, hyperlipidaemia and smoking*

†Adjusted in addition to the above for baseline Gensini score

Association with MI (case v control)

Finally, we attempted to replicate the association between these 8 variants and MI using two distinct sets of controls (Table 10). When compared to traditional control subjects (without MI or CAD (MI-/CAD-)) the associations were replicated for 5 SNPs (at 9p21 ($p=2.7x10^{-3}$), 6p24 ($p=1.1x10^{-3}$), 1q41 ($p=5x10^{-3}$), 21q22 ($p=8.3x10^{-3}$) and 2q33 ($p=0.06$)) (Table 10). However, when compared to control subjects without MI but *with* CAD (MI-/CAD+), we only observed trends towards significance for association with two SNPS. Given the potential for case-misclassification bias, whereby CAD patients may develop subsequent MI and are therefore not truly controls, we performed a pre-specified secondary analysis, with younger cases (<60 years) and older controls (>70years). In this analysis, those two SNPs achieved statistical significance (at 2q33 ($p=7.1x10^{-3}$) and 21q22 ($p=0.02$)), even after adjustment for covariates (Table 10).

Due to the limitations of power in this study we were unable to demonstrate significant associations with the remaining 3 SNPs (3q22.3, 10q11, 12q24) with either CAD or MI phenotypes.

Table 10: Association between CHD risk variants and MI phenotypes

MI	***MI v Traditional Controls***		***MI v Controls with CAD***		***MI v Controls with CAD - Age Selected***		
Locus	MI+ versus MI-/CAD- N = 1110 v 518		MI+ versus MI-/CAD+ N = 1110 v 965		MI+ <60yrs versus MI-/CAD+ >70yrs N = 692 v 367		
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	P-adj*
1q41	1.27 (1.07-1.50)	$5.0x10^{-3}$	1.06 (0.92-1.23)	0.39	1.24 (1.01-1.53)	0.040	0.10
2q33	1.22 (0.99-1.52)	0.05	1.16 (0.98-1.38)	0.10	1.46 (1.11-1.92)	$7.1x10^{3}$	0.01
3q22.3	1.02 (0.84-1.25)	0.82	1.05 (0.88-1.24)	0.61	1.0 (0.78-1.28)	0.98	-
6p24	1.30 (1.11-1.52)	$1.1x10^{-3}$	0.96 (0.84-1.1)	0.58	1.11 (0.91-1.35)	0.30	-
9p21	1.26 (1.08-1.47)	$2.7x10^{-3}$	1.07 (0.94-1.21)	0.31	1.14 (0.95-1.38)	0.16	-
10q11	1.15 (0.92-1.44)	0.22	1.0 (0.82-1.22)	0.99	0.99 (0.74-1.33)	0.96	-
12q24	0.97 (0.83-1.14)	0.74	1.02 (0.90-1.16)	0.71	0.99 (0.82-1.20)	0.96	-
21q22	1.36 (1.08-1.71)	$8.3x10^{-3}$	1.20 (1.0-1.44)	0.05	1.39 (1.05-1.83)	0.02	0.02

**adjusted for age, gender, BMI, diabetes, hypertension, hyperlipidaemia, smoking*

CAD allelic risk score

Given that 3 SNPs associated significantly with CAD, we explored the potential clinical relevance of this finding and constructed a simple cumulative allelic risk score using these three SNPs and examined its association with CAD burden or severity assessed by the Gensini score (Figure 14). We demonstrated that each additional risk allele out of a maximum of 6 was associated with a 2-3 point greater median Gensini score (non parametric p<0.001), which is, for example, the equivalent of a 50% lesion in the proximal RCA. Similarly, each additional risk allele was associated with an OR of 1.27 (1.17-1.39) for presence/absence of CAD.

Figure 14: CAD severity and risk allele burden

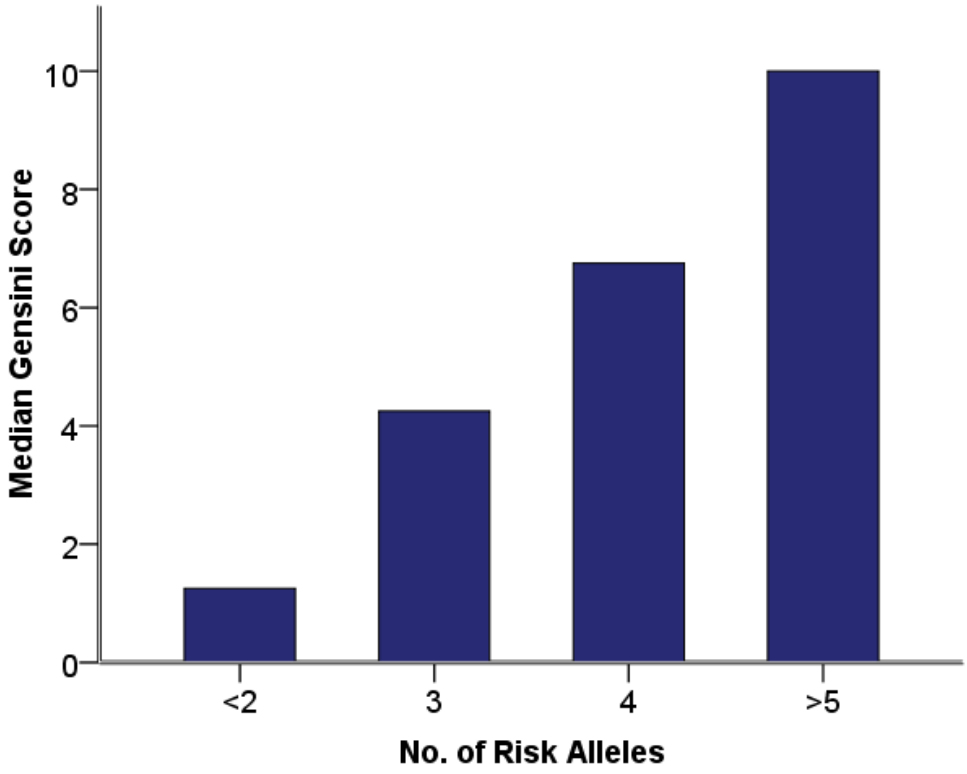


Figure illustrating the median Gensini Score by number of cumulative risk alleles for the 3 variants associating with CAD phenotypes (rs10757278 (9p21), rs12526453 (6p24) and rs17465637 (1q41); P for trend is <0.001.

Discussion

Coronary heart disease (CHD) is a complex composite phenotype and includes both CAD and MI. Using detailed coronary angiographic data, we sought to refine the phenotype for 8 novel CHD risk loci identified through GWAS and whose mechanism of action is currently unknown. Using this exploratory approach, we demonstrate differential associations between some of these loci and CAD and MI phenotypes, suggesting that these variants may act at different stages in the spectrum of CHD.

We postulated that examination of the CAD phenotype in a binary manner may identify loci which lead to initiation of disease, by for example promoting vessel wall abnormalities, whereas the phenotypes of severity/extent may provide additional information on processes promoting multiple lesions or burden, while association with progression gives further information on possible expansive or proliferative processes. Used together, they can provide more complete information on the atherosclerotic phenotype as well as its downstream complication of plaque rupture and MI.

We examined association between these CAD phenotypes and 8 risk variants. SNPs at three loci, (1q41, 6p24 and 9p21) demonstrated significant association with both CAD presence and severity/extent, independently of association with MI. The most robust association identified was for 6p24 and 9p21, with the latter being additionally associated with CAD progression. The association between these CAD phenotypes and 9p21 has been described by our group and others, but is novel for the 6p24 locus.(Dandona et al. 2010; Patel et al. 2010) Furthermore when these 3 CAD variants are combined into an allelic risk score, there is a cumulative effect on CAD severity, although this of course may be overestimated by virtue of using data from the same cohort

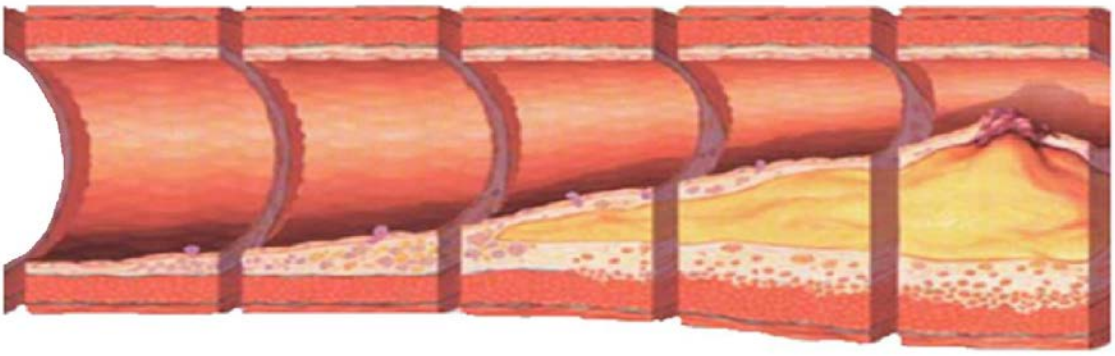
We also re-examined the associations with MI, using traditional MI controls and controls advocated by Reilly et al and Horne et al who proposed that comparing MI subjects, who will almost always have CAD, to traditional controls without CAD will confound the findings by also demonstrating positive association with CAD.(Horne et al. 2008; Reilly et al. 2011) Thus, to investigate an association with the phenotype of MI that is usually a result of plaque destabilization or thrombosis, one should compare subjects with MI (and CAD) to subjects without MI but who also have stable CAD. In this study, using traditional controls we replicated association between MI and 5 loci, but none were significant when using the CAD+/MI- control group. This is consistent with

the negative findings of both Horne et al for 9p21 as well as Reilly et al who examined 11 variants including 7 of the 8 in this study.(Horne et al. 2008; Reilly et al. 2011) However, in contrast to the latter study, we also performed secondary analyses using older controls and younger cases (MI<60, CAD+/MI- >70) to try and mitigate the obvious case misclassification which may occur, given that CAD patients are at high risk of developing MI at a later date and thus becoming cases. With this approach we demonstrated significant association with MI+/CAD+ for at least two variants at 2q33 and 21q22. This suggests that these two loci could act via mechanisms to precipitate MI *after* CAD has developed.

Although overall we showed differential association with MI and CAD phenotypes, with the limitations in power we are unable to conclusively state that specific variants associate *only* with certain CAD sub-phenotypes. Our post hoc power calculations suggest that we may have missed smaller effect sizes. This may also explain why we were unable to demonstrate associations for three loci (3q22.3; 10q11; 12q24), all of which have been found to have effect sizes much lower than the original GWAS, in recent large scale studies. For example the Reilly et al study identified an association with CAD for 3q22.3 with an OR of 1.15 (1.03-1.27), while we only had power to detect this down to an OR of 1.27.(Reilly et al. 2011) Yet, the same study provides corroboration for our lack of association with 10q11. In addition, it is of interest that the two most significant associations with angiographic CAD in their study were for 6p24 and 9p21, which confirms our findings.(Reilly et al. 2011)

Emerging evidence including data from a mouse model, suggests that the 9p21 locus may promote a proliferative phenotype leading to atherosclerosis, which is in agreement with our findings.(Visel et al. 2010) Functional studies are ongoing to elucidate the mechanisms of the other variants identified in this study and may be guided by findings such as these. For example, at the 1q41 locus the MIA3 gene is thought to regulate hematopoietic cell migratory processes such as monocytes, a key process in atherosclerosis and confirmed to represent a CAD phenotype in this study.

Figure 15: Stages of atherosclerosis in relation to CHD phenotypes



Stage of Atherosclerosis:	*CAD Initiation*	*CAD Extent*	*CAD Progression*	*MI/ Plaque Event*
Angiographic Phenotype:	*CAD Presence/ Absence*	*Gensini Score*	*Gensini Rate/yr*	*MI Risk in Presence of CAD*

Schematic representation of the main stages of atherosclerosis leading to myocardial infarction with the corresponding angiographic phenotypes attempting to capture each of these stages; Figure modified from the original illustration found at http://en.wikipedia.org/wiki/File: End_ dysfunction_Athero.PNG. Permission not required under the GNU free documentation license.

Strengths and Limitations

In an attempt to conserve phenotypic clarity our sample size was restricted and we may have missed associations with some SNPs due to a lack of statistical power. This is unfortunately a necessary trade off until appropriate and very large collections of highly phenotyped populations can be collated. Additionally, the nature of this study hinges on multiple testing, of both variants and phenotypes. Given the sample size, modest effect sizes and exploratory nature of this work, we concede that some associations may not remain significant after Bonferonni adjustment. However, the consistency of association across related CAD phenotypes and their partial corroboration by other studies suggests that these are unlikely to be spurious associations, and that the principal of refining the phenotypes is robust and merits testing in further larger studies.

Strengths of this study include use of a large angiographic cohort with a detailed panel of CAD phenotypes including a semi-quantitative score which avoids

misclassification bias due to the time sensitive nature of CAD and gives a better indication of lifelong cumulative burden of disease. A further strength is the use of MI-/CAD+ controls for assessment of MI risk distinct from CAD risk along with age stratification with older controls and younger cases to mitigate misclassification bias.

Implications

This work has implications for our approach to understanding complex disease, perhaps by promoting a paradigm for use of broad phenotypes in large scale epidemiology studies to identify important or "hot" regions and then following up with smaller studies that refine the phenotypic signals to help identify potential mechanistic clues. From a clinical perspective, identification of the correct underlying phenotype is critical for risk prediction and may explain why some genomic markers do not predict incident events despite strong associations with disease in cross- sectional studies.

Conclusions

In conclusion, we have shown that refining the phenotype is a useful approach to begin to understand underlying mechanisms in novel risk loci. Specifically, we demonstrate that 5 out of 8 CHD risk loci, whose function is still unknown, are associated differentially with CAD and MI phenotypes, suggesting potentially different mechanisms of action for each in ultimately conferring risk of MI. However further work is needed to confirm these findings and to follow up with functional studies to elucidate specific molecular mechanisms suggested by these studies.

Table 11: Patient characteristics by case-control status for CAD phenotypes

	All Subjects			***Without MI***	
	CAD- Controls (n=518)	CAD+ Cases (n=2075)	P value	CAD+/MI- Cases (n=965)	P value
Age, yrs	58.4 (11.1)	65.6 (10.6)	<0.001	65.6 (10.2)	<0.001
Male, %	47.6	74.3	<0.001	73.4	<0.001
Body Mass Index, kg/m^2	29.8 (6.8)	29.2 (5.7)	0.045	29.2(5.8)	0.1
Diabetes, %	18	32.6	<0.001	31.2	<0.001
Hypertension, %	54.5	72.3	<0.001	73.2	<0.001
Hyperlipidaemia, %	51.9	75.7	<0.001	75	<0.001
Smoking, %	49.3	64.5	<0.001	63.8	<0.001
Myocardial Infarction, **%**	0	52.6	<0.001	0	<0.001

Means, standard deviations or % presented.

Table 12: Patient characteristics by case-control status for MI phenotypes

	CAD- Controls			*CAD+ Controls*		*Age Selected CAD+ Controls*		
	MI+ Cases (n=1110)	CAD-/MI- Controls (n=518)	P value	CAD+/MI- Controls (n=965)	P value	CAD+/MI+ <60 Cases (n=692)	CAD+/MI- >70 Controls (n=367)	P value
Age, yrs	65.1 (10.9)	58.4 (11.1)	<0.001	65.6 (10.1)	0.33	48.8 (7.7)	75.7 (4.2)	<0.001
Male, %	73.4	47.6	<0.001	73.3	0.956	77.3	69.5	0.006
Body Mass Index, kg/m^2	29.2 (5.7)	29.8 (6.8)	0.09	29.2 (5.7)	0.99	29.8 (5.9)	27.6(4.7)	<0.001
Diabetes, %	32.7	18	<0.001	31.6	0.59	34.4	28.6	0.054
Hypertension, %	70.9	54.5	<0.001	73.6	0.18	69.4	75.3	0.040
Hyperlipidaemia, %	76.0	51.9	<0.001	75.1	0.69	78.2	71.8	0.021
Smoking, %	64.7	49.3	<0.001	64.1	0.73	69.4	57.7	<0.001
Myocardial Infarction, %	100	0	<0.001	0	<0.001	100	0	<0.001

Means, standard deviations or % presented.

Table 13: Power calculations for detectable effect sizes (aim 1b)

		With the given N (cases), we had 80% power at α=0.05 to detect these approximate effect sizes under a log additive model (per allele)			
Locus	*SNP ID, rs number*	*CAD v No CAD (OR)*	*Severity (Gensini Score)*	*Progression (Gensini rate/yr)*	*MI v No MI/CAD (OR)*
1q41	17465637	1.24	5	4.8	1.25
2q33	6725887	1.32	6.6	6.3	1.35
3q22.3	9818870	1.32	6.2	6	1.35
6p24	12526453	1.23	4.8	4.6	1.24
9p21	10757278	1.21	4.5	4.4	1.23
10q11	1746048	1.32	6.6	6.3	1.35
12q24	3184504	1.21	4.6	4.4	1.23
21q22	9982601	1.32	6.6	6.3	1.35

Chapter 4: Results, GWAS Variants and Intermediate CHD Phenotypes

The results for specific aims 2a and 2b are presented in this chapter. These studies follow on from the previous chapter by exploring associations with traditional and novel intermediate phenotypes to again identify clues towards mechanism of risk. Study 1 examines the association between the 8 variants without known mechanism of risk and quantitative measures of traditional risk factors including lipid profile, glucose, obesity, inflammation and blood pressure. Study 2 follows on by examining association with a non-traditional risk factor of arterial stiffness/wave reflection indices derived from pulse wave analysis.

Contribution:

I conceived the ideas for both of these studies and performed all relevant analyses. My contribution to the methodology is described in detail in chapter 2, but pertinent to these studies included management of PMR and META-Health study staff, assisting in collection of pulse wave analysis measurements (blinded to genotype), coordination of sample retrieval for genotyping and collation and quality control of all results and data. I then performed all genetic and statistical analysis.

Study 1: Association between novel genetic variants and intermediate phenotypes representing traditional risk factors

Summary:

In this study we explore the associations between the 8 MI risk variants described in the previous chapter whose mechanism of risk is currently unknown with quantitative intermediate phenotypes of traditional cardiovascular risk factors. First, in a population of subjects without CAD we identified that two variants at 1q41 and 12q24 were associated with several phenotypes representative perhaps of metabolic syndrome. However we were unable to replicate this finding in a larger CAD population. Our findings confirm other reports that these 8 risk loci are unlikely to have a direct effect on traditional risk factors and their mechanism of risk is likely to be novel.

Introduction

Early unbiased genome wide approaches have led to the identification of more than a dozen novel risk loci for coronary heart disease in predominantly Caucasian cohorts.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; 2007; Erdmann et al. 2009; Gudbjartsson et al. 2009) Many loci have replicated in large cohorts, yet of these only four appear to have a mechanistically relevant basis through lipid metabolism pathways (1p13, 1p32, 19p13, 6q26).(Samani et al. 2008a; Kathiresan et al. 2009b) The remaining loci span genomic regions that as yet have no clearly defined mechanism to explain CHD risk.

Valuable insights into the functions of these variants may be learned by studying their effects on related or intermediate phenotypes which may be causal in the pathway to developing MI. This is best illustrated by the initial association of 1p13 with MI and then with the intermediate phenotype of lipid levels, with subsequent functional studies identifying its role in cellular cholesterol transport through the sortillin protein.(Musunuru et al. 2010b) Whether other risk loci also associate with intermediate phenotypes representing traditional risk factors is not clearly described.

We therefore systematically investigated the association between variants at eight CHD risk loci, whose mechanisms of action are currently unknown, with

intermediate phenotypes representative of traditional risk factors for CVD including fasting lipid and glucose levels; measures of obesity and inflammation and brachial blood pressure and heart rate. We examined these associations first in a Caucasian population consisting of both healthy and general population subjects without CAD and then attempted to replicate positive findings in a larger cohort of CAD subjects. We hypothesized that some or all of these markers would demonstrate association with these intermediate phenotypes thereby providing mechanistic insights into their functional roles.

Methods

Subjects

For the primary analysis we examined subjects from two populations without CAD to reduce confounding from association with CAD itself as well as with therapies which modulate the phenotypes of interest. Group 1 was derived from a healthy sample of subjects free of CVD risk factors enrolled in the Predictive Medicine (PreMed) study.(Patel et al. 2011a), while group 2 was derived from the Morehouse and Emory Team up to Eliminate Health Disparities (META-Health) study, which is a cross-sectional survey of residents in the Atlanta metropolitan area who were invited for assessment of their blood pressure, vascular health, biomarkers and anthropometric measures, in addition to a series of health questionnaires.(Amyre Morris et al. 2011). Full details about study criteria, design and definitions for these two cohorts are described in chapter 2. To maximize power we pooled the healthy subjects from the PreMed study and general population subjects (without CAD) from the META-Health study. The total sample size consisted of 403 subjects.

We utilized the Genebank population of CAD subjects as a replication cohort to examine significant associations in those with CAD. Full details of this population are again to be found in chapter 2. Patients were sampled consecutively from each study and all studies were approved by the Emory University or Morehouse Institutional Review Boards, and informed consent was obtained from all subjects.

Intermediate Phenotypes

The phenotypes studied in this analysis included (1) total cholesterol; LDL; HDL; TG and glucose (2) BMI, waist, weight, serum leptin and CRP (3) systolic BP; diastolic BP; mean BP; pulse pressure and heart rate. Full details on collection and ascertainment of these phenotypes are available in chapter 2. In the replication cohort, phenotypes on body fat composition were unavailable.

SNP selection and Genotyping

A review of published genome wide association studies identified variants at 16 loci associating with CAD and/or MI through June 2009 which had met a GWAS threshold of $5x10^{-8}$ in discovery studies (for 1 million tests of association). Of these, 3 did not replicate in larger follow up studies (2q36, 15q22, 6q25).(Samani et al. 2009) Another 4 loci have potential functional relevance to lipid metabolism (1p13 –SORT1; 19p13 – LDLR; 1p32 – PCSK9; 6q26- LpA) and were therefore not included in this study, the purpose of which was to examine those loci without a defined mechanism. (Samani et al. 2008a; Clarke et al. 2009; Kathiresan et al. 2009b) One SNP at 12q24.3 was not included due to its small effect size in the discovery study.(Erdmann et al. 2009) Lead single nucleotide polymorphisms (SNPs) at the remaining 8 loci of interest, which are independent of traditional risk factors, were thus genotyped and are listed in Table 15. Genotyping methods for all three cohorts are given in methods chapter 2.

Statistical methods

All continuous variables are described as mean ± standard deviation, while categorical variables are presented as proportions. The distribution of all variables was tested for normality and only plasma triglyceride; CRP and leptin levels required log transformation for parametric analysis. All SNPs were tested for Hardy-Weinberg equilibrium using PLINK.(Purcell et al. 2007) Association tests for quantitative traits were performed using linear regression, assuming an additive model of inheritance.

We tested genotypes for association with each intermediate phenotype in the pooled group of PreMed and META-health subjects to increase statistical power and

reduce type 2 error. We then examined all nominal positive associations ($p<0.1$) in the Genebank cohort in an attempt to replicate findings. Two tailed P values of <0.05 were considered statistically significant. All analyses were performed using PLINK(Purcell et al. 2007) and SPSS v17.0 statistical package (SPSS, Illinois).

Results

Patient characteristics for the Caucasian subjects in the pooled cohort consisting of PreMed and META-health subjects is presented in Table 14 (n=403). These subjects included overall 45% males and had a mean age of 55. None of the subjects had a documented MI or known coronary artery disease. The characteristics of the Genebank cohort included in this study are also presented in Table 14 (n=2442). These subjects were derived from a CAD population and were expected to have significantly greater risk factors. The distribution of genotypes and allele frequencies in each group for the 8 risk variants were in Hardy-Weinberg equilibrium, with allele frequencies similar to published data for Caucasians (Table 15).

Table 14: Subject characteristics for PMR, META-Health and Genebank (aim 2a)

	PMR (n=133)	***META (n=270)***	***Pooled (n=403)***	***GB (n=2442)***
Age	44.8 (14.0)	51.6 (9.27)	49.2 (11.6)	63.8 (11.3)
Male %	58.9	37.4	44.4	67.7
CHD,%	0	0	0	79.6
Smokers,%	0	13.3	8.9	15.3
Hyperlipidaemia,%	0	37.2	24.8	70.7
Total Chol, mg/dL	178.2 (26.8)	202.1 (40.7)	194.2 (38.3)	169.7 (43.5)
LDL, mg/dL	99.57 (21.5)	118.34 (32.4)	112.1 (30.5)	96.0 (35.3)
HDL, mg/dL	63.4 (17.2)	58.93 (18.9)	60.4 (18.5)	41.2 (12.7)
TG, mg/dL	76.1 (35.1)	134.2 (84.8)	114.4 (76.9)	168.4 (138.1)
Diabetes, %	0	5.5	3.7	30.1
Glucose, mg/dL	85.6 (8.1)	92.3 (23.0)	90.2 (19.7)	122.1 (46.3)
Hypertensive, %	0	31.5	21.1	68.3
Systolic BP, mmHg	115.6 (11.9)	118.2 (16.3)	117.3 (15.06)	136.6 (22.8)
Diastolic BP, mmHg	68.1 (9.26)	76.1 (10.5)	73.5 (10.8)	75.7 (11.9)
MAP, mmHg	83.9 (9.1)	90.1 (11.5)	88.1 (11.7)	95.9 (13.5)
PP, mmHg	47.5 (9.8)	42.1 (11.5)	43.8 (11.3)	60.9 (19.6)
Heart Rate, bpm	60.5 (9.60)	61.0 (9.64)	59.2 (9.7)	72.2 (14.3)
Waist, cm	79.1 (9.3)	96.3 (16.7)	91.5 (16.9)	n/a
Weight, kg	69.2 (11.8)	81.6 (21.9)	77.6 (20.1)	88.5 (20.2)
BMI, kg/m2	23.1 (2.5)	28.1 (6.4)	26.5 (5.9)	29.4 (6.1)
CRP, mg/L	1.17 (3.17)	2.74 (3.74)	2.23 (3.64)	4.0 (16.5)*
Leptin, pg/ml	7698 (9753)	28702 (33191)	21607 (29315)	n/a

**Only in a small subset (n=160); % proportions or mean (SD) are given unless stated*

CHD Coronary Heart Disease; MAP mean arterial pressure; PP pulse pressure; BMI body mass index; CRP C reactive protein; BP blood pressure; TG triglycerides; LDL low density lipoprotein; HDL high density lipoprotein

Table 15: Details of CHD risk variants genotyped in PreMed and META-Health

					PreMed		META-Health	
Locus	SNP	Nearest Gene	Risk allele	MAF reported	MAF observed PreMed	HWE *P* value	MAF observed META	HWE P Value
1q41	rs17465637	MIA3	C	0.28	0.44	0.47	0.42	0.08
2q33	rs6725887	WDR12	C	0.14	0.07	0.51	0.10	0.51
3q22.3	rs9818870	MRAS	T	0.15	0.14	0.26	0.14	0.98
6p24	rs12526453	PHACTR1	C	0.35	0.24	0.81	0.27	0.22
9p21	rs1333049	CDKN2A/B	G	0.50	0.35	0.41	0.41	0.45
10q11	rs1746048	CXCL12	C	0.16	0.31	0.41	0.25	0.25
12q24	Rs653178	SH2B3	T	0.47	0.24	0.07	0.36	0.1
21q22	rs9982601	SLC5A3-MRPS6-KCNE2	T	0.13	0.16	0.75	0.15	0.64

Lead variants at each risk locus as identified in original genome wide association studies are listed, with their risk alleles and minor allele frequencies. SNP = Single Nucleotide Polymorphism; MAF = Minor Allele Frequency; HWE = Hardy Weinberg Equilibrium

Association analyses

Association between the 8 risk variants and lipid and glucose parameters revealed no significant association between total cholesterol, LDL or HDL levels with any of the variants (Table 16). There was a modest association between the variant at 1q41 (rs17465637) and total triglyceride levels (log transformed). Under an additive model each risk allele at this locus increased triglyceride levels by 1.1 mg/l (estimated by reverse log transformation). There was also a modest association between the variant at 12q24 with fasting plasma glucose levels, such that each copy of the risk allele was associated with an increase of 3.3mg/l under an additive model.

For the second category of phenotypes consisting of measures of obesity and inflammation, the most striking observation was the series of associations between 12q24 and waist, weight, BMI and plasma leptin levels. For each risk allele waist was increased by 2.5cm, weight by 2.7kg and BMI by 1 point. Other nominal associations noted in this group included 6p24 with weight; 1q41 and 10q11 with serum leptin; 1q41, 6p24 and 10q11 with BMI. No associations were noted with any of the 8 markers and plasma CRP (Table 17).

Finally for the group of phenotypes representing blood pressure and heart rate, several variants were associated with resting heart rate including those at 1q41, 3q22.3, 9p21, 10q11 and 12q24. The variants at 10q11 and 12q24 were also associated with diastolic blood pressure and 1q41 with pulse pressure. There were no associations noted for MAP or systolic BP (Table 18).

Taken together the findings for two loci appeared to stand out. There were trends towards association for 1q41 and 12q24 with features of the metabolic syndrome. The variant at 1q41 associated with plasma triglycerides, BMI and serum leptin levels while the variant at 12q24 appeared to show trends of association with glucose, waist, weight, BMI, serum leptin and diastolic blood pressure.

Table 16: Association between CHD risk variants and lipid/glucose phenotypes

	Lipids and Glucose PMR META				
Risk Locus	*TC*	*TG*	*LDL*	*HDL*	*Glucose*
1q41	2.71 (2.60), 0.30	**0.03 (0.02), 0.04**	1.66 (2.11), 0.43	-0.82 (1.26), 0.52	1.56 (1.36), 0.25
2q33	-3.95 (4.60), 0.40	-0.04 (0.07), 0.54	-3.51 (3.70), 0.34	1.95 (2.20), 0.39	1.51 (2.47), 0.54
3q22.3	-1.20 (3.90), 0.78	-0.01 (0.05), 0.87	-0.60 (3.11), 0.84	-0.25 (1.90), 0.89	-1.28 (2.11), 0.54
6p24	0.85 (3.00), 0.78	-0.02 (0.04), 0.53	0.70 (2.40), 0.77	2.30 (1.45), 0.11	0.63 (1.59), 0.69
9p21	3.18 (2.80), 0.26	0.05 (0.04), 0.18	1.35 (2.27), 0.55	0.56 (1.37), 0.68	1.23 (1.40), 0.40
10q11	0.31 (2.90), 0.92	0.05 (0.04), 0.28	0.05 (2.36), 0.98	-1.06 (1.45), 0.47	1.63 (1.59), 0.30
12q24	3.16 (2.60), 0.23	0.04 (0.04), 0.27	2.61 (2.10), 0.22	0.02 (1.29), 0.98	**3.29 (1.40), 0.02**
21q22	0.16 (3.70), 0.97	-0.01 (0.05), 0.99	-0.04 (2.90), 0.99	0.64 (1.80), 0.72	0.84 (1.95), 0.67

Beta (SE), p values are given for each association test

TC - Total Cholesterol; TG – triglycerides; LDL – low density lipoprotein; HDL – high density lipoprotein

Table 17: Association between CHD risk variants and obesity/inflammation

	Obesity and Inflammation Phenotypes				
Risk Locus	*Waist*	*Weight*	*Log(leptin)*	*Log(CRP)*	*BMI*
1q41	1.40 (1.21), 0.24	1.72 (1.36), 0.21	**0.23 (0.09), 0.02**	0.02 (0.04), 0.60	**0.82 (0.40), 0.04**
2q33	1.22 (2.10), 0.56	0.43 (2.39), 0.86	-0.21 (0.17), 0.24	0.05 (0.08), 0.52	-0.06 (0.73), 0.93
3q22.3	-0.83 (1.81), 0.65	-0.70 (2.07), 0.73	0.09 (0.14), 0.49	0.05 (0.07), 0.45	-0.11 (0.63), 0.86
6p24	-0.30 (1.37), 0.83	**-3.42 (1.58), 0.03**	-0.03 (0.11), 0.79	0.01(0.05), 0.87	**-0.80 (0.48), 0.07**
9p21	0.80 (1.27), 0.51	1.62(1.45), 0.26	0.10 (0.10), 0.34	0.02 (0.05), 0.74	0.59 (0.44), 0.18
10q11	1.48 (1.35), 0.27	2.16(1.58), 0.17	**0.19 (0.11), 0.09**	-0.01 (0.05), 0.99	**0.95 (0.46), 0.04**
12q24	**2.51 (1.20), 0.04**	**2.67 (1.40), 0.06**	**0.25 (0.09), <0.01**	0.03 (0.04), 0.49	**1.13 (0.41), 0.01**
21q22	1.00 (1.69), 0.55	-1.66 (1.97), 0.40	-0.01 (0.13), 0.92	-0.01 (0.06), 0.79	0.10 (0.59), 0.86

Beta (SE), p values are given for each association test

CRP – C reactive protein; BMI – body mass index

Table 18: Association between CHD risk variants and BP and HR phenotypes

	Blood Pressure and Heart Rate Phenotypes				
Risk Locus	*sBP*	*dBP*	*PP*	*MAP*	*HR*
1q41	**-**0.64 (1.00), 0.52	0.78 (0.73), 0.39	**-1.40 (0.74), 0.08**	0.26 (0.76), 0.73	**1.53 (0.66), 0.02**
2q33	0.13 (1.78), 0.94	0.81 (1.29), 0.53	-0.60 (1.33), 0.63	0.70 (1.34), 0.60	0.22 (1.16), 0.84
3q22.3	-1.80 (1.50), 0.22	-0.45 (1.09), 0.68	-1.34 (1.12), 0.23	-0.90 (1.14), 0.41	**2.86 (0.98), <0.01**
6p24	0.55 (1.16), 0.63	0.31(0.84), 0.71	0.19 (0.87), 0.83	0.45 (0.87), 0.61	-0.36 (0.76), 0.64
9p21	0.38 (1.09), 0.72	0.68 (0.79), 0.39	-0.30 (0.81), 0.71	0.50 (0.82), 0.54	**1.61 (0.70), 0.02**
10q11	0.45 (1.15), 0.69	**1.73 (0.82), 0.04**	-1.23 (0.85), 0.15	1.27(0.86), 0.14	**1.67 (0.76), 0.03**
12q24	0.07 (1.01), 0.94	**1.29 (0.73), 0.08**	1.09 (0.75), 0.15	0.83 (0.77), 0.27	**1.30 (0.67), 0.04**
21q22	0.93 (1.44), 0.51	-0.02 (1.05), 0.98	0.85(1.08), 0.43	0.27 (1.09), 0.80	1.28 (0.94), 0.19

Beta (SE), p values are given for each association test

sBP – systolic blood pressure; dBP – diastolic blood pressure; PP – pulse pressure; MAP –mean arterial pressure; HR – heart rate

Replication analysis

Given the use of multiple testing and the likelihood of false positive associations we attempted to replicate these signals in a larger population consisting of patients undergoing investigation or treatment for CAD. Of the positive signals noted in the PreMed/META-Health group however, none were replicated successfully in the Genebank cohort. Only the association with heart rate for 12q24 showed a modest non significant association (p=0.07) (Table 19). In fact there were no new significant associations noted in the Genebank cohort when all variants were tested with all phenotypes to minimize a type 2 error in the PreMed/META-Health analysis (data not shown).

Table 19: Replication of nominal associations in the Genebank cohort (aim 2a)

Locus	*Phenotype*	*Beta (SE), p value*
1q41	Triglycerides	0.02 (0.02), 0.31
1q41	Body Mass Index	0.29 (0.19),0.12
1q41	Pulse Pressure	0.66 (1.01), 0.51
1q41	Heart Rate	0.57 (0.79), 0.46
3q22.3	Heart Rate	-0.86 (0.93, 0.35
6p24	Weight	-0.28 (0.64), 0.66
6p24	Body Mass Index	-0.07 (0.18), 0.69
9p21	Heart Rate	0.57 (0.74), 0.44
10q11	Body Mass Index	0.43 (0.26),0.10
10q11	Diastolic Blood Pressure	0.56 (0.86), 0.51
10q11	Heart Rate	-0.20 (1.00), 0.87
12q24	Glucose	0.87 (1.36), 0.52
12q24	Weight	-0.41 (0.58), 0.48
12q24	Body Mass Index	0.09 (0.19),0.61
12q24	Diastolic Blood Pressure	0.26 (0.63), 0.68
12q24	Heart Rate	1.42 (0.79), 0.07

Beta (SE), p values are given for each association test; Serum leptin not available in Genebank

Discussion

In this study we have demonstrated that the 8 validated CHD variants without known mechanisms of risk do not appear to convincingly associate with quantitative intermediate phenotypes representative of traditional risk factors. These findings are in keeping with other studies which reported no significant association between these variants and traditional risk factors although suggestive trends for at least one SNP may warrant further attention.

Genotype-phenotype associations were initially tested in a group without CAD in order to minimize potential confounding from the disease-association itself and from medical interventions which modulate for example lipids and blood pressure. In this group, despite some subjects taking cardiovascular medications, findings suggested nominal associations between 1q41 and triglycerides, body mass index and serum leptin and for 12q24 with glucose, weight, waist, BMI, diastolic blood pressure and serum leptin. There was thus a suggestion that these loci may influence some feature common to the metabolic syndrome as a mechanism of risk. The association with several physiologically related phenotypes suggests that these associations warrant further study and replication.

Rs17465637at the 1q41 locus has been identified through two separate GWAS (Samani et al. 2007; Kathiresan et al. 2009a)and confirmed in a further 3 studies as associating with CHD.(Hiura et al. 2008; Koch et al. 2011; Wang et al. 2011a) Only one study failed to find an association with the same variant but instead reported another variant rs3008621in a separate haplotype block but also in the 1q41 region.(Samani et al. 2009) Indeed in our own Genebank data, (described in chapter 3; study 2) we have shown that the 1q41 locus associates significantly with CAD and CAD severity and that atherosclerosis may be its underlying phenotype. We noted that the association was modest and weakened after adjustment for risk factors which in itself suggests a possible interaction with risk factors. In this study, the association with TG, BMI and leptin suggests a strong link to the metabolic syndrome. Interestingly another recently published study also found a modest association with CAD while also finding an association with HDL. (Xie et al. 2011)

The variant is located in the intronic region of the melanoma inhibitory activity family, member 3 (MIA3) gene (also known as TANGO). The MIA3 gene, coding for a 14 kDa protein of so far unknown function, was originally identified as a new member of

MIA gene family. Experimental evidence suggested that MIA3 reduced the attachment to fibrinogen or other cell adhesion molecules such as ICMA-1 and human micro-vascular endothelial cells (HMECS).(Arndt et al. 2007) This process is fundamental for the formation and progression of atherosclerotic plaque and also for plaque instability. Whether other functions of MIA3 contribute to metabolic disturbances remain to be identified.

A second promising finding was the association of the SNP at 12q24 with glucose, weight, waist, BMI, diastolic blood pressure and serum leptin. This SNP was identified in a GWAS of blood counts, associating with eosinophil count but also with MI.(Gudbjartsson et al. 2009) Two SNPs in complete LD with similar effects were reported as rs3184504 a non-synonymous SNP (R262W) in exon 3 of SH2B3 (also known as LNK) and rs653178 which lies in intron 1 of the *ATXN2* gene which lies adjacent to the *SH2B3* gene. Interestingly, rs3184504 has previously been shown to associate with type 1 diabetes through genome wide association.(Todd et al. 2007) Although in the discovery study a trend toward risk for hypertension was observed for rs3184504 no association was observed between rs3184504 and other traditional risk factors for myocardial infarction including high-density lipoprotein, low-density lipoprotein, type 2 diabetes and smoking initiation.(Gudbjartsson et al. 2009) However no other formal reports, to our knowledge, have examined the association between this locus and more detailed intermediate phenotypes as in this study.

SH2B3 is a member of the APS family of adaptor proteins and acts as a broad inhibitor of growth factor and cytokine signalling pathways. *SH2B3* encodes the T-cell adapter protein Lnk, which regulates T-cell receptor-, growth factor- and cytokine receptor-mediated signalling. It has been shown to be a negative regulator of tumour necrosis factor α signalling in human endothelial cells.(Fitau et al. 2006) SH2B3 is expressed in human vascular endothelial cells, where it promotes inflammation and so this could represent a potential mechanism by which variants could contribute to the progression of plaques in coronary arteries leading to MI.

However, despite these suggestive associations and plausible mechanistic explanations, we were unable to replicate both of these finding in the larger Genebank replication cohort. We did not have the phenotypes of waist and serum leptin in this cohort and so these associations remain to be confirmed. Several reasons may exist why both the 1q41 and 12q24 findings were not replicated in the larger Genebank cohort. These include the fact that the Genebank CAD subjects (1) had a significantly

different risk factor profile including diabetes and smoking (2) were older and genetic effects may have been attenuated by a greater lifetime exposure to environmental influences such as diet (3) most were taking medications to control risk factors such as statins and (4) were a selected group with inherent biases.

Alternatively, these associations may simply be weak and are epiphenomenon, a conclusion which would be in agreement with other published studies. These suggested that the association between novel variants and CHD was not affected by traditional risk factors and further sub-analyses did not show an effect on the intermediate phenotypes themselves. Hingorani et al, have recently attempted a comprehensive analysis of intermediate phenotypes with GWAs discovered variants.(Angelakopoulou et al. 2011) In a cohort of approximately 25000 they examined CHD and lipid and diabetes SNPs. They did not find any associations for 1q41, which is in keeping with published data and our negative replication study. However, they only included SNPs discovered up until 2008 and therefore only included 2 of the 8 SNPs (9p21 and 1q41) in this study making direct comparison difficult. Importantly, this study did not include 12q24. While the discovery study for 12 q24 reported no association with traditional risk factors they did not examine the full breadth of quantitative intermediate phenotypes and so this particular variant may still warrant further investigation.

Limitations:

There were several limitations of this study. First, our primary analysis group was relatively small including only 400 subjects and it is possible that we may have missed some associations (type 2 error), although association analysis in the larger Genebank group did not identify any new associations in a group 8 times larger. Although it is still possible we may have been underpowered to detect very small effect sizes, the overall the lack of association is consistent with published literature. Secondly, we tested multiple variants and phenotypes and the risk of a type 1 error was large. However, our use of a replication cohort ameliorated this risk and although we did not use Bonferroni adjustment we had planned to do so if we had identified significant associations. Other limitations include absence of serum leptin and waist measurements in the Genebank cohort which meant we were unable to replicate the association of 12q24 in this cohort and so those associations' remains plausible. Finally, we only included 8 variants and did not re-examine associations with lipid parameters

for the 4 lipid variants. However, our intention was to try and identify mechanisms for unknown variants and so this was not performed.

Conclusions

In conclusion, this study has demonstrated that the 8 risk variants without known mechanisms of risk do not associate with quantitative measures of intermediate phenotypes representative of traditional risk factors. Although one variant may warrant further investigation due to its association with a breadth of related phenotypes, this study on the whole confirms the negative findings from the literature. It is therefore likely that hitherto unknown mechanisms account for CHD risk from these variants, raising the possibility of novel therapeutic discoveries and molecular understanding.

Study 2: A novel genetic variant associating with myocardial infarction in the PHACTR1 gene is associated with impaired central haemodynamic indices

Summary:

Given that the 8 CHD risk variants do not associate with traditional risk factors we sought to examine if they could be associated with a novel risk pathology for CHD, that of arterial stiffness and central pressure augmentation through arterial pressure wave reflections. Of the 8 GWAS variants examined, only one at 6p24 was associated with the tested central haemodynamic indices in two separate populations. This variant resides is in the PHACTR1 gene and may have a role in modulating arterial elasticity/ wave reflections.

Introduction:

Heritable factors play a key role in the development of CHD.(Damani and Topol 2007) Early unbiased genome wide approaches have led to the identification of more than a dozen novel risk loci for myocardial infarction (MI) in predominantly Caucasian cohorts.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; WTCC 2007; Erdmann et al. 2009; Gudbjartsson et al. 2009; Kathiresan et al. 2009a) Many loci have replicated in large cohorts, yet of these only four appear to have a mechanistically relevant basis through lipid metabolism pathways (1p13, 1p32, 19p13, 6q26).(Samani et al. 2008a; Kathiresan et al. 2009b) The remaining loci span genomic regions that as yet have no clearly defined mechanism to explain MI risk.

Valuable insights into the functions of these variants may be learned by studying their effects on related or intermediate phenotypes which may be causal in the pathway to developing MI. This is best illustrated by the initial association of 1p13 with MI and then with the intermediate phenotype of lipid levels, with subsequent functional studies identifying its role in cellular cholesterol transport through the sortillin protein.(Musunuru et al. 2010b) Another intermediate phenotype of interest in vascular disease is abnormal pressure wave reflection within the arterial tree. Abnormalities in these arterial tree properties occur due to complex composite changes in parameters such as arterial stiffness and vasomotor tone, ultimately leading to increased left

ventricular after-load(Toprak et al. 2009) and cardiovascular events such as myocardial ischaemia,(Ohtsuka et al. 1994) MI,(Laurent and Boutouyrie 2005) and stroke.(Laurent and Boutouyrie 2007) and have been confirmed as significant risk factors in a recent meta-analysis.(Vlachopoulos et al. 2010a)

We therefore investigated the association of variants at eight MI risk loci, whose mechanisms of action are currently unknown, with non-invasively derived pulse wave analysis indices including the magnitude and timing of reflected arterial pressure waveforms in two separate Caucasian populations. The first consisting of very healthy subjects, free of confounding risk factors in order to enable an assessment of direct genetic risk free from confounding and the second, a community-based population that allowed determination of genetic effects in the context of common risk factors. We hypothesized that some or all of these markers would demonstrate association with indices of adverse arterial wave reflection.

Methods

Subjects

Two populations without known CAD were studied (Table 20). Group 1 was derived from a healthy sample of subjects free of CVD risk factors enrolled in the Predictive Medicine (PreMed) study.(Patel et al. 2011a) This group was chosen to minimize confounding from CAD, medication usage and other risk factors. Healthy non-smoking, normal weight (Body Mass Index (BMI) <26 kg/m^2) volunteers aged 20-70 years were recruited after careful screening for absence of risk factors including hypertension (SBP<130 or DBP<90 mmHg x3), hyperlipidaemia (total cholesterol <200 mg/dL, LDL <120 mg/dL), and impaired fasting glucose or diabetes (fasting glucose <100 mg/dL). Further details are available in chapter 2. A total of 133 eligible Caucasian subjects underwent vascular studies and blood draws for genotyping.

Group 2 was derived from the Morehouse and Emory Team up to Eliminate Health Disparities (META-Health) study, which is a cross-sectional survey of residents in the Atlanta metropolitan area who were invited for assessment of their blood pressure, vascular health, biomarkers and anthropometric measures, in addition to a series of health questionnaires. Full details have been published previously.(Amyre Morris et al. 2011) and details are also available in chapter 2. History of diabetes, hypertension,

cardiovascular disease (CVD; MI or stroke) and smoking status were defined by participant self report. Subjects of self-reported non-Caucasian race or with documented CVD were excluded, allowing inclusion of 270 Caucasian subjects.

Patients were sampled consecutively from each study, both of which were approved by the Emory University or Morehouse Institutional Review Boards, and informed consent was obtained from all subjects.

Central Haemodynamic Indices

All indices were estimated using non invasive Sphygmocor© technology (Atcor Medical, Australia) in a quiet temperature controlled room after an overnight fast. Pulse Wave Analysis was performed by recording sequential high quality pressure waveforms at the radial artery using a highly sensitive tonometer as described in chapter 2. The device applies a proprietary transfer function to this peripheral measurement to estimate central (proximal) aortic pressure waveforms. This permits estimation of central (aortic) pressures, pressure augmentation secondary to wave reflections (augmented pressure, AP) and the augmentation index (augmented pressure/total central pulse pressure, AIx), which is widely considered to be a complex composite measure of wave reflection properties of the arterial tree. Due to its sensitivity to heart rate, a standardized value to 75bpm was used for the purposes of this study as advocated by Wilkinson et al.(Wilkinson et al. 2000) Time to Reflected Wave (TrW) is an indirect measure of stiffness derived from analysis of the pressure wave form and is a measure of the time taken (in ms) for the reflected wave to return from the periphery and merge with the incident wave, where a shorter time indicates faster wave travel, and thus greater arterial stiffness.

Quality control indices were evaluated at the time of study and non acceptable readings discarded and repeated. Three readings were taken for each subject and averaged for analysis. Reproducibility studies in our laboratory on 9 subjects on consecutive days, by the same operators performing these studies, have demonstrated a coefficient of variation of 20.3% and 2.2% for AIx and TrW respectively. Operators were blinded to genotype results.

SNP selection and Genotyping

SNP selection and genotyping procedures have been described in chapter 2. As described in the previous studies, lead single nucleotide polymorphisms (SNPs), defined by the original GWAS reports, at the 8 loci of interest, which are independent of traditional risk factors and have no known mechanism, were thus genotyped. Details of the genotyped variants and their Hardy Weinberg distributions are given in Table 15 in the previous study.

Statistical methods

All continuous variables are described as mean ± standard deviation, while categorical variables are presented as proportions. The distribution of all variables was tested for normality and none required prior transformation. All SNPs were tested for Hardy-Weinberg equilibrium using PLINK.(Purcell et al. 2007) Association tests for quantitative traits were performed using linear regression, assuming an additive model of inheritance. We tested genotypes for association with the arterial indices separately in each subject group and then in the total combined group to increase statistical power and reduce type 2 error. Bonferroni adjustment for 8 genotypes and 5 phenotypes (p value x 13) was performed in the pooled analyses, as well as adjustment for age, mean arterial pressure (MAP) and height in model 1 which are major determinants of PWA indices, in addition to gender, glucose, total cholesterol, BMI and smoking status in model 2.

Two tailed P values of <0.05 were considered significant. All analyses were performed using PLINK(Purcell et al. 2007) and SPSS v17.0 statistical package (SPSS, Illinois).

Power calculations

We estimated the effect sizes that would be observed at a fixed power of 80% and an alpha of 0.05. Varying the risk allele frequency from the least frequent to most frequent, 0.10 to 0.45, we estimated that under an additive model our pooled sample

size (n=403) would allow us to identify effect sizes per risk allele of 2.7 to 4.4% for AIx; 1.0 to 1.7mmHg for AP and 3.5 to 5.5ms for TrW.

Results

Patient characteristics for the Caucasian subjects in PreMed (n=133) who were healthy and free of all cardiovascular risk factors and for those in META-Health (n=270) are presented in the previous section in Table 14, along with values for the pooled population. Compared to PreMed, subjects in META-Health were older, more likely to be female and almost a third had either hypertension and/or hyperlipidaemia, 5% were diabetic, and 13% were current smokers. As expected the central haemodynamic indices were more impaired for subjects in META-Health compared to those in PreMed (all $p<0.01$) (Table 20). The distribution of genotypes and allele frequencies in each group for the 8 risk variants were in Hardy-Weinberg equilibrium, with allele frequencies similar to published data for Caucasians and are also presented in the previous section (Table 15).

Association with vascular traits

We analyzed the association of these variants with the main quantitative vascular traits, AIx and AP (both standardized to a heart rate of 75bpm, AIx/AP), time to reflected wave (TrW), and central systolic and diastolic BP in each study population separately and then combined. The association findings for AIx, AP and TrW are presented in Tables 21-23.

Table 20: Central haemodynamic indices for PMR and META-Health

	PreMed ***Healthy Subjects*** ***n =133***	***META-Health*** ***Community*** ***n = 270***	***Pooled Cohort*** ***n= 403***
Pulse Wave Analysis Indices			
Augmented Pressure, mmHg	2.9 (5.4)	7.0 (4.6)	5.6 (4.9)
Augmentation Index, %	8.5 (14.9)	20.7 (11.3)	16.5 (13.6)
Time to Reflected Wave, ms	155.3 (20.5)	144.0 (16.3)	148.6 (18.0)
Central SBP, mmHg	102.4 (13.2)	110.9 (15.6)	108.0 (15.5)
Central DBP, mmHg	70.0 (9.7)	76.7 (10.8)	(74.5 (10.9)

Mean (SD) and % are shown. Augmented Pressure and Index values are heart rate adjusted

(1) PreMed

The most prominent finding in the PreMed group was the association of the variant at 6p24 with 3 of the 5 studied central haemodynamic parameters (Tables 21-23). The MI risk allele (C) was associated with worse (higher) AIx ($p<0.001$), (higher) AP ($p=0.001$) and (lower) TrW ($p=0.023$), but not central blood pressure (systolic $p = 0.36$, diastolic $p=0.42$). The strongest association was with AIx for which each copy of the risk allele increased the AIx by nearly 7% (Beta 6.88, $p<0.001$) (Table 21)

Additionally the variant at the 21q22 locus had significant association with AIx ($p=0.002$), AP ($p=0.024$) and TrW ($p=0.037$) but not central blood pressure, while the variant at 12q24 showed a borderline trend towards association with AIx ($p=0.06$) and AP (p-0.09) (Tables 21-23).

(2) META-Health

Of the 8 SNPs tested in this population, only the 6p24 variant showed significant association with AIx ($p=0.005$), AP ($p=0.049$), TrW ($p=0.013$) (Tables 21-23). Again, this association was not significant for central systolic ($p= 0.37$) or diastolic blood pressure ($p= 0.45$). Neither the 12q24 nor 21q22 loci which had demonstrated modest

association in the PreMed cohort, nor any other loci showed any significant association with any phenotypes in this cohort.

(3) *Combined analysis*

To minimize a type 2 error we combined the two cohorts for a final analysis. The pooled cohort of 403 subjects showed a significant association between the 6p24 variant and AIx (p=0.001), AP (p=0.005), TrW (p=0.002) (Tables 21-23). The risk allele C conferred a 3.5 % increase in AIx per risk allele. This equates to a mean AIx value of 18.4% in the homozygote risk group compared to 12.7% in the referent non risk GG group (Figure 16). The association remained significant after adjustment for multiple testing for 8 variants and 5 phenotypes (Table 21-23). Furthermore, after adjustment for age, mean arterial pressure and height, which are the strongest determinants of these parameters (model 1), the association persisted for AIx and TrW but was weakened for AP (Table 21-23). After additional adjustment for model 1 parameters and other risk factors which could influence these indices, including gender, glucose, total cholesterol, BMI and smoking (model 2), only AIx remained significantly associated with the marker at 6p24, while the association was attenuated to borderline for TrW and non-significance for AP (Tables 21-23). Finally, we did not observe significant interactions between the 6p24 marker and the tested covariates (data not shown).

Of the remaining variants tested for association in the larger pooled cohort (n=403) no further significant associations were identified with any phenotype (Tables 21-23).

Table 21: Association between CHD risk variants and Augmentation Index

Augmentation Index (AIx)									
	PreMeD (n=133)		META-Health (n=270)		Pooled (n=403)		P values		
Risk Locus	Beta (SE)	p	Beta (SE)	p	Beta (SE)	p	Bonferroni*	Model 1	Model 2
1q41	-0.17 (1.8)	0.93	-1.3 (0.9)	0.16	0.50 (0.9)	0.59			
2q33	-4.91 (3.4)	0.15	-0.95 (1.5)	0.53	-0.99 (1.6)	0.54			
3q22.3	1.09 (2.6)	0.68	0.13 (1.4)	0.93	0.37 (1.4)	0.80			
6p24	6.88 (2.0)	<0.001	2.80 (1.0)	0.005	3.50 (1.1)	0.001	0.013	0.026	0.030
9p21	1.40 (1.9)	0.45	-0.52 (1.0)	0.60	0.80 (1.0)	0.30			
10q11	1.63 (1.9)	0.40	-0.58 (1.1)	0.59	1.0 (1.0)	0.35			
12q24	-3.90 (2.0)	0.05	1.00 (0.9)	0.25	1.41 (1.0)	0.14			
21q22	7.30 (2.3)	0.002	1.40 (1.3)	0.31	2.66 (1.3)	0.04	0.52		

*The beta (B) coefficients are presented in the direction of the effect of the risk allele (i.e., a positive value indicates a higher level in carriers of the risk allele). * Bonferroni adjusted for 8 variants + 5 phenotypes. Model 1 adjusted for age, MAP, height; Model 2 adjusted for model 1 + gender, cholesterol, glucose, BMI and smoking*

Table 22: Association between CHD risk variants and Augmented Pressure

Augmented Pressure (AP)									
	PreMeD (n=133)		META-Health (n=270)		Pooled (n=403)		P values		
Risk Locus	Beta (SE)	p	Beta (SE)	p	Beta (SE)	p	Bonferroni*	Model 1	Model 2
1q41	-0.33 (0.6)	0.61	-0.56 (0.4)	0.12	-0.01 (0.3)	0.98			
2q33	-1.41 (1.2)	0.25	-0.51 (0.6)	0.41	-0.43(0.6)	0.47			
3q22.3	0.36 (0.1)	0.71	-0.16 (0.6)	0.78	0.06 (0.5)	0.90			
6p24	2.30 (0.7)	0.001	0.80 (0.4)	0.049	1.10 (0.4)	0.005	0.06	0.09	0.13
9p21	0.68 (0.7)	0.30	-0.10 (0.4)	0.83	0.39 (0.4)	0.26			
10q11	0.40 (0.7)	0.54	-0.27 (0.4)	0.54	0.22 (0.4)	0.57			
12q24	-1.20 (0.7)	0.09	-0.36 (0.4)	0.32	0.39 (0.4)	0.23			
21q22	1.95 (0.9)	0.024	0.87 (0.5)	0.10	1.05 (0.5)	0.03	0.39		

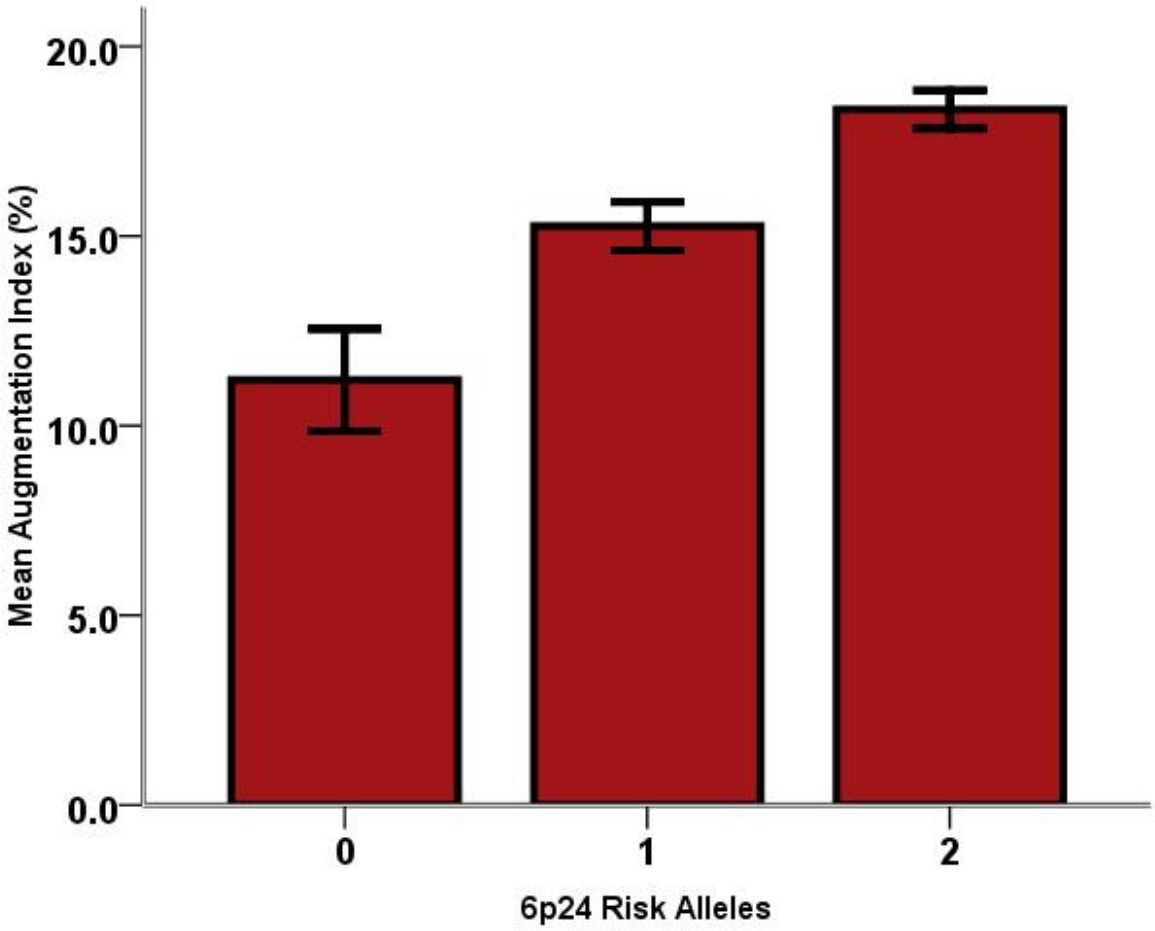
*The beta (B) coefficients are presented in the direction of the effect of the risk allele (i.e., a positive value indicates a higher level in carriers of the risk allele). * Bonferroni adjusted for 8 variants + 5 phenotypes. Model 1 adjusted for age, MAP, height; Model 2 adjusted for model 1 + gender, cholesterol, glucose, BMI and smoking*

Table 23: Association between CHD risk variants and Time to Reflected Wave

Time to Reflected Wave (TrW)									
	PreMED (n=133)		**META-Health (n=270)**		**Pooled (n=403)**		**P Values**		
Risk Locus	Beta (SE)	p	Beta (SE)	p	Beta (SE)	p	Bonferroni*	Model 1	Model 2
1q41	-3.61 (2.5)	0.14	1.20 (1.3)	0.37	-1.20 (1.2)	0.30			
2q33	5.45 (4.7)	0.25	2.40 (2.2)	0.28	2.4 (2.1)	0.26			
3q22.3	1.42 (3.5)	0.69	-1.56 (2.1)	0.45	-0.6 (1.9)	0.74			
6p24	-6.60 (2.9)	0.023	-3.70 (1.5)	0.013	-4.30 (1.4)	0.002	0.026	0.045	0.062
9p21	-0.60 (2.6)	0.82	-0.23 (1.4)	0.87	-0.89 (1.3)	0.50			
10q11	-3.90 (2.6)	0.15	0.95 (1.6)	0.55	-1.17 (1.4)	0.42			
12q24	2.20 (2.8)	0.43	-1.30 (1.3)	0.32	0.52 (1.3)	0.68			
21q22	-7.10 (3.3)	0.037	-3.30 (1.9)	0.10	-4.11 (1.7)	0.01	0.13		

*The beta (B) coefficients are presented in the direction of the effect of the risk allele (i.e., a positive value indicates a higher level in carriers of the risk allele). * Bonferroni adjusted for 8 variants + 5 phenotypes. Model 1 adjusted for age, MAP, height; Model 2 adjusted for model 1 + gender, cholesterol, glucose, BMI and smoking*

Figure 16: Mean augmentation index and 6p24 risk allele carriage



Mean augmentation index (HR 75 standardized) per risk allele at 6p24, represented by SNP rs12526453 (risk allele C). Pooled cohort (n=403). Values are adjusted for mean arterial blood pressure and age. SE bars shown.

Of the remaining variants tested for association in the larger pooled cohort (n=403) no further significant associations were identified (Tables 21-23).

Discussion

In order to explore potential mechanistic pathways of newly identified genetic variants associating with MI, whose function is currently unknown, we investigated their relationship with the intermediate phenotype of pulse wave analysis indices. We demonstrate that the MI risk locus at chromosome 6p24 in the PHACTR1 gene, identified through GWAS of early onset MI, is significantly associated with abnormal wave reflection properties of the arterial tree in two independent cohorts.

In this study, the risk allele (C) of the 6p24 variant (rs12526453) was associated with greater AIx, AP, and TrW in a healthy cohort free from confounding risk factors or medications, and replicated in a larger community population sample. The association with AIx appeared most robust, with homozygotes having an average value of 18.6% compared to the referent group mean of 12.7%. Augmentation index is considered a complex measure of wave reflections and elasticity of the muscular arteries, all of which could be considered as representing small vessel or systemic arterial stiffness. Furthermore, the time to reflected wave is also considered a surrogate of large artery stiffness.("Atcor Medical; Wave Reflection Tutorials" 2011) Importantly, abnormalities in central haemodynamic indices including AIx are powerful predictors of cardiovascular morbidity and mortality. A recent meta-analysis of population cohorts confirms that a 10% rise in AIx confers an approximate 30% increase in risk for all cause mortality.(Vlachopoulos et al. 2010a) Thus, the independent association of this 6p24 variant with several related central haemodynamic indices suggests that it may mediate risk of MI by modulating arterial elastic properties and pressure wave reflections.

Interestingly we did not observe any association with central systolic or diastolic blood pressure in either study cohort. This is perhaps not surprising, given that AIx has been shown to be considerably more heritable than blood pressure. For example in a study of twins, Snieder et al demonstrated a heritability of 37% for AIx, but only 13 to 25% for blood pressure traits, while others have found estimates as high as 62% for AIx.(Snieder et al. 2000; Cecelja et al. 2009) This is likely due to the fact that arterial elastic properties are just one determinant of blood pressure and genetic variants are more likely to explain greater variance in these upstream components than in the final composite phenotype.

While there were promising initial associations between at least two other SNPs at 12q24 and 21q22 with these parameters, none were successfully replicated in the second cohort with risk factors. Other variants did not associate significantly with any phenotype although this could be explained by lack of statistical power to detect much smaller effect sizes.

The rs12526543 SNP resides on the short arm of chromosome 6, in the un-translated portion of the PHACTR1 gene but is in LD with several neighboring SNPs within this gene (Figure 17). PHACTR1 codes for phosphatase and actin regulator 1, which is one of many regulating proteins for the protein phosphatase 1 (PP1) enzyme. (Allen et al. 2004) The latter is a ubiquitously expressed enzyme involved in a wide array of physiological processes, including gene expression, muscle contraction, and glycogen metabolism as well as being a critical negative regulator of Ca^{2+} cycling and contractility in smooth muscle cells and cardiomyocytes, which may be pertinent in the context of vasomotor tone, arterial elasticity and wave reflections.

Genetic polymorphisms have been widely studied and associated with indices of pressure wave reflection.(Yasmin et al. 2006; Lacolley et al. 2009) Our study is unique in (1) following the GWAS paradigm of looking backwards to identify causal pathways, rather than an a priori candidate gene hypothesis (2) for using a strategy of refining the original phenotype to identify the underlying mechanistic pathway. Furthermore, a recent GWAS in 4,600 subjects identified a hundred variants associating with a related phenotype of arterial stiffness using PWV. Two of these are in fact located in the PHACTR1 gene, just 30 kb from and in close LD to rs12526453 (Figure 17).(Tarasov et al. 2009) Our findings, taken with these support the role of this gene and locus in adversely influencing arterial elastic properties as a potential mechanism leading to MI, possibly through calcium cycling and altered smooth muscle contractility.

Limitations

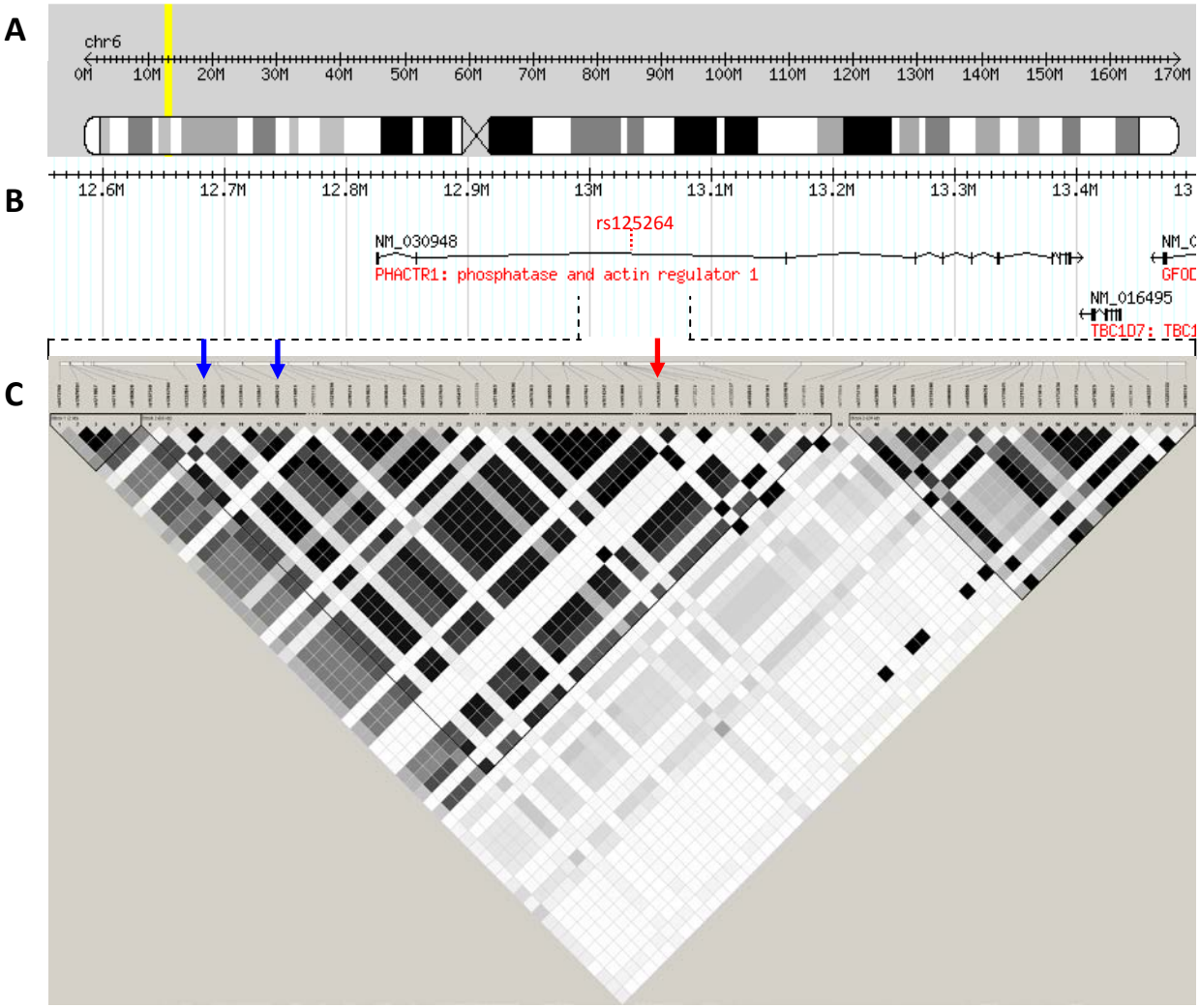
Limitations of our study include its cross-sectional design which restricts the conclusions we can draw regarding causality, although refining the phenotype in this manner provides some insight into potential risk pathways. Second, our restricted sample size meant that we may have missed SNP effects of a smaller magnitude than our power calculations suggested or those that have interactions with environmental

factors. While we combined cohorts to maximize power in the pooled analysis, we accept that the significant baseline differences in the characteristics of the cohorts may have introduced some heterogeneity and bias. We were also unable to adjust for other possible confounders which could influence the studied indices such as menopause status of female participants or specific medication effects for those in the META-Health group with risk factors. On the other hand the nature of our study necessitates multiple testing, which could lead to false positive findings. However, some degree of confidence in our findings is provided by their persistence despite conservative Bonferroni adjustment, as well as the fact that the association with 6p24 was with several linked parameters in two distinct populations which was apparent even with relatively small sample sizes. Finally, we did not have sufficient data to examine association with pulse wave velocity, an independent and gold standard measure of large artery stiffness, to differentiate if the observed effects were due primarily to enhanced stiffness.(Tarasov et al. 2009)

Conclusion

In conclusion, we have shown that the 6p24 marker, rs12526453, which was recently identified as conferring risk of MI through genome wide association, also associates with impaired central haemodynamic indices in two distinct populations independent of age and blood pressure. This finding could have implications for understanding the mechanism of risk for this locus as well as opening up possible therapeutic interventions in those with the risk allele.

Figure 17: Genomic map of the 6p24 locus



Genomic and LD map for the 6p24 risk region. Panel A: Ideogram demonstrating the location of the risk locus (yellow highlight) on the short arm of chromosome 6. Panel B: This region is magnified to show the Entrez gene representation of the PHACTR1 gene and the intronic position of rs12526453. Panel C: HapMap LD plot demonstrating LD between rs12526453 and neighboring SNPs in a 100kb interval. The darker squares represent an r2 closer to 1 and thus greater LD. The red arrow indicates the location of the rs12526453 SNP, while the blue arrows represent the rs7750679 and rs9296512 SNPs recently identified as top 100 hits in a GWAS of pulse wave velocity.(Tarasov et al. 2009) The two SNPs are in LD with rs12526453 each with an r^2 value of 0.88.

Chapter 5: Results, GWAS Variants and Genetic Risk Prediction

The results for specific aims 3a and 3b are presented in this chapter. In these studies we attempt to evaluate the novel variants for the purposes of CHD risk prediction, specifically for secondary risk prediction. Study 1 examines the association of 9p21 with incident risk of MI and death in the Emory Genebank cohort. Study 2 follows by assessing a multi marker genetic risk score composed of the known 11 risk variants, for association with prevalent MI as well as incident MI and death. In both studies we explore the added value of the genetic data to traditional risk factor information.

Contribution:

I conceived the ideas for both of these studies and performed all analyses. My contribution to the methodology is described in chapter 2, but relevant to these studies included coordination and management of collection of all follow up data. In addition I was responsible for verifying and validating this data through an outcomes committee. Using the available genotype data I then performed all analyses and created the relevant figures.

Study 1: The 9p21 risk variant does not predict adverse incident events in a population enriched for CAD

Summary:

In this study we attempted to evaluate the predictive value of 9p21 status on future risk of incident events in a cohort of patients with coronary disease. During a median follow up of 2.7 years 137 events were noted from 2597 patients. Despite substantial evidence for cross sectional associations with MI, no significant association was observed with 9p21 and incident events in this setting. In younger subjects (<60yrs at enrolment) there was a non-significant trend towards increased risk with homozygotes. Overall, these findings are in agreement with other data published on 9p21 and *secondary* risk prediction.

Introduction

The first generation of Genome Wide Association Studies (GWAS) for coronary heart disease (CHD) has identified the 9p21 locus as associating with MI and CAD in large scale populations with robust initial statistical validation and subsequent replication.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007) There is now almost universal consensus that 9p21 is associated with prevalent MI and CAD. However given that (1) causality is difficult to infer from these cross sectional studies and (2) an urgent need exists for novel CHD risk prediction strategies, prospective studies are now required to expand on these initial findings.

Several studies have so far examined association between this risk genotype and prospective risk of events in a population cohort with varying results. However little data exists on whether 9p21 associates with future events in a high risk population in whom secondary risk stratification is currently inadequate. (Weiner and Rabbani 2010) We therefore sought to examine the association of 9p21 genotype with incident cardiovascular risk in a cohort of subjects prospectively followed up after undergoing coronary angiography.

Methods:

Study Sample & Definitions

In 2597 Caucasian participants, recruited from the Emory Genebank that enrolled patients undergoing cardiac catheterization between 2003 and 2010, we documented demographic characteristics, medical histories, and behavioural factors. Specific details regarding risk factor definitions and coronary angiographic phenotyping have been described previously in chapter 2. Family history (FH) of CAD was defined as having any first degree relative (parents, siblings) with MI or coronary revascularization prior to the age of 60 years. As these CHD variants have not yet all been validated in non-Caucasian populations, we excluded those with self reported non-Caucasian ancestry. Patients with heart transplantation and missing or incomplete phenotype data were also excluded. The study was approved by the Institutional Review Board and all subjects provided written informed consent.

All subjects were prospectively followed by telephone interview, chart abstraction and through state vital records data to document major cardiovascular events and death. Follow-up was performed at time periods ranging from 1-5 years. Further details are provided in chapter 2. Cardiovascular death was defined as due to sudden or immediate cardiac death, stroke or acute vascular disease. Incident MI, including recurrent or first MI was documented and verified using hospital records using standard criteria. The primary endpoint for incident event analysis was the composite of cardiovascular death and MI.

SNP Selection and Genotyping

We selected the rs10757278 variant as the marker of the 9p21 risk locus, based on previous work by our group.(Helgadottir et al. 2007) This marker is in tight LD with the other 9p21 risk variants examined in other studies (eg rs1333049; rs47446). Genotyping was performed using the Centaurus (Nanogen) platform at deCODE genetics, Reykjavik, Iceland (Bell et al. 2002; Kutyavin et al. 2006) Genotyping rate was >97%. The quality of each Centaurus SNP assay was evaluated by genotyping each assay on the Caucasian (CEU) samples and comparing the results with the HapMap data. All assays had a mismatch rate <0.5%.

Statistical Analysis

Continuous variables are presented as means (SD) and categorical variables as proportions (%) with one way ANOVA and chi-squared tests used to determine differences by genotype.

Incident event risk analysis: Kaplan Meier and Cox regression analyses were applied to identify association between genotype and risk of incident MI and combined death and MI during follow-up. Models were constructed to identify univariate predictors of events with multivariate models incorporating all significant univariate predictors. Hazard Ratios (HR) were estimated for homozygotes and heterozygotes referenced to the non-risk genotype (AA). In sub group analysis, we examined for risk of incident events in younger subjects (<60 years at enrolment) as well as stratification for prior history of MI, and baseline CAD. A 2-tailed *P* value <0.05 was considered significant. PLINK statistical software was used to estimate Hardy-Weinberg equilibrium and minor allele frequency for the 9p21 variants.(Purcell et al. 2007) All remaining statistical analyses were performed using SPSS 17.0 (Chicago, IL).

Results

The rs10757278 variant was in Hardy Weinberg equilibrium with a MAF of 0.49 which is similar to published and Hap-Map data. Of 2597 self-reported Caucasian, 2533 (mean age 63.9 ± 11.1, range 24 to 90 years, 67% male), were successfully followed up for a median of 2.7 years. Patient group characteristics are presented in Table 24 by risk genotype. There were no major differences by genotype in gender or major risk factors. Interestingly there was a borderline trend in age, with older subjects more likely to be in the non risk AA group. In addition, as previously described Prior MI and baseline CAD were significantly associated with 9p21. We also observed a borderline association between 9p21 and family history of CAD prior to age 60, with more subjects homozygote for the risk allele reporting a positive family history.

Table 24: Patient characteristics by 9p21 genotype for Genebank (aim 3a)

N=2597	*Overall*	*AA*	*AG*	*GG*	*P value*
Age, yrs	63.9 (11.3)	64.9 (10.9)	63.4(11.3)	64.0(11.3)	0.02
Male Gender,%	67.7	67.8	68.4	66.2	0.65
Body Mass Index, kg/m^2	28.9 (5.7)	29.2(5.8)	29.5(6.1)	29.0(5.4)	0.32
Diabetes,%	30.4	29.9	31.6	28.7	0.42
Hypertension,%	68.2	67.1	68.4	69.0	0.75
Hyperlipidaemia,%	70.2	68.6	70	72.2	0.38
Ever Smoked,%	61.2	60.1	61.3	62.0	0.78
Family History CAD <60yrs,%	15.3	13.2	14.8	18.0	0.05
Median Gensini Score (IQR)*	13.0 (0-52)	10 (0-47)	16 (1.5-60)	17(1-74)	0.007*
Prior MI,%	40.3	37.1	40.4	43.2	0.05

*Mean and standard deviations or % presented unless otherwise indicated. *The Gensini score represents coronary artery disease burden.*

Association between 9p21 genotype and incident cardiovascular risk

During the follow up period there were 137 composite endpoint outcomes of CV death and MI. We did not observe a significant association between 9p21 genotype and any of the outcomes of incident MI, CV death or the composite of both. There were approximately equal numbers of events in the homozygote risk and non risk groups (GG and AA respectively) (Table 26), with no separation in Kaplan Meier curves over time (Log rank p=NS; Figure 18a).

In contrast a history of diabetes, myocardial infarction, hypertension, smoking and baseline CAD were predictive of the composite outcome in univariate analysis. In multivariate models, all of these, except hypertension remained independently associated with risk of future CV death/MI. Family history was not associated with future risk of events (Table 25).

Table 25: Univariate and multivariate predictors of CV death/MI

	Univariate	*p*	*Multivariate*	*p*
Age (yr)	1.01 (0.99-1.03)	0.06	-	-
Gender (1=male)	0.94 (0.66-1.34)	0.72	-	-
Diabetes	1.89 (1.35-2.65)	<0.001	1.72 (1.20-2.46)	0.003
Hypetension	1.56 (1.06-2.30)	0.026	1.29 (0.85-1.97)	0.236
Hyperlipidaemia	1.11 (0.76-1.62)	0.57	-	-
Smoking	1.64 (1.14-2.39)	0.008	1.50 (1.03-2.20)	0.036
FH CAD<60	1.07 (0.68-1.71)	0.76	-	-
Prior MI	2.30 (1.60-3.30)	<0.001	1.65 (1.14-2.38)	0.008
Baseline >50% CAD	3.30 (1.73-6.30)	<0.001	2.20 (1.07-4.48)	0.031

Presence of condition coded as 1 against reference of 0. Hazard ratios presented (95% confidence intervals)

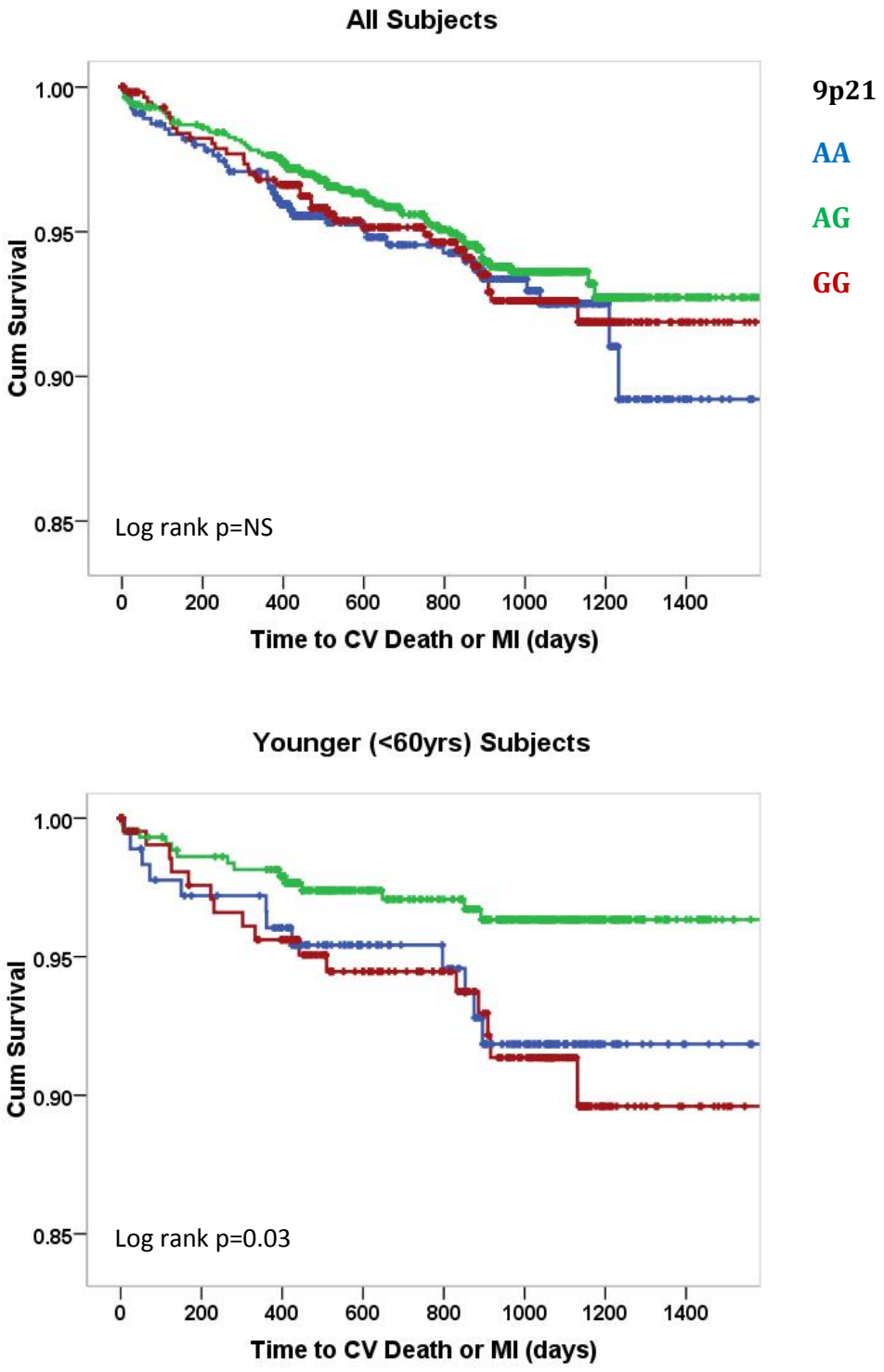
Given that genetic effects may already have manifested in patients with prior CAD, we repeated analysis after stratifying for prior MI or obstructive CAD. In the analysis with subjects who had never had prior MI (n=1043), with the AA group as reference, the risk estimates were AG=0.81 (0.42-1.58) and GG=1.19 (0.58-2.43) in 1371 cases. Likewise in the group which did not have obstructive CAD at baseline the risk estimates were, AG=1.13 (0.38-3.38) and GG= 0.71 (0.17-2.99) with 660 cases. Thus there was no significant association with future events in both of these sub-groups.

Finally we examined association between 9p21 genotype and incident events in young subjects, defined as being <60 years of age at enrollment. Again, there were no significant associations, aside from a weak trend towards more CV deaths/MI in the homozygous risk group (log rank p = 0.03; Figure 18b) (Table 26).

Table 26: 9p21 and incident CV risk in Genebank subjects

	9p21 Genotype (rs10757278)		
ALL	AA	AG	GG
CV death (50/2328)	13	24	13
HR (95% CI)	*1.0 (ref)*	*0.86 (0.44-1.70)*	*0.94 (0.44-2.03)*
MI (87/2328)	24	40	23
HR (95% CI)	*1.0 (ref)*	*0.78 (0.47-1.29)*	*0.90 (0.5-1.6)*
Composite (134/2328)	36	62	36
HR (95% CI)	*1.0 (ref)*	*0.81 (0.53-1.22)*	*0.95 (0.60-1.50)*
Patients <60 at enrollment	AA	AG	GG
CV death (11/857)	2	3	6
HR (95% CI)	*1.0 (ref)*	*0.61 (0.1-3.6)*	*2.59 (0.52-12.8)*
MI (32/857)	10	12	10
HR (95% CI)	*1.0 (ref)*	*0.44 (0.19-1.03)*	*0.83 (0.35-2.0)*
Composite (42/857)	12/6.6	14/3.1	16/7.2
HR (95% CI)	*1.0 (ref)*	*0.5 (0.2-1.0)*	*1.13 (0.53-2.4)*

Figure 18: 9p21 genotype and risk of incident CV death/MI



Kaplan Meier analysis for relationship between 9p21 genotype (A=non risk, G=risk) and risk of CV death/MI in (a) All Caucasian patients and (b) in subjects younger than the age of 60 at enrolment.

Discussion

Genome wide association studies (GWAS) have identified variants at the 9p21 locus as conferring risk for CHD including MI and CAD in cross-sectional studies. (Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; Schunkert et al. 2008; Erdmann et al. 2009; Gudbjartsson et al. 2009; Kathiresan et al. 2009a; Samani et al. 2009) Few studies until recently have sought to examine the predictive value of the 9p21 genotype for future risk prediction. In this study we examined the association of 9p21 with incident CV events, specifically in an older cohort with CAD in whom there is a need for better secondary risk prediction strategies. However, we were unable to demonstrate a significant association with incident CV risk over a short follow-up period, although a trend towards an association was apparent in the younger subgroup.

Shortly after the publication of the 3 seminal studies identifying 9p21 as a risk marker, Talmud et al demonstrated in a population cohort of 2742 men with 270 composite events followed for a period of 15 years that 9p21 was associated with incident risk. Subjects who were homozygous for the risk variant had a 60% greater risk than the referent group (HR 1.60, 1.12-2.28). They went on to show that 9p21 could enhance risk reclassification although it did not improve the area under the curve (AUC) when added to models with traditional risk factors.(Talmud et al. 2008) Among other population based studies examining 9p21 and cardiovascular events (Table 27), the largest study (22,219 women) by Paynter et al also demonstrated association with incident risk albeit with a slightly lower HR for homozygotes (HR 1.32, 1.07-1.63). They too found that 9p21 did not add to the AUC when added to traditional risk models.(Paynter et al. 2009) Both studies were limited by each being restricted to a single gender.

In contrast, we examined a more heterogeneous population with the intention of determining if 9p21 status could predict short term incident risk in individuals enriched for CAD and undergoing coronary evaluation. Risk prediction in this group is currently inadequate, with a high proportion of events occurring in the short term. As such we looked at a population of over 2400 patients with a mean age of 64 years and a prevalence of obstructive CAD of over 70% with a third having had a prior MI. This is in contrast to the populations studied for a much longer period by both Talmud and Paynter, which were 10-15 years younger and were derived from the general population as opposed to a cath lab demographic.(Talmud et al. 2008; Paynter et al. 2009)

Our negative findings could of course be explained by a lack of statistical power rather than an absence of true association. Preliminary power calculations suggested we had 80% power to detect HR down to 1.4, which would be adequate to identify an effect size as reported by Talmud et al. In our analysis, traditional risk factors which were independently associated with events were of this magnitude and therefore it is possible that we may have been unable to detect smaller effect sizes like those reported by Paynter et al. (Talmud et al. 2008; Paynter et al. 2009)

An alternative explanation could be that genetic effects are attenuated with advancing age and as our population as relatively old this may account for absence of observed effect especially if an already small effect size exists. Indeed significant evidence exists that demonstrates greater effect size in younger subjects and for heritability of premature MI. The average age of patients in the Ridker and Talmud studies was 56 and 52 respectively, whereas in our study it was 64. Furthermore, one group found no association with incident risk in an elderly cohort.(Dehghan et al. 2008) We anticipated this a priori and tested subgroup analysis with subjects <60 years at enrolment. In this group we observed a trend towards more events, even though the total n was lower, adding some weight to this hypothesis.

A further possibility for the lack of an association is that the risk phenotype for 9p21 is not MI per se and is rather a tendency to atherosclerosis as described by our group and others.(Dandona et al. 2010; Patel et al. 2010; Reilly et al. 2011) As such 9p21 would not be expected to cause an increase in fatal and non fatal MI rates but rather of new coronary artery disease. Both the Ridker and Talmud studies included revascularization as part of a composite endpoint, and this perhaps accounts for their positive associations. Interestingly in the former study, 9p21 was not associated with incident MI in a separate analysis of endpoints.(Talmud et al. 2008) Therefore, given that most patients in our cohort already had established CAD (79%), it could be argued that genetic effects in them had already manifested and would thus contribute little to further CV risk.

This latter point is of interest given recent findings that other groups have also failed to find association between 9p21 risk alleles and incident risk in CAD enriched populations (Table 27) in contrast to population based studies which overall have observed association with 9p21 and incident events. Somewhat paradoxically, one study even found a trend towards a protective effect for the risk allele of rs10757278 in a Caucasian cohort of similar size and demographic character to ours.(Gong et al. 2011)

A possible explanation for this could be that subjects already diagnosed with CAD or MI are likely to be aggressively treated with prognosis altering medications such as statins. Detailed medication records would assist in teasing out this possibility.

Finally, our follow up time was short with a median of 2.7 years. The population studies described above had follow up for 12-15 years. Genetic predisposition likely requires time to exert effects, with potential requirements for environmental interactions and other epigenetic factors, maybe occurring over decades. This may also explain the lack of association and if this were to be confirmed then it would suggest that risk genotypes are unlikely to be useful for predicting short term risk.

Taken together, and within the contexts of power limitations, we interpret our findings as suggesting that (1) the 9p21 locus likely confers a modest risk for incident CV events, (2) that genetic risk is most likely attenuated with age, (3) is unlikely to be significant in short term risk prediction and (4) defining the exact phenotype is of critical importance in order to perform and understand genetic risk prediction.

Limitations & Strengths

There are some limitations of our study. As described in previous chapters, our cohort is highly selected and not typical of the general population and results may not generalise to community cohorts. Second, we only included one 9p21 SNP based on work from our group which, while other groups have reported different SNPs. However this SNP is in very high linkage disequilibrium with other 9p21 published variants. Third we only examined death and MI as events and did not have sufficient information to examine other endpoints such as hospital admissions for chest pain or revascularization, which would have given more statistical power to the study. However by restricting our phenotypes to hard endpoints we were less liable to identify spurious associations.

Major strengths of our study include firstly, detailed CAD phenotyping which permitted us to examine association with incident MI in the context of CAD which has recently been highlighted as an important distinction.(Reilly et al. 2011) Second, we had a sample size comparable to other published studies with carefully defined and adjudicated events. Our cohort is also one with disease and this permitted us to examine the question of secondary risk prediction.

Table 27: Prospective studies for 9p21 and CHD risk

Study		Cohort	Size	Primary Outcome*	Follow up (yrs)	HR (95% CI) Per Risk Allele	HR (95% CI) Risk Homozygotes v Reference	Improvement in C statistic (9p21+Model)	Risk Reclassification	Overall assoc
Bruneck	(Ye et al. 2008)	Population	769	M/D/ R/S/P	10	1.35 (1.02-1.78)				+
Northwick Park	(Talmud et al. 2008)	Population	2,742	M/D/R	15		1.57 (1.10-2.25)	No	Some	+
Women's Health Study	(Paynter et al. 2009)	Population	22,129	M/D/R/S	10.2	1.15 (1.03-1.27	1.32 (1.07-1.63)	No	No	+
ARIC+	(McPherson et al. 2007; Brautbar et al. 2009)	Population	9,998	M/D/R	14.6	1.20 (n/a)	1.29 (1.09-1.52)	Yes	Some	+
Copenhagen Heart Study	(McPherson et al. 2007)	Population	10,578	M/D/R	15		1.38 (1.19-1.60)			+
MORGAN project	(Karvanen et al. 2009)	Population	2,928	M/D/R/S/U	5-10	1.20 (1.07-1.34)				+
Rotterdam	(Dehghan et al. 2008)	Elderly	6,720	M/D/R	9.5	1.03 (0.90-1.18)	1.0 (0.79-1.26)			-
Cardiovascular Health Study	(Shiffman et al. 2011)	Elderly	3,651	M	12.6	1.22 (1.03-1.45)		No	No	+
UTAH Heart Study	(Horne et al. 2008)	CAD	1,748	M/D	6.7	0.94 (0.82-1.08)				-
cohorts 1&2		CAD	1,014	M/D	3.6	0.91 (0.77-1.09)				-
CDCS Study	(Ellis et al. 2009)	CAD	1,054	D/H	4.0		0.78 (0.52-1.16)#			-
PMI Study		ACS	733	D/H	9.1		1.08 (0.74-1.60)#			-
GRACE	(Buysschaert et al. 2010)	ACS	3,247	M/D	0.5	1.49 (1.03-1.98)	-	No		+
Peng et al	(Peng et al. 2009)	ACS	520	M/D/U/F	2.4	NS	NS			-
INVEST-GENES	(Gong et al. 2011)	CAD + HT	2,364	M/D/S	2.8	0.81 (0.66-1.00)				-
INFORM	(Gong et al. 2011)	ACS	557	D/H	3	0.75 (0.59-0.95)				-
CABG Genomics	(Muehlschlegel et al. 2010)	Post CABG	845	D	5		1.70 (1.10-2.70)#	No		+

Summary of prospective studies examining association between 9p21 and incident CHD risk, showing hazard ratios as well as assessments of area under the curve analysis and risk reclassification based on traditional models. ACS = Acute Coronary Syndromes; CAD = Coronary Artery Disease; CABG= Coronary Artery Bypass Grafts; CHD = Coronary Heart Disease; MI = Myocardial Infarction; HF = Heart Failure; HT = Hypertension; NS = Non Significant

*Outcome Abbreviations: D = Death; M = Myocardial Infarction; R = Revascularization; S = Stroke; P = Peripheral Arterial Disease; H = Hospitalization; U = Unstable Angina; F = Heart Failure; # Recessive model – Risk homozygotes v (heterozygote + non risk homozygote); + Reported in two studies

Conclusion

In conclusion, the 9p21 locus was not associated with incident risk of cardiovascular death, MI or a composite endpoint of both in a CAD enriched population. We explore potential explanations for this and conclude that the effect size of 9p21, if present, is likely small; that it is likely greatest in younger subjects; may only be apparent over a longer time frame and that the underlying phenotype is of critical importance if genetic risk prediction and personalized medicine are to come to fruition.

Study 2: Association of a genetic risk score with both prevalent and incident myocardial infarction in subjects undergoing coronary angiography

Summary:

Following on from the assessment of a single genetic variant for risk prediction in a cohort with angiographic phenotyping, we next sought to determine the value of a multi-marker risk score for this purpose. In this study we describe the construction of a risk score using 11 established markers and then examine its association with both prevalent and incident MI. We demonstrate that the genetic risk score (GRS) is significantly associated with prevalent MI occurring before the age of 70 years with improvement of the C-statistic in models of prevalent MI. However, when examined in subjects with >50% angiographic CAD (cases and controls) there was no significant association. Furthermore, the GRS did not predict risk of near term incident MI or death over a median 2.5 years. This study highlights the impact of a high burden of genetic risk variants for MI but also suggests that the prognostic value of such a GRS remains uncertain for secondary risk prediction.

Introduction:

Genetic susceptibility for myocardial infarction (MI), the potentially fatal consequence of coronary artery disease (CAD), is due at least in part, to the cumulative effect of common polymorphisms with each risk variant conferring a small additive effect.(Hemminki et al. 2008; McCarthy et al. 2008) Recent genome wide association studies (GWAS) have identified multiple single nucleotide polymorphisms (SNPS) associating with both MI and CAD.(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; Erdmann et al. 2009; Gudbjartsson et al. 2009; Kathiresan et al. 2009a; Samani et al. 2009) Combination of these variants into a risk score may thus permit examination of the cumulative effect of risk SNP burden on prevalent and incident MI.

Kathiresan et al. demonstrated that a genetic risk score based on the 9 variants identified in their GWAS, was linearly associated with their primary phenotype of early onset MI.(Kathiresan et al. 2009a) However, as the score was based on the same

discovery cohort the effect size may have been overestimated. Replication in independent cohorts is therefore required to demonstrate that (1) risk scores derived from large GWAS can be applied to other independent populations, (2) such a score is also associated with non premature MI and (3) risk scores that predict prevalent MI or CAD are able to also predict future incident major adverse events.

Recent large prospective studies in younger low risk community cohorts have either confirmed or refuted the value of such a genetic risk score in prediction of *incident* MI over 10-12 years.(Paynter et al. 2010; Ripatti et al. 2010; Thanassoulis et al. 2012) This discrepancy may be because these variants associate primarily with CAD and mechanisms underlying development of atherosclerosis, but not with MI per se. Attempts to dissect these two phenotypes require study of cohorts with detailed phenotyping for both MI and CAD.(Horne et al. 2008; Reilly et al. 2011) However, to date, no studies have examined the association between a genetic risk score and either prevalent or incident MI in the context of detailed CAD phenotyping. Furthermore, little data exists on use of such a risk score to predict future prospective risk of adverse events in a CAD cohort in whom secondary risk prediction algorithms are currently inadequate.(Weiner and Rabbani 2010)

We therefore sought to examine the association of a genetic risk score based on 11 CHD variants discovered through GWAS with (1) *prevalent* MI with onset <70 years of age and (2) *incident* MI and death in a cohort of subjects undergoing coronary angiography.

Methods:

Study Sample & Definitions

In 2597 Caucasian participants, recruited from the Emory Genebank that enrolled patients undergoing cardiac catheterization between 2003 and 2010, we documented demographic characteristics, medical histories, and behavioral factors. Specific details regarding risk factor definitions and coronary angiographic phenotyping are available in chapter 2 and have been published previously.(Gensini 1975; Patel et al.) Family history (FH) of CAD was defined as having any first degree relative (parents, siblings) with MI or coronary revascularization prior to the age of 60 years. As these CHD variants have not yet all been validated in non-Caucasian populations, we excluded

those with self reported non-Caucasian ancestry. Patients with heart transplantation and missing or incomplete phenotype data were also excluded. The study was approved by the Institutional Review Board and all subjects provided written informed consent.

Prevalent MI

MI was consistently defined according to standard criteria from medical records as documented history of acute (at enrolment) or remote MI.(Thygesen et al. 2007) Age at first MI was recorded to the closest year. Subjects with MI onset at age <70 years were defined as cases. To mitigate case misclassification bias, whereby younger control subjects could become cases by developing MI in later life, we selected subjects >70 years at enrolment without a history of prior MI or during the study period, as controls. We also specified a-priori subgroup analyses to explore association with more premature MI defined as cases with MI <60 and <50 years of age. Finally, given that our statistical test of MI versus no MI would also test the hypothesis of CAD versus no CAD, we utilized previously described methods to examine the effect of the risk score on odds of MI in the presence of significant CAD determined as angiographic CAD >50% luminal stenosis in any major epicardial vessel (MI+/CAD+ versus MI-/CAD+).(Horne et al. 2008; Reilly et al. 2011)

Incident MI

All subjects were prospectively followed by telephone interview, chart abstraction and through state vital records data to document major cardiovascular events and death. Follow-up was performed at time periods ranging from 1-5 years. Incident MI, including recurrent or first MI was documented and verified using hospital records using standard criteria. The primary endpoint for incident event analysis in this study was defined as MI, using standard criteria as described above. The composite outcome of all cause death and MI was defined as a secondary endpoint.

Table 28: Details of CHD variants used to compile genetic risk score

Locus	SNP ID	Nearest Gene	Risk allele	Published risk allele frequency	Observed risk allele frequency	HWE p value	Reported allelic MI risk (OR)	Reference*
1p13	rs646776	CELSR2-PSRC1-SORT1	T	0.81	0.80	0.18	1.19	(Kathiresan et al. 2009a)
1p32	rs11206510	PCSK9	T	0.81	0.82	0.19	1.15	(Kathiresan et al. 2009a)
1q41	rs17465637	MIA3	C	0.72	0.73	0.89	1.14	(Kathiresan et al. 2009a)
2q33	rs6725887	WDR12	C	0.14	0.14	0.33	1.17	(Kathiresan et al. 2009a)
3q22.3	rs9818870	MRAS	T	0.15	0.16	0.89	1.15	(Erdmann et al. 2009)
6p24	rs12526453	PHACTR1	C	0.65	0.67	0.09	1.12	(Kathiresan et al. 2009a)
9p21	rs10757278	CDKN2A/B	G	0.50	0.50	0.50	1.29	(Helgadottir et al. 2007)
10q11	rs1746048	CXCL12	C	0.84	0.87	0.66	1.17	(Kathiresan et al. 2009a)
12q24	rs3184504	SH2B3	T	0.47	0.49	0.30	1.13	(Gudbjartsson et al. 2009)
19p13	rs1122608	LDLR	G	0.75	0.75	1.0	1.15	(Kathiresan et al. 2009a)
21q22	rs9982601	SLC5A3-MRPS6-KCNE2	T	0.13	0.13	0.25	1.20	(Kathiresan et al. 2009a)

**References relate to studies utilizing myocardial infarction as the primary phenotype, from which values in the table are derived, except 3q22.3 for which a combined phenotype was used.*

SNP Selection and Genotyping

We selected CHD risk variants discovered through GWAS and published before June 2009, whose primary phenotype was MI or CHD and which satisfied the genome wide association threshold of $5x10^{-8}$, (reflecting a million tests of association). The selected SNPs are listed in Table 28 along with risk allele frequencies and published effect sizes. The LpA locus was not included for logistical reasons despite publication in 2009.(Clarke et al. 2009; Tregouet et al. 2009) Genotyping was performed using the Centaurus (Nanogen) platform at deCODE genetics, Reykjavik, Iceland or with the SNPstream (Beckman Coulter) platform at Emory University, Atlanta, GA as described in chapter 2.(Bell et al. 2002; Kutyavin et al. 2006) Genotyping rates for all SNPS was >97%, except rs646776 (73.2%), rs11206510 (72.1%) and rs1122608 (72.9%). The quality of each Centaurus SNP assay was evaluated by genotyping each assay on the Caucasian (CEU) samples and comparing the results with the HapMap data. All assays had a mismatch rate <0.5%. For the SNPstream array, the GenomeLab SNPstream Genotyping System Software Suite v2.3 (Beckman Coulter, Inc., Fullerton, CA) was used for array imaging and genotype calling. To ensure genotyping accuracy and reproducibility two internal quality control samples were included and each run in triplicate, on each of the 384-well arrays.

Genetic Risk Scores

A *weighted* genetic risk score was calculated based on the average number of risk alleles (0, 1 or 2) in the SNPs genotyped weighted by the effect size published in larger scale studies (natural log of the OR x no. of risk alleles for each SNP), using the "Profile Scoring" option in PLINK.(Purcell et al. 2007) Thus, 2 copies of the risk allele 9p21 (published OR per risk allele 1.3) contributed proportionately more to the overall risk score for an individual than two copies of the risk allele 6p24 (OR 1.1). Missing genotype data for individual SNPs was accounted for by mean imputation based on overall risk allele frequency. Details of missingness are provided in Table 29. We performed validation analysis using only data from subjects with the full complement of genotyped variants to ensure imputation did not adversely influence our results.

Table 29: Table of missingness for the 11SNPs comprising the risk score

Number of variants successfully genotyped	Number of patients
11	1661
10	1817
9	1875
8	2402
7 or more	2597

Genotyping rates for all SNPS was >97%, except rs646776 (73.2%), rs11206510 (72.1%) and rs1122608 (72.9%). As a consequence there was a significant number of subjects who had genotype data for 8 markers, but fewer with data on 9-11 variants. Mean imputation based on risk allele frequency was used to estimate score data for all patients. Validity of imputation was tested by repeating primary analyses with un-imputed data in 1661 subjects.

Statistical Analysis

Continuous variables are presented as means (SD) and categorical variables as proportions (%) with one way ANOVA and chi-squared tests used to determine differences between groups.

Prevalent MI association analysis: Logistic regression models were constructed to test the effect of the weighted risk score, as a continuous variable on prevalent MI risk (case= MI<70yrs; controls = no MI>70yrs). We further examined the association with younger cases, defined as MI<60 years and MI<50 years against the same control group. For illustrative purposes, we also categorized the score into quintiles to derive estimates for effect size (OR) for the top quintile compared to the first quintile as the referent category. We then tested for association between the continuous risk score and MI in all patients *with* CAD (MI+/CAD+ versus MI-/CAD+). This was performed using the same cases (MI<70yrs) but selecting controls (>70 yrs) without prior MI but *with* significant stable angiographic CAD (defined as >50% stenosis). Analyses were performed before and after adjusting for gender, body mass index, diabetes, hypertension, hyperlipidaemia, smoking and family history (FH) of premature MI/CAD. Interaction terms were tested for association between the risk score and risk factors including FH, with stratified analysis to evaluate any significant interactions.

Incremental predictive value of the risk score for prevalent MI occurring before the age of 70 was tested using ROC analysis and estimated through the c-statistic. Models were constructed with and without the risk score using (1) traditional risk factors (gender, BMI, diabetes, hypertension, hyperlipidaemia, smoking and FH), and (2) with gender and FH only, which are the only variables likely to be known in early life.

Incident MI risk analysis: Cox regression analyses were applied to identify association between the GRS and risk of incident MI and combined death and MI during follow-up. Models were constructed using a categorized risk score, with and without traditional risk factors and presence of baseline angiographic CAD (>50%). Hazard Ratios (HR) were estimated for the top quintile of risk score referenced to the first quintile as before. Subgroup analyses included stratifying by history of MI at enrollment to examine risk of recurrent and first MI during follow up and examining incident risk in younger subjects <60 years.

A 2-tailed *P* value <0.05 was considered significant. PLINK statistical software was used to estimate Hardy-Weinberg equilibrium and minor allele frequencies for all SNPs.(Purcell et al. 2007) All remaining statistical analyses were performed using SPSS 17.0 (Chicago, IL).

Results

For the 11 SNPs genotyped, the observed frequencies for each risk allele were similar to published and Hap-Map data and were consistent with Hardy Weinberg equilibrium (Table 28). A total of 2597 self-reported Caucasians (mean age 63.9 ± 11.1, range 24 to 90 years and 67% male) were genotyped for this study.

Table 30: Patient characteristics by case-control status (aim 3b)

	Overall N=2597	Cases (MI<70 yrs) N=889	Controls (no MI<70 yrs) N=449	P value	Controls (no MI<70 yrs *With* CAD N=370	P value
Age, yrs	63.9 (11.3)	62.9 (10.0)	75.7 (4.20)	<0.001	75.8 (4.2)	<0.001
Male Gender, %	67.6	76.2	59.0	<0.001	65.4	<0.001
Body Mass Index, kg/m^2	28.9 (5.7)	29.7 (5.8)	27.6 (5.3)	<0.001	27.7 (5.1)	<0.001
Diabetes, %	30.2	35.9	27.2	0.001	28.4	0.011
Hypertension, %	70.6	70	73.9	0.148	75.7	0.048
Hyperlipidaemia, %	68.2	77.8	69.0	0.001	71.9	0.029
Ever Smoked, %	61.2	67.8	55.6	0.001	57.2	0.001
Family History CAD <60yrs,%	15.3	20.5	8.7	<0.001	7.0	<0.001

Mean and standard deviations or % presented unless otherwise indicated.

Prevalent MI association with a genetic risk score

For prevalent MI analysis (cross sectional), we examined 889 cases of MI<70 years and 449 controls >70 years without prior MI (Table 30). Cases were significantly younger than controls by design, and expected significant differences in other risk factors were also observed (Table 30). In our primary analysis there was a significant association between the weighted risk score and prevalent MI, when modeled as a continuous variable ($p=9.74x10^{-5}$) (Table 31). In further analysis of the Emory cohort, risk scores were grouped into quintiles and analyzed as categorical variables. Each increase in quintile of risk score was associated with a statistically significant increase in odds of MI after adjustment for covariates including CAD. Thus, in comparison to the lowest quintile, the adjusted OR for MI for an individual in the highest quintile of weighted risk score was 1.81 (1.21-2.70) (Figure 19). Furthermore, the effect sizes were greater in cases with MI <60 years or <50 years of age compared to older controls. Thus, the adjusted OR for MI for the highest quintile of weighted risk score compared to the lowest was 1.90 (1.24-2.92) for MI<60 years and 2.37 (1.44-3.91) for MI<50 years (Figure 19). Interestingly, it appears that a significant increase in odds of MI was only observed in the highest quintiles, with no apparent increase between the first 3 quintiles (Figure 19).

Table 31: Association between a CHD genetic risk score and prevalent MI

	Association with GRS		
Case-Control Group	Beta	SE	P
MI <70yrs v no MI>70 yrs (All)	17.05	4.37	$9.74x10^{-5}$
MI<70 yrs v no MI>70 yrs (CAD+)	11.73	4.96	0.02

GRS= Genetic Risk Score, weighted using the published effect sizes of individual variants (Table 28).
**Adjusted for gender, BMI, diabetes, hypertension, hyperlipidema, smoking, family history and angiographic CAD.*

We did not observe significant interactions between the risk score and covariates in the association with MI <70 years. Most pertinent was the lack of interaction for FH of CAD (p for interaction 0.63). Overall, the mean weighted risk score was marginally higher in subjects with a positive FH compared to those with a negative or unknown FH (0.085±0.01 v 0.083±0.01 respectively, p=0.05).

Given the significant association with prevalent MI, we examined the additive value of the genetic risk score when modeled with existing risk factors for prevalent MI risk at <70 years using the area under the curve statistic. There was a statistically significant increase in the c-statistic when the risk score was added to a model containing gender, BMI, traditional risk factors and FH (0.681 (0.015) v 0.693 (0.015), p=0.012) (Table 32). Similarly, when modeled for MI at younger ages, there was again a significant increase in the c-statistic (<60yrs 0.699 (0.016) v 0.712 (0.016) p=0.03; <50yrs 0.716 (0.018) v 0.733 (0.018), p=0.04) (Table 32). The difference between the AUC was even greater when the score was modeled with the fixed risk factors of FH and gender (difference in AUC = 0.027; 0.030; 036 for MI <70; <60 and <50yrs respectively, all p<0.005) (Table 32).

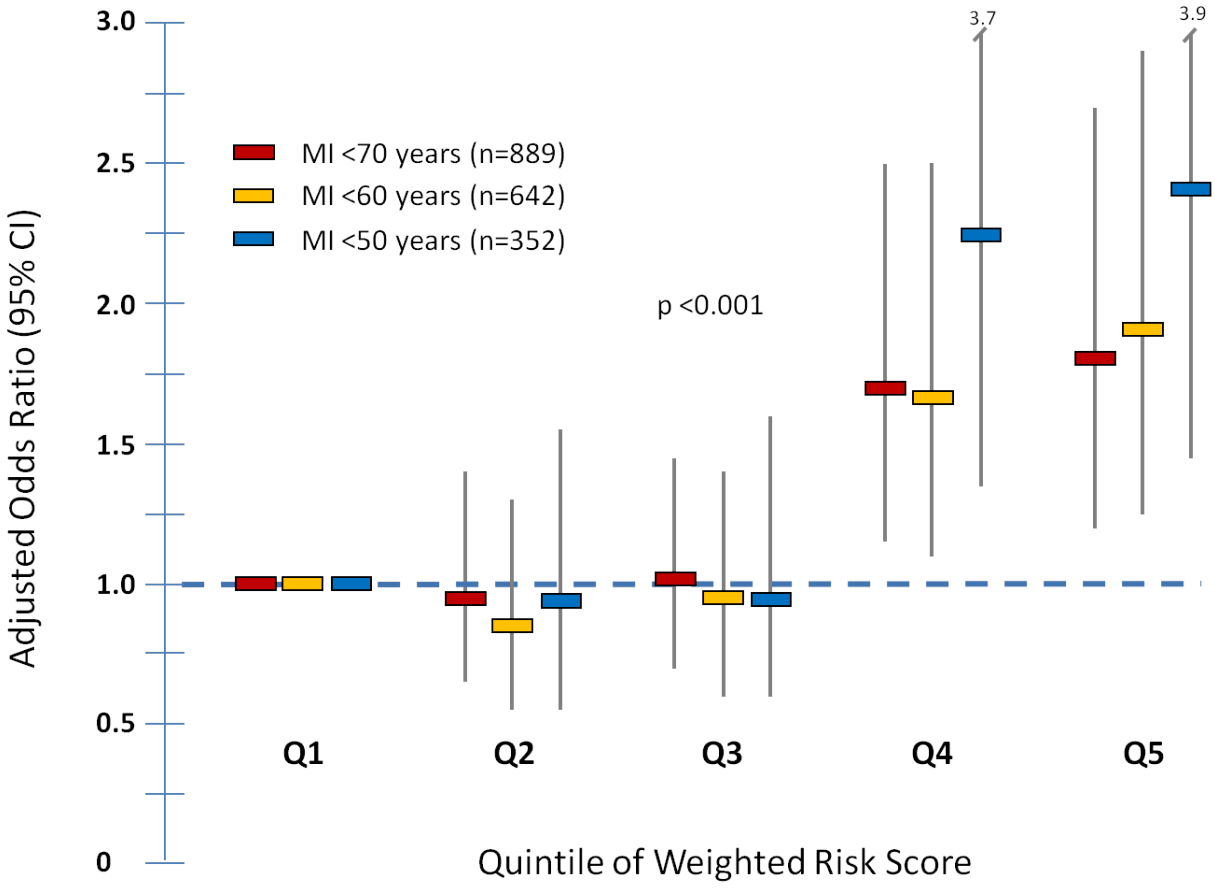
Table 32: Discriminatory analysis using AUC for prevalent MI

	Traditional RFs	Traditional RFs + GRS	Improvement in C statistic	P value	Early RFs	Early RFs + GRS	Improvement in C statistic	P value
MI70	0.681(0.015)	0.693(0.015)	0.012	0.032	0.612	0.639	0.027	0.002
MI60	0.699(0.016)	0.712(0.016)	0.013	0.016	0.625	0.655	0.030	0.002
MI50	0.716(0.018)	0.733(0.018)	0.017	0.040	0.639	0.675	0.036	0.001

Traditional Risk Factors (RFs) = gender, BMI, diabetes, hypertension, hyperlipidaemia, smoking and family history (FH) CAD; Early RFs = Gender and FH CAD only; GRS = Genetic Risk Score; MI70/60/50 = myocardial infarction occurring before respective age compared to older controls without prior MI; P values for comparison of AUC.

Finally, to determine if these variants confer risk of MI (by plaque rupture or thrombosis) over and above the risk of developing CAD, we repeated the main analysis using older CAD+ controls (methods). We examined 889 cases (MI<70yrs) and 370 controls (no MI>70 years with CAD+). Overall the results were significantly attenuated with a modest association only (p=0.02) (Table 31).

Figure 19: Genetic risk score and prevalent MI



Association between quintiles of genetic risk score and MI in different age groups; Adjusted Odds Ratios and 95% confidence intervals are presented. Covariates included gender, BMI, risk factors, family history of CAD and angiographic CAD.

Incident MI: Association with a genetic risk score

For *incident* MI (prospective) analysis we followed the entire Emory cohort of 2597 subjects for adverse outcomes. Demographic data by quintile of risk score for the Emory cohort are presented in Table 33, for which there was a statistically significant difference in age with increasing risk score, whereby those with lower scores were older than those with higher scores (p trend = 0.001). Also, those with the highest scores tended to have higher incidence of hyperlipidaemia.

After a median of 2.5 years follow-up, 101 subjects experienced an MI and 358 the composite endpoint of death or MI. Kaplan Meier analysis did not reveal a significant association between quintiles of the GRS and events, despite a suggestion of more events in the highest quintile for the subgroup of younger patients (Figure 20). When compared to the lowest quintile, subjects in the highest quintile trended towards more events with an adjusted HR of 1.08 (0.57-1.99) for MI (Table 34). For the composite endpoint of Death/MI, the adjusted HR was paradoxically protective at 0.68 (0.49-0.94). Other significant multivariate predictors in the latter model included age (HR 1.03 (1.02-1.04)), diabetes (HR 1.51 (1.20-1.90)), hypertension (HR 1.28 (1.00-1.65)), smoking (HR 1.39 (1.10-1.75)) and baseline significant angiographic CAD (HR 1.31 (1.0-1.73)). A history of hyperlipidaemia was noted to be protective (HR 0.78 (0.61-0.99)). In subgroup analysis, there was no association between the GRS and future recurrent or first MI during follow up (p= 0.67 and p=0.21 respectively)

Finally, we repeated the analyses for prevalent and incident MI using only subjects with full genotype data for all 11 variants (Missingness presented in Table 29) and identified similar findings, thereby confirming that mean imputation did not appreciably influence our results (data not shown).

Table 33: Demographics by quintiles of risk score for the Genebank cohort

Total N=2597	Quintile 1 N=521	Quintile 2 N=518	Quintile 3 N=522	Quintile 4 N=518	Quintile 5 N=518	P value
Genetic Risk Score†	0.50 (0.05)	0.59 (0.02)	0.64 (0.02)	0.70 (0.02)	0.77 (0.04)	-
Age, yrs	64.1 (11.3)	64.6 (11.7)	64.9 (11.0)	63.5 (11.1)	62.1 (10.9)	0.001
Male Gender, %	68.5	65.1	66.9	66.4	71.2	0.26
Body Mass Index, kg/m^2	29.6 (6.2)	29.1 (6.3)	29.3 (6.0)	29.1 (5.6)	29.5 (5.6)	0.57
Diabetes, %	29.8	30.8	33.5	28.8	28.2	0.36
Hypertension, %	65.6	69.8	69.1	70.4	66.3	0.37
Hyperlipidaemia, %	64.4	70.1	68.9	73.1	76.3	0.001
Ever Smoked, %	61.3	59.4	59.5	60.8	65.0	0.35
Family History of CAD<60yrs, %	15.2	13.1	14.6	15.6	18.0	0.29
Prior Myocardial Infarction, %	36.2	36.6	42.1	40.5	45.6	0.01

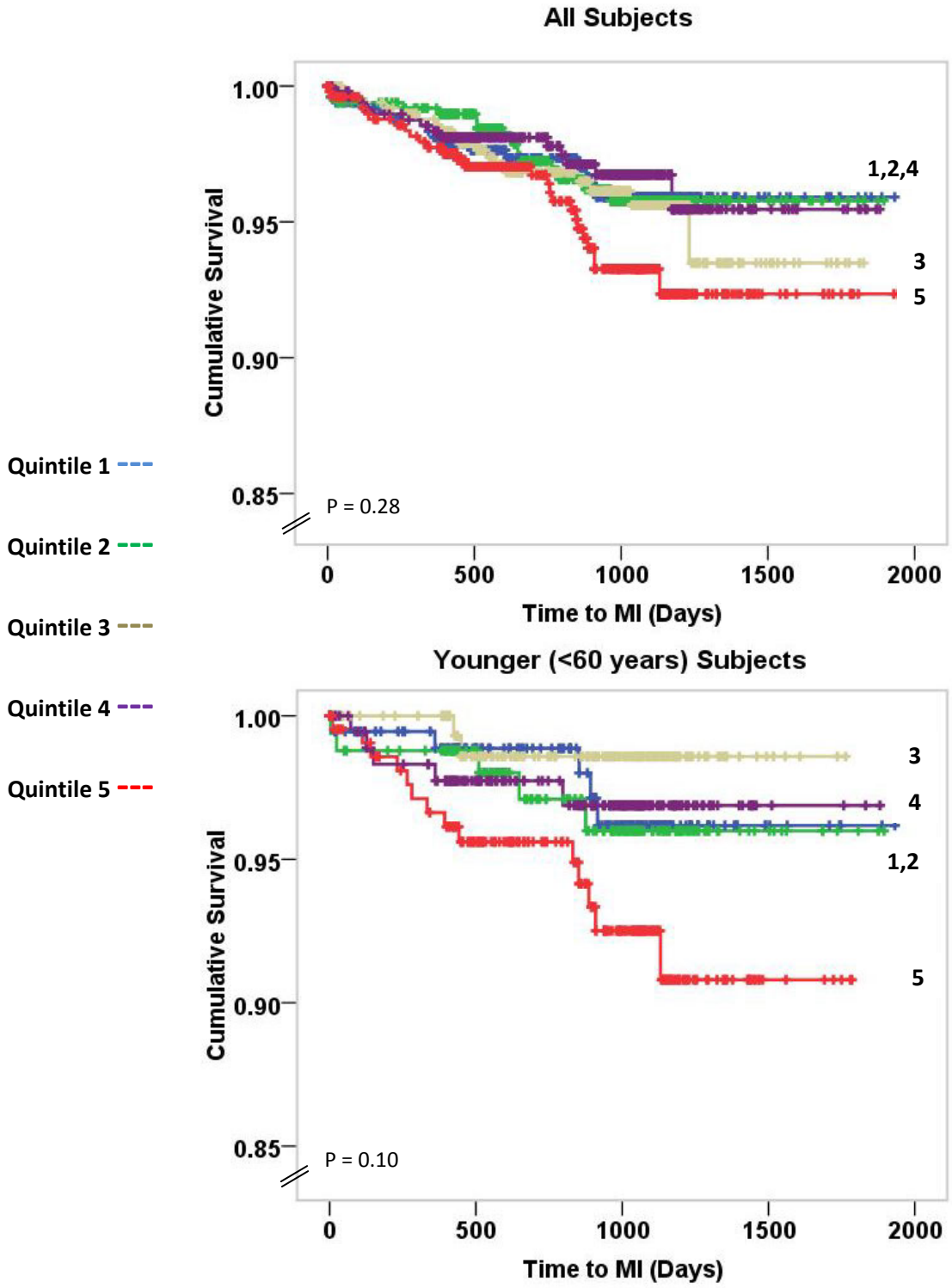
*Mean and standard deviations or % presented unless otherwise indicated. *The Gensini score represents coronary artery disease burden. †Genetic risk score is calculated based on average number of risk alleles per SNP (see methods)*

Table 34: Association between a CHD genetic risk score and incident events

	Incident Risk Prediction						
	Events by quintile					Top v Bottom Quintile	
	Q1	Q2	Q3	Q4	Q5	HR (95% CI)	HR (95% CI) Adjusted
All subjects	N=2597						
MI	18	20	17	20	26	1.24 (0.67-2.29)	1.08 (0.57-1.99)
Death/MI	82	74	73	63	66	0.72 (0.51-0.99)	0.65 (0.46-0.91)
< 60 yrs	N=970						
MI	6	6	8	3	14	2.25 (0.86-5.88)	2.02 (0.75-5.40)
Death/ MI	18	12	20	19	22	1.16 (0.62-2.17)	1.02 (0.54-1.93)

**Adjusted for age, gender, BMI, risk factors, prior MI, baseline CAD and family history of CAD.*

Figure 20: Genetic risk score and incident MI



Kaplan Meier graphs for association between quintiles of genetic risk score and incident MI. Separate analysis are presented for (A) the total cohort (n=2597, 101 events) and (B) the younger (age <60 years at enrollment) cohort (n=970, 37 events). Log-Rank p values presented. Numbers 1 to 5 correspond to risk score quintile categories.

Discussion

In a study of high risk angiographically phenotyped patients we demonstrate that a cumulative risk score generated from published GWAS CHD variants is: (1) significantly associated with MI before age 70 years in a Caucasian cohort independent from the discovery samples; (2) not associated with MI when both cases and controls have established CAD; and (3) not a strong predictor of incident near term adverse events.

Although genetic risk scores using variants identified through genome wide linkage scans and candidate gene studies have been previously studied, the first attempt to devise a cumulative risk score from GWAS variants was by Kathiresan et al, using 9 risk variants for premature MI. (Kathiresan et al. 2009a) They found that subjects within the highest quintile of a weighted risk score had two-fold higher odds of premature MI than those in the lowest quintile. Our study validates the use of such a score in an independent cohort after addition of two extra variants that have since been identified.(Erdmann et al. 2009; Gudbjartsson et al. 2009) In comparison, our cohort was significantly older, but we still showed that subjects within the highest quintile of risk score also had an almost two fold greater odds of MI prior to the age of 70 years than those in the lowest quintile, with an even greater risk in subjects with more premature disease (MI <60 and <50 years), thereby confirming that hereditary effects are greatest in younger age. Interestingly the association observed for prevalent MI in our study was non-linear, with only the highest quintile of risk score significantly associating with MI. These findings may reflect possible gene-gene interactions or simply more extensive CAD prone to complications, such as a greater lipid core or thin cap fibro-atheroma arising as a consequence of inheriting a greater than average number of these atherosclerosis predisposing risk variants.

Use of the broad phenotype of CHD by early large scale GWAS has raised concerns as to whether these risk variants confer actual risk of MI since the processes involved in atherosclerosis that may not necessarily lead to MI. Our group and others have shown that 9p21, for example, associates primarily with CAD and atherosclerosis, with functional studies also suggesting this.(Dandona et al. 2010; Patel et al. 2010; Visel et al. 2010) With others we have also demonstrated that in subjects with angiographically significant CAD, the 12 risk variants individually do not associate with MI.(Reilly et al. 2011) Thus, at the pathophysiologic level, these variants are unlikely to be associated with thrombosis or plaque rupture, and that MI risk observed in prior

studies is likely secondary to development of greater CAD burden. This study extends these observations by demonstrating that the association between the cumulative risk score and MI is considerably attenuated. In contrast to the study of Reilly et al, where controls were younger and thus subject to possible case-misclassification, use of older controls is a major strength of this study, given the critical time sensitive nature of the MI phenotype. Although this finding is of major interest for mechanistic understanding, MI is a more tangible clinical endpoint and as such association of a risk score with MI, even by proxy may still be clinically relevant.

Although subjects reporting a positive FH of CAD/MI had a marginally higher risk score, the cross sectional association between the risk score and MI was independent of FH, and furthermore, there was no interaction with FH, suggesting that genetic risk is mediated both in those with and without a FH of premature CAD. Despite the challenges related to accurate documentation of FH, this serves to highlight the complexity of heritability of MI in relation to common variants. Identification of rarer variants with more Mendelian characteristics through next generation sequencing may yield further insights into the role of FH in relation to complex diseases.(Cirulli and Goldstein 2010)

Genetic risk score and incident MI: Three large studies have examined the value of genetic risk scores in predicting first MI in asymptomatic populations. In a prospective study of 19,000 community based women, no association was observed between a panel of 12 MI risk variants and future adverse CV events after 12 year follow-up.(Paynter et al. 2010) However, Ripatti et al found that a risk score of 13 variants was associated with prevalent and incident CV events in a community based population of 31,000 men and women; subjects in the highest quintile were at 1.66-fold greater risk of adverse events during a 10-year follow-up period compared to those in the lowest quintile.(Ripatti et al. 2010) An association between incident MI risk and 13 SNPs was also observed in the Framingham cohort.(Thanassoulis et al. 2012) In contrast to our population who had established CAD or were at high risk of CAD where future risk prediction is particularly challenging, all of these studies examined primary risk of MI.

In this study, although we observed a very marginal trend towards more prospective MI in those with the highest quintile of risk score, the final association results were not statistically significant. This is likely to reflect the fact that the primary

phenotype for most of these variants is atherosclerosis, which had already developed by the time of enrolment into a cardiac catheterization cohort. Interestingly we noted that the distribution of risk scores was related to age with older subjects trending towards lower scores, potentially indicating that those with high scores were at greater risk for premature fatal events. This finding may help to explain the paradoxically protective association for those with the highest risk scores with the composite outcome of MI/ death. Given that a history of hyperlipidaemia was also protective in the same model, it is plausible that unmeasured treatment effects such as intensive statin use may also be responsible. Overall, the observation of a lack of association between genetic variants and incident events in a diseased cohort has also been described in other studies whereby for example the 9p21 risk locus has shown convincing association for incident risk prediction in primary prevention population cohorts but not in those with diagnosed CAD (See previous study).(Gong et al. 2011)

Finally, we also tested the discriminatory value of the genetic risk score for identifying cases of prevalent MI, which may be greater than for single SNPS as advocated by Davies et al.(Davies et al. 2010) Indeed, after addition of the genetic risk score to traditional risk factors, we found a modest, but statistically significant improvement in the c-statistic for prevalent MI <70 yrs as well as more premature MI. However, genetic data is unique and provides risk information at birth, unlike the majority of acquired risk factors that impact later in life. When the genetic risk score was added to a model containing the fixed risk factors of gender and FH (the only ones likely to be known in adolescence), we found that it markedly enhanced the c-statistic, with the greatest discriminatory value for more premature MI. Use of genomic data in this manner would represent a shift in genetic biomarker assessments and requires further exploration.

Limitations & Strengths

There are some limitations of our study. Firstly, we had a selected population with CAD and thus our findings may be susceptible to unmeasured bias. Secondly, our controls were not healthy, although risk allele frequency would likely be even lower in healthier controls and would strengthen the observed statistical associations. Thirdly, we used imputation to estimate risk scores for those with missing data, although an analysis using non imputed data revealed similar overall results. Fourthly, while we

may have been underpowered for analysis of incident events, our study was powered sufficiently to be able to detect the effects of age/10 yr to a HR of 1.16. Finally, we only included 11 MI SNPs in our score and have not included variants in relevant metabolic pathways or newer and other recently discovered CHD variants.(Clarke et al. 2009; Tregouet et al. 2009; Schunkert et al. 2011) However, the recent study by Thannassoulis et al demonstrates that addition of 16 newer variants to a 13 SNP model did not impact on their association findings.(Thanassoulis et al. 2012) Moreover, some of these SNPs may not to be true risk SNPs in a particular locus, possibly attenuating the results.(Musunuru et al. 2010b)

Major strengths of our study include (1) study of an independent cohort from those used in discovery GWAS (2) the use of elderly controls in the cross sectional analysis to mitigate the important problem of case-misclassification bias (3) examination of the association with the risk score in the context of proven CAD to determine if there was MI risk over and above association with atherosclerosis, which to our knowledge has not been performed previously and is important given the overlap in phenotypes and (4) study of both prevalent and incident MI in the same cohort to avoid concerns including those of population stratification.

Conclusion

In conclusion, in a cohort of subjects undergoing coronary angiography, we demonstrate that a high burden of CHD risk variants is associated with prevalent MI but not significantly with near term incident MI or death. We propose that cumulatively these variants confer risk of MI through development of CAD, but once disease has developed the association with future risk of adverse events is attenuated. As such the clinical utility of a GRS may be of limited value in secondary risk prediction. These findings have implications for understanding the clinical utility of genetic risk scores for secondary as opposed to primary risk prediction

Chapter 6: Results, Genomic Discovery and Novel Therapy for CHD

The results for specific aim 4 are presented in this chapter. This pilot study explores therapeutic possibilities arising from genomic discovery. As described previously, the majority of GWAS variants have no known function, but a genome wide linkage scan has identified variants in the leukotriene pathway that associate with risk of MI. This study examines whether drug intervention targeted to the leukotriene pathway in carriers of the risk variants is feasible, and can improve surrogate markers of cardiovascular risk.

Contribution:

A preceding association study for leukotriene haplotypes and MI had been performed and published using the Emory Genebank by Helgadottir et al. A provisional protocol had been formulated for this intervention study but not initiated. I was responsible for refining this and then performing the study. I identified and contacted suitable subjects carrying the risk haplotypes and screened and consented them at Emory. I was assisted by a study coordinator to manage the visits and compile a database of results. Research staff and other investigators performed the FMD and biomarker assessments. On completion of the study, I performed all appropriate statistical analyses.

Study 1: The 5-Lipoxygenase inhibitor zileuton improves endothelial function in carriers of coronary heart disease risk haplotypes in the ALOX5AP and LTA4H leukotriene pathway genes

Summary

Just prior to the emergence of genome wide association, genome wide linkage studies identified variants in the leukotriene synthesis pathway as associating with risk of coronary heart disease (CHD) and were also associated with greater levels of this pathway metabolite, leukotriene B4 (LTB4), an inflammatory mediator. In this study, we demonstrate that administration of an orally active leukotriene synthesis inhibitor to subjects carrying the leukotriene risk haplotypes reduces LTB4 levels, and in parallel improves endothelial function. This finding, stimulated by genetic discovery, raises the possibility of a novel therapy for CHD as well as demonstrating the feasibility of tailored therapy based on genotype.

Introduction

Using an unbiased genome wide linkage approach, variants in the leukotriene pathway genes were identified as associating with risk of MI. This discovery appeared to validate developing mouse and human data on the significant role of these inflammatory mediators in atherosclerosis, while raising exciting possibilities for a novel class of therapies for vascular disease.

In an Icelandic cohort, Helgadottir et al demonstrated an association with a haplotype (combination of SNPs) in the ALOX5AP gene, which encodes 5-Lipoxygenase activating protein, with 2 fold risk of MI in 296 families (713 individuals with MI) and reported a second haplotype (hapB), associating with MI and ischaemic stroke in a second British population.(Helgadottir et al. 2004; Helgadottir et al. 2005) A further study identified variants in the LTA4H gene in the same pathway as also conferring modest risk of MI and stroke in Caucasians, but an almost 3 fold risk in African Americans.(Helgadottir et al. 2006) Several studies have replicated this finding for MI, coronary disease and ischaemic stroke with varying degrees of success.

Leukotrienes are powerful inflammatory mediators and it was believed that greater activity in the leukotriene synthesis pathway may account for the mechanisms

behind this genetic risk. Indeed functional studies by Helgadottir et al revealed that males with MI who were also carriers of hapA had greater levels of neutrophil stimulated LTB4 than controls without MI and subjects with MI without the risk genotype.(Helgadottir et al. 2004) Given these findings, there is an immediate opportunity to devise therapy based on leukotriene synthesis inhibition or receptor blockade to ameliorate cardiovascular risk, particularly in subjects with a genetic predisposition to enhanced leukotriene activity.

Leukotriene synthesis and receptor blockers have been licensed for use in asthma but have not yet been utilized in treating vascular disease. In this study we sought to determine whether therapy with an orally active 5-lipoxygenase (5-LO) inhibitor in stable CAD patients carrying the leukotriene risk haplotypes would reduce LTB4 synthesis and improve endothelial function, a surrogate marker of vascular risk.

Methods:

Study Design

Subjects were recruited from those enrolled within the Emory Genebank study, details of which are presented in chapter 2. These were patients undergoing coronary angiography for known or suspected coronary disease. A subset of 1340 consecutive patients has previously been genotyped for leukotriene risk variants for an association study with myocardial infarction.(Helgadottir et al. 2006) From those who had been genotyped and had haplotype estimation we identified all those with at least one copy of either of the risk haplotypes hapA, hapB or hapK. Figure 21 demonstrates the recruitment flow.

All eligible subjects living within a 50 mile radius of the study site were then invited to participate in the drug study. The first 12 subjects responding to invitations attended a screening visit. Thereafter eligible subjects were consented and commenced the study protocol. Subjects underwent a brief history and physical examination at each visit with measurement of heart rate, blood pressure and BMI. At the baseline visit, patients underwent phlebotomy for blood sample collection and endothelial function testing. Blood samples were processed for inflammatory markers, oxidative stress markers and ionophore neutrophil stimulation assessment of LTB4 production. These tests were performed at baseline, 2, 4, 6 and 8 weeks after enrolment. Study drug was

administered at the first visit (baseline) and continued for 4 weeks, with the last dose on the morning of the 4 week visit (Figure 21). A final week 20 visit was scheduled post hoc to ensure values had returned to baseline.

Figure 21: Subject selection flow and study design for aim 4a

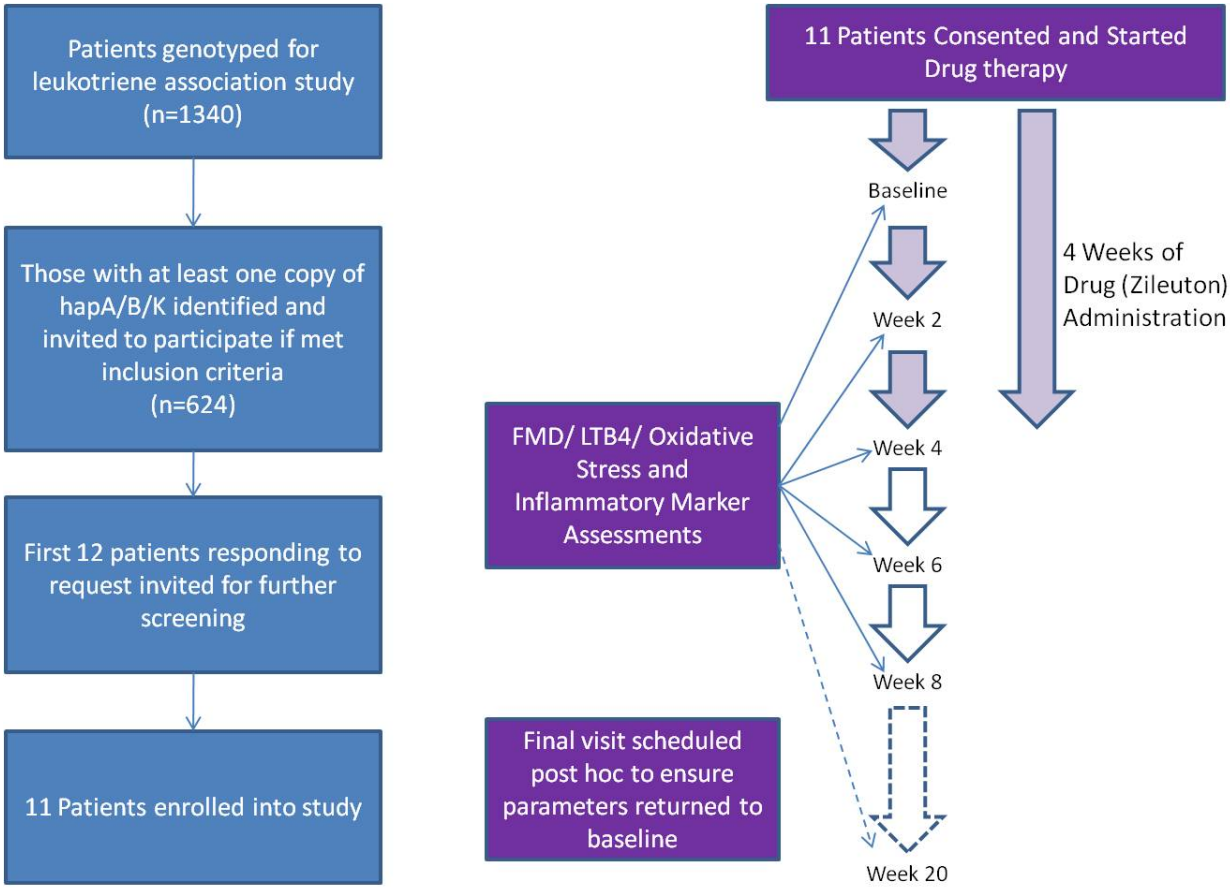


Figure illustrating subject recruitment flow: Screening of the Genebank cohort identified those genotyped for leukotriene variants; from these further selection identified a smaller pool of156 subjects who were contacted and the first responders then recruited sequentially

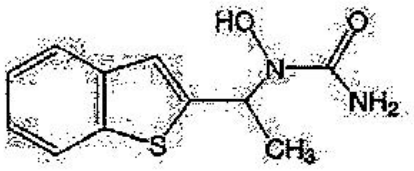
Exclusion criteria included: Inability to give informed consent; Acute MI during index admission or within 3 months diagnosed as >2-fold increase in CPK-MB or troponins >0.1mg/dL; Use of nitroglycerin 2 hours before vascular function testing; Age <21 or >80 years; premenopausal females with potential for pregnancy; current neoplasm; LFT elevation >2X upper limit of normal; any chronic systemic inflammatory disease; NYHA

Functional Class III or IV heart failure. Additionally, patients in whom there was to be an imminent change in medications or procedures were not recruited.

Study drug

Zileuton is an oral inhibitor of 5-LO that has recently received FDA approval for the treatment of asthma (Figure 22) (Appendix). Since both LTB_4 and the CysLTs have potential pathophysiologic roles in the development of atherosclerosis and its complications through different mechanisms, we hypothesized that 5-LO inhibition with zileuton, and therefore decreased LTB_4 and CysLT production, in patients with at-risk polymorphisms of the FLAP and LTA_4-H genes will reduce inflammation and oxidative stress and improve endothelial function. The CysLT receptor antagonists montelukast and zafirlukast may also improve inflammatory and vascular function parameters, but the effect of these agents is likely to be less pronounced as they do not have an effect on LTB_4 activity. Zileuton has been well tolerated when administered to patients with asthma. Dosing is recommended by the manufacturer at 600mg QID.(Zileuton (Zyflo) package insert) For the purposes of this study we selected this dose as it was the maximum dose based on drug safety trials for asthma. All subjects required 2 weekly liver enzyme tests with study drug termination if enzymes >3ULN.

Figure 22: Chemical structure of zileuton (Zyflo©)



Zileuton has the chemical name (±)-1-(1-Benzo[b]thien-2-ylethyl)-1-hydroxyurea and the following chemical structure:

Quantification of LTB4 levels

Leukotriene measurements were taken ex-vivo using a neutrophil stimulation technique. Neutrophils were first isolated from 12 ml of peripheral venous blood collected in EDTA vacutainers. Subjects were seen in the morning in a fasting state, and blood samples were stored at room temperature for no longer than 90 min before being

processed. Percoll gradient centrifugation was used to isolate neutrophils as previously described.(Wyche et al. 2004)

LTB4 concentrations in neutrophils were quantified with a commercially available LTB4 enzyme immunoassay. Assay Designs EIA assay kits (Ann Arbor, MI, USA) were used to measure the analytes and the assays were performed as per the manufacturer's instructions. The principle of this assay has been described previously.(Cuthbertson et al. 2000) Briefly, samples were added to a 96-well plate coated with monoclonal antibody against LTB4 and incubated for 2h at room temperature. Subsequently, the samples were removed and washed three times with buffer and an enzyme-linked antibody specific for LTB4 was added to each well and incubated at 37C for 2 h. Then the reaction was terminated by adding a "stop" solution. The optical density was determined by using a plate reader at 405 nm to determine the concentration of LTB4 with correction between 570nm and 590 nm. The mean concentration for each sample was obtained from the duplicate measurements.

Flow Mediated Dilatation:

Endothelial function was assessed with ultrasound based flow mediated dilatation of the brachial artery. A detailed description of the technique is given in methods chapter 2. Brachial artery diameter was assessed before and after 5 minutes of arterial occlusion to induce shear stress. The difference in diameter was expressed as a percentage of the baseline diameter. Measurements were taken at each clinic visit, in a quite temperature controlled room prior to any regular medications being taken in the morning.

Biomarkers:

We estimated several plasma biomarkers at each visit. These included plasma lipids (total cholesterol, HDL, LDL and triglycerides) and inflammatory markers (CRP and IL6). Full details of the techniques used to assess these biomarkers are given in chapter 2.

Major aminothiol compounds, indices of non-free radical oxidant stress, play a critical role in redox signalling and can be quantified in plasma to assess oxidative stress burden in-vivo.(Jones 2006) Of these, cysteine constitutes the major thiol pool *extracellularly* that reacts readily with oxidants to form its oxidized disulphide cystine. *Intracellularly*, glutathione is a major antioxidant that helps eliminate peroxides and maintain cellular redox, and its oxidized form is GSSG.(Jones 2006) Increased oxidative stress, measured as lower levels of glutathione, higher levels of cystine, or altered ratios of reduced to oxidized thiols, is associated with risk factors for cardiovascular disease (CVD), subclinical vascular disease and with adverse outcomes.(Moriarty et al. 2003; Ashfaq et al. 2008; Patel et al. 2009) We measured plasma cysteine, its oxidized form cystine, glutathione, and its oxidized disulphide (GSSG) in all subjects as described previously.(Jones and Liang 2009) Ratios of cysteine/cystine and glutathione/GSSG are expressed as redox potentials, E_hCySS and E_hGSSG, respectively, where a more oxidized value has a more positive numeric value.(Jones and Liang 2009) Briefly, samples were collected directly into tubes containing a preservative to retard auto-oxidation, centrifuged, and stored at -80°C, which shows no significant loss for 1 year. Analyses by high performance liquid chromatography were performed after dansyl derivatization on a 3-aminopropyl column with fluorescence detection. Metabolites were identified by co-elution with standards and quantified by integration relative to the internal standard, with validation relative to external standards as previously described.(Jones and Liang 2009) The coefficients of variation (CV) for glutathione was 5%, GSSG was 9.7%, cysteine 3.8%, and cystine 3.2%.

Statistics:

All continuous variables are described as mean ± standard deviation, while categorical variables are presented as proportions. The distribution of all variables was tested for normality and only plasma triglyceride and CRP levels required log transformation for parametric analysis.

We tested differences in flow mediated dilatation between visits using paired t-tests and with repeated measures linear models. Two tailed P values of <0.05 were considered statistically significant. All analyses were performed using SPSS v17.0 statistical package (SPSS, Illinois).

Results:

Population characteristics:

Of the 1340 genotyped patients, 624 were identified as carrying at least one copy (heterozygote or homozygote) of a risk haplotype (HapA,B or K). Approximately 10% of these patients were Black. Similarly HapB subject numbers were small, with prevalence in the population of around 5%. Of the 624 subjects, we excluded those living outside of the state or greater than 50 miles from the study site, those whose physicians requested exclusion and those who had requested not to be contacted as part of their original consent to participate in the Genebank cohort. The remaining 156 eligible participants were invited to participate (Table 35). Sequential recruitment based on first responders was commenced until 12 subjects had been recruited. After the baseline screening visit one subject was found to be ineligible due to recent and planned changes in medication. Thus, 11 subjects commenced the study drug and included 10 males and 1 female.

Table 35: Patient recruitment by leukotriene genotype

Haplotype	Ethnicity	Heterozygotes	Homozygotes	Total
HapA	White	260	21	281
	Black	25	2	27
HapB	White	45	1	46
	Black	16	2	18
HapK	White	209	26	235
	Black	17	0	17
Total risk carriers				624
Exclusions				468
Final recruitment pool				156

Enrolled patient characteristics are presented in Table 36. The mean age of subjects was 59 ± 5 years. The distribution of risk factors is given in Table 36. All subjects had at least one risk haplotype and included 5 with HapA; 0 with HapB; 9 with hap K and 3 with both hapA and K.

Table 36: Patient characteristics for the 5-LO drug study

Patient characteristics (n=11)	
Age, yrs	59.1 (5.55)
Male Gender, %	91
Caucasian, %	91
BMI, kg/m2	29.39 (5.28)
Hypertension,%	50
Systolic BP, mmHg	127.4 (17.2)
Diastolic BP, mmHg	77.0 (13.9)
Diabetes, %	40
Glucose, mg/dL	99.7 (14.1)
Smoking, %	90
Hyperlipidaemia, %	80
Total Cholesterol, mg/dL	177.4 (51.1)
Myocardial Infarction, %	50
Coronary Artery Disease, %	70
Median Gensini (IQR)	66.5 (3.5-138)

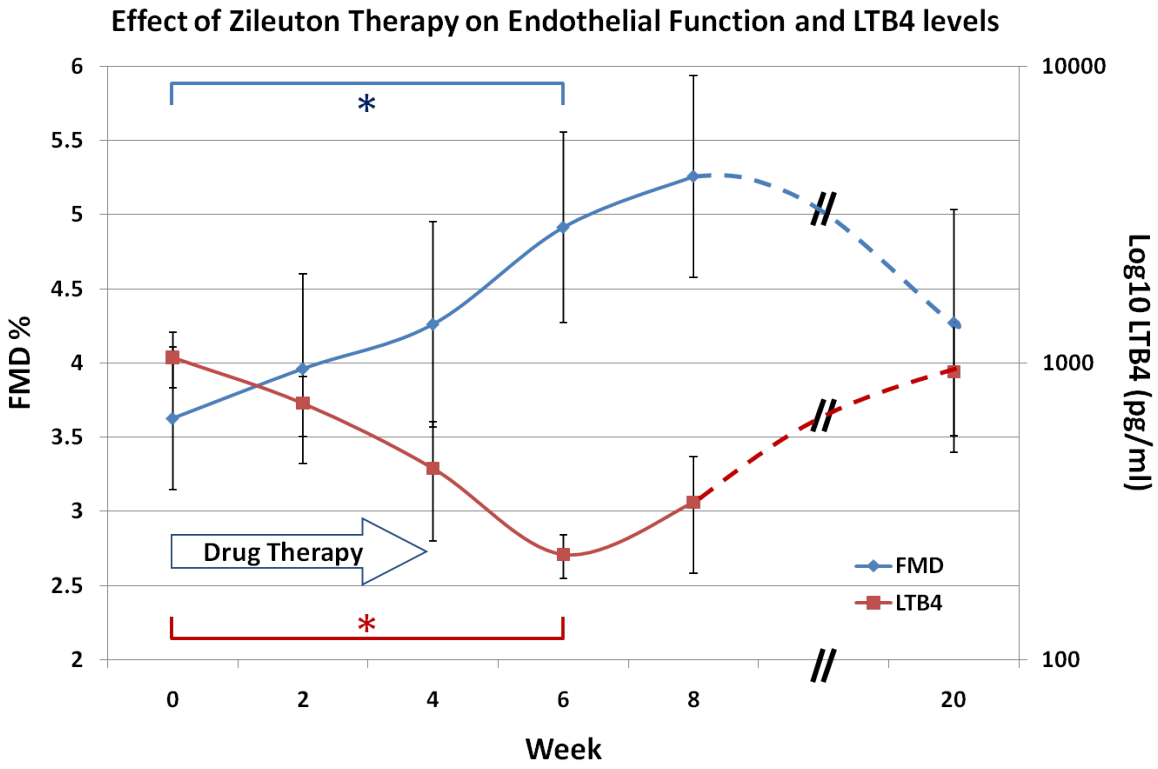
Demographic details for 11 study subjects enrolled. Value given are % or mean (standard deviation) unless stated. Gensini score of coronary disease severity/burden

Safety and efficacy - LTB_4 levels

Of the 11 subjects commenced on Zileuton, one subject developed elevated liver enzymes greater than 3x baseline and had to stop the drug after 2 weeks of therapy. The patient was withdrawn from further assessments and liver enzymes monitored until return to baseline at 3 months without clinical consequences. The remaining 10 (male) subjects completed the duration of drug therapy and study measurements.

Ionophore neutrophil stimulated leukotriene B4 counts dropped significantly during and after study drug administration. Levels had fallen by 29% by week 2, 58% by week 4 and 78% at week 6 when they reached a trough before returning towards baseline. There was a statistically significant decrease in LTB4 from baseline to week 4 (p=0.017) and week 6 (p=0.018), with no difference compared to week 8 (p=0.113) or 20 (p=0.798) (Figure 23).

Figure 23: Effect of zileuton on endothelial function and leukotriene levels



Graph illustrating changes in neutrophil stimulated LTB4 and endothelial function with time after 4 weeks of oral zileuton therapy

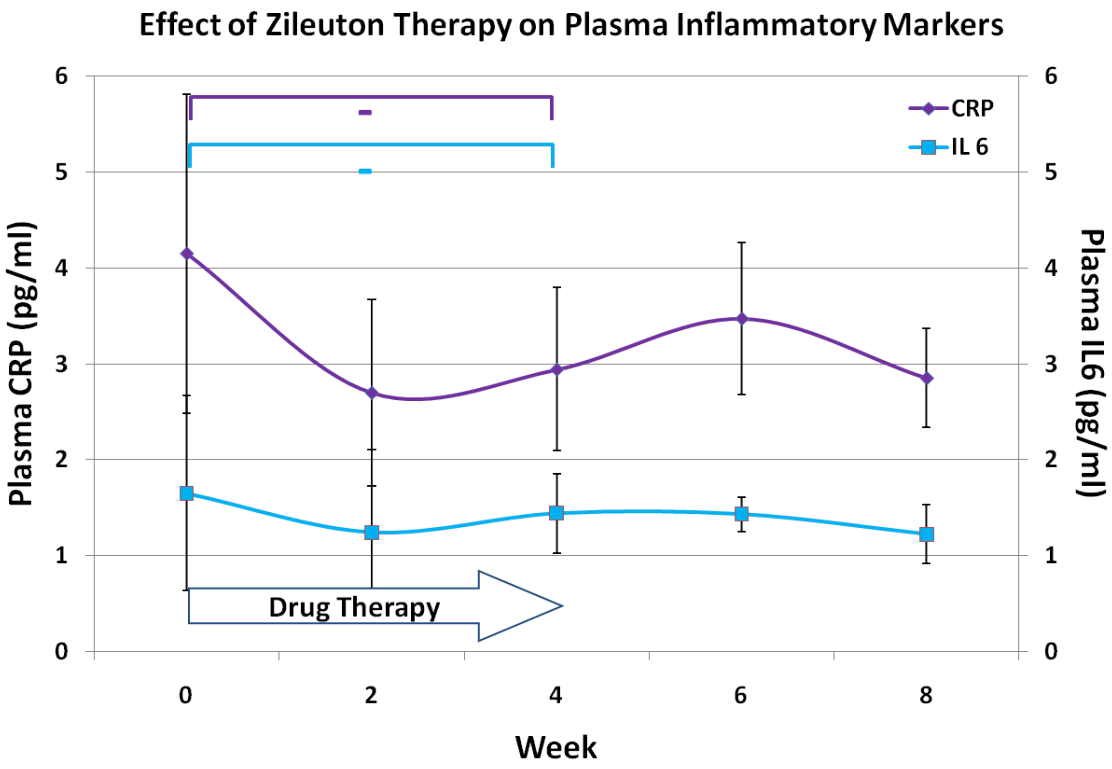
Endothelial function

Endothelial function, as measured by flow mediated dilatation progressively improved during and after drug therapy. The mean FMD for the group had increased from baseline by 9%, 17.5%, 35.5% and 44% at weeks 2, 4, 6 and 8. There was a significant difference in FMD between baseline and week 6 (p=0.022) and week 8 (p=0.007), and by week 20, it had returned towards baseline, such that there was no significant difference compared to baseline at this time point (p=0.28) (Figure 23).

Inflammation

While there was an early decrease in plasma CRP and IL6 levels after 2 weeks of Zileuton therapy. However there were no statistically significant differences in plasma levels compared to baseline at any of the measured time points (Figure 24).

Figure 24: Effect of zileuton on plasma inflammatory markers

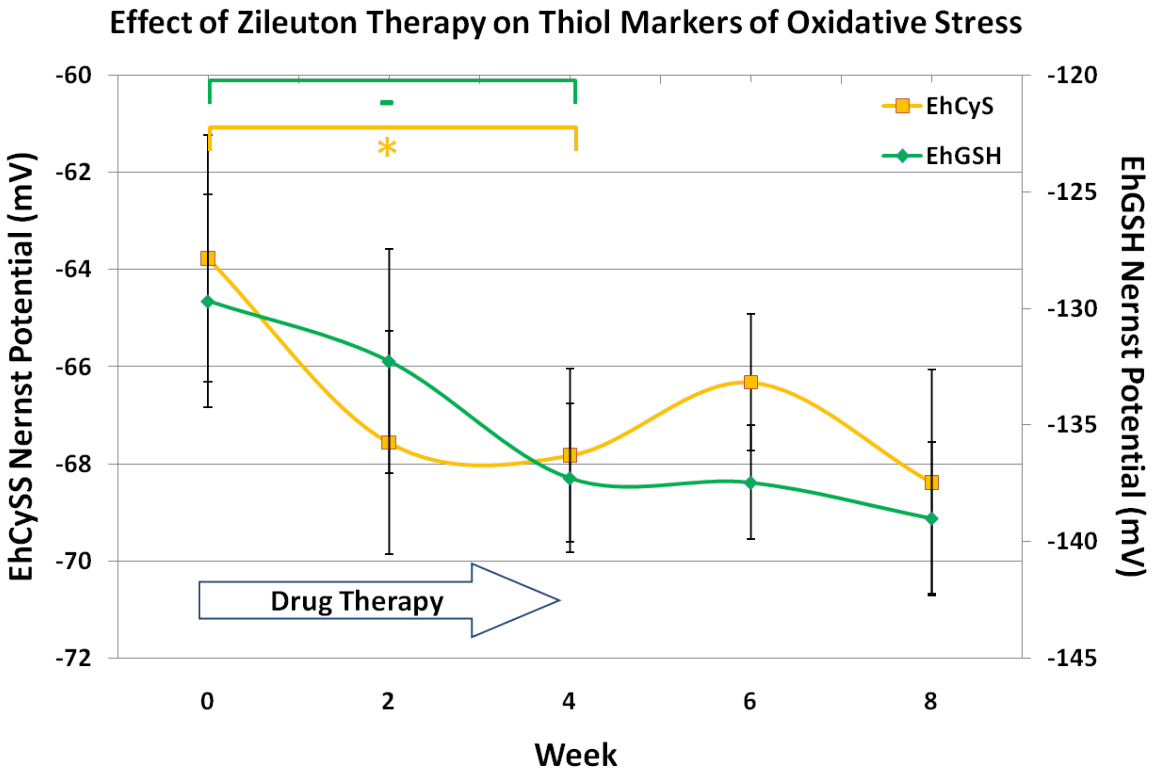


Graph illustrating changes in inflammatory markers after 4 weeks of oral zileuton therapy

Oxidative Stress

The redox potential of glutathione and cysteine, representing oxidative stress, demonstrated a trend towards improvement (more negative) during drug therapy. After 4 weeks of zileuton both redox potentials had improved by approximately 6% compared to baseline (EhGSH, p= 0.066; EhCySS p =0.029). However, values remained low up to week 8, with non significant differences compared to baseline (Figure 25).

Figure 25: Effect of zileuton on markers of oxidative stress



Graph illustrating changes in thiol markers of oxidative stress (redox values of CySS and GSH) after 4 weeks of oral zileuton therapy

Discussion

In this pilot study, we demonstrated that administration of a 5-LO inhibitor to CAD subjects carrying leukotriene risk genotypes, reduces leukotriene levels and in parallel improves endothelial function. These findings support the idea that genomic findings may yield novel therapies and also demonstrate that it is feasible to target therapy based on genetic risk.

The association between the leukotriene pathway and cardiovascular risk has been postulated based on multiple lines of in vitro, animal and human evidence. For example, atherogenic mouse models with deletions of loci containing the 5-LO gene or BLT receptors demonstrate protection against atherosclerosis, while studies of carotid plaque have identified substantial over expression of the 5-LO/ LT pathway in advanced

lesions and with greater expression of the enzyme in symptomatic or unstable plaque.(Mehrabian et al. 2002; Subbarao et al. 2004; Cipollone et al. 2005) In addition, LTB4 and its receptors play a key role in chemotaxis and monocyte migration across the endothelium as well as foam cell formation, while genetic variants in the pathway were identified to associate with greater carotid intima thickness (Aiello et al. 2002; De Caterina and Zampolli 2004; Subbarao et al. 2004).

The landmark genome wide linkage studies by Helgadottir et al, demonstrated variants in the ALOX5AP gene and the LTA4H gene as conferring risk of MI and stroke in different populations. (Helgadottir et al. 2004; Helgadottir et al. 2005; Helgadottir et al. 2006) Functional studies by the same group also demonstrated that subjects with the risk haplotypes who had experienced an MI had greater levels of leukotriene than controls and those with MI but without the genotype. Although one study has failed to validate greater leukotriene levels in young healthy subjects with leukotriene risk haplotypes, greater leukotriene activity is believed to represent the mechanism of risk.(Maznyczka et al. 2007)

These findings have raised excitement about the possibility of therapeutic intervention to reduce CV risk. Hakonarson et al demonstrated that treatment of 268 MI patients with at-risk variants of the FLAP or LTA_4-H genes with the FLAP inhibitor DG-031 reduced LTB_4 production and plasma MPO and CRP levels. (Hakonarson et al. 2005). The highest dose of the drug showed reduction of plasma MPO levels within 6 hours after the dose by 31% despite the use of statins in 90% of the cohort. A recent phase 2 trial in ACS patients has announced results for another agent called VIA 2291, a selective reversible blocker of 5-LO. The study was designed to establish the optimal dosing and safety data in 191 ACS patients, treated daily for 12 weeks with one of 3 dosing regimes.(Tardif et al. 2010) The authors noted a significant reduction in LTB4 measurements in a dose dependent manner. A further coronary CT based sub-study reported preliminary evidence of fewer new plaques in the treated group compared to placebo.(Tardif et al. 2010) However, large phase 3 trials with sufficient outcomes will need to be performed to demonstrate any benefit on CV risk.

In the meantime, studies such as ours have attempted to explore the effect of therapy on surrogate markers of risk such as endothelial function. We demonstrate for the first time a clear directional relationship between LTB4 suppression and improvement in endothelial function which resolved on cessation of drug therapy. This is perhaps unsurprising given that LTB4 has been shown to be potent vasoconstrictor in

guinea pig vascular preparations,(Back et al. 2004; Sakata et al. 2004) while selective inhibition of the BLT2 receptor in apoE knockout mice reduces vascular oxidative stress and improves endothelial function.(Hoyer et al. 2011) However, surprisingly few clinical studies have explored the effect of leukotriene antagonism in human endothelial function. In one study, Montelukast given to subjects exercising in high air pollution environments ameliorated the impairment of endothelial function.(Rundell et al. 2010) Another study is ongoing to examine the effect of Montelukast on endothelial function in patients after experiencing an acute coronary syndrome (clinicaltrials.govNCT00351364). Our study is unique in examining 5-LO synthesis antagonism thereby blocking both LTB4 and CysLT production as opposed to Montelukast which only blocks CysLT receptors and may not have obvious vascular effects, although some studies have shown that genetic variants in LTC4 synthase also confer some risk of CVD.

We did not however observe any significant impact on inflammatory markers with 5-LO inhibition. This is in contrast to the study by Hakonarson, and to that of Allayee et al who observed that in 60 pts followed for 6months as part of a RCT comparing Montelukast and theophyliine, subjects taking Montelukast had significantly lower levels of hsCRP and lipids.(Allayee et al. 2007) It is possible that the benefits of Zileuton are beyond inflammatory pathways although equally it is possible that we did not have sufficient power to detect significant changes or that we did not treat subjects for as long as these other studies to observe such effects. Another study is ongoing to also examine the effect of Montelukast on inflammatory mediators after ACS which may provide more answers to this question (clinicaltrials.gov).

Finally, we demonstrated that it is feasible to treat patients based on genotype. We selected from a subset of patients enrolled in the Genebank study, those carrying the risk variants and offered them specific therapy based on genetic findings suggesting that they could be at higher risk of events. However, it is currently unclear how genetic data in this context could be used in clinical settings. Potentially one day physicians may have access to complete patient genomic and variant data with cross referencing to databases of known risk genotypes to flag those conditions requiring specific therapy. If leukotriene variants were highlighted potentially drugs such as zileuton could be administered in a primary prevention setting.

Strengths and Limitations

A major limitation of this study is the lack of a control group, or subjects taking placebo. However the size of the effect and bidirectional nature of decreasing LTB4 and rising FMD suggests this effect is unlikely to be a placebo effect. Importantly, we were also unable to ascertain whether subjects with the risk genotype benefitted more from the drug than non carriers. However, this study was designed as a pilot or feasibility study and as such this is not a significant concern as these questions will now need to be addressed using well powered randomized trials.

Conclusion

In conclusion we have shown in this study that 1) it is possible to administer zileuton safely and obtain meaningful reductions in LTB4 levels and 2) that 5-LO antagonism is particularly beneficial to endothelial function. Further work is required to determine if this effect is only significant in carriers of risk variants or in all subjects. Either way, this exciting therapeutic option has arisen as a consequence of unbiased genome wide findings and as such genomic discovery may have started to fulfill one of its key promises.

Chapter 7: General Discussion, Conclusions and Future Work

General discussion

The overall aim of this thesis was to investigate mechanisms underlying risk from genetic variants for CHD discovered in recent genome wide association and linkage studies by refining the associated phenotypes. Genomic medicine promises to facilitate: 1) Identification of novel mechanisms of disease, 2) Enhanced individual risk prediction and 3) Novel therapeutic strategies with personalized genotype-based therapeutics.

Discovering novel mechanisms of CHD risk

Genome wide association is a hypothesis free approach which permits discovery of genetic variants outside of known candidate genes and is therefore able to deliver on this promise. The first wave of GWAS for CHD identified a dozen novel variants of which only 3 were considered to modulate lipid phenotypes (Chapter 1, Table 3). The remainder had no known function or obvious mechanism by which they could cause CHD.

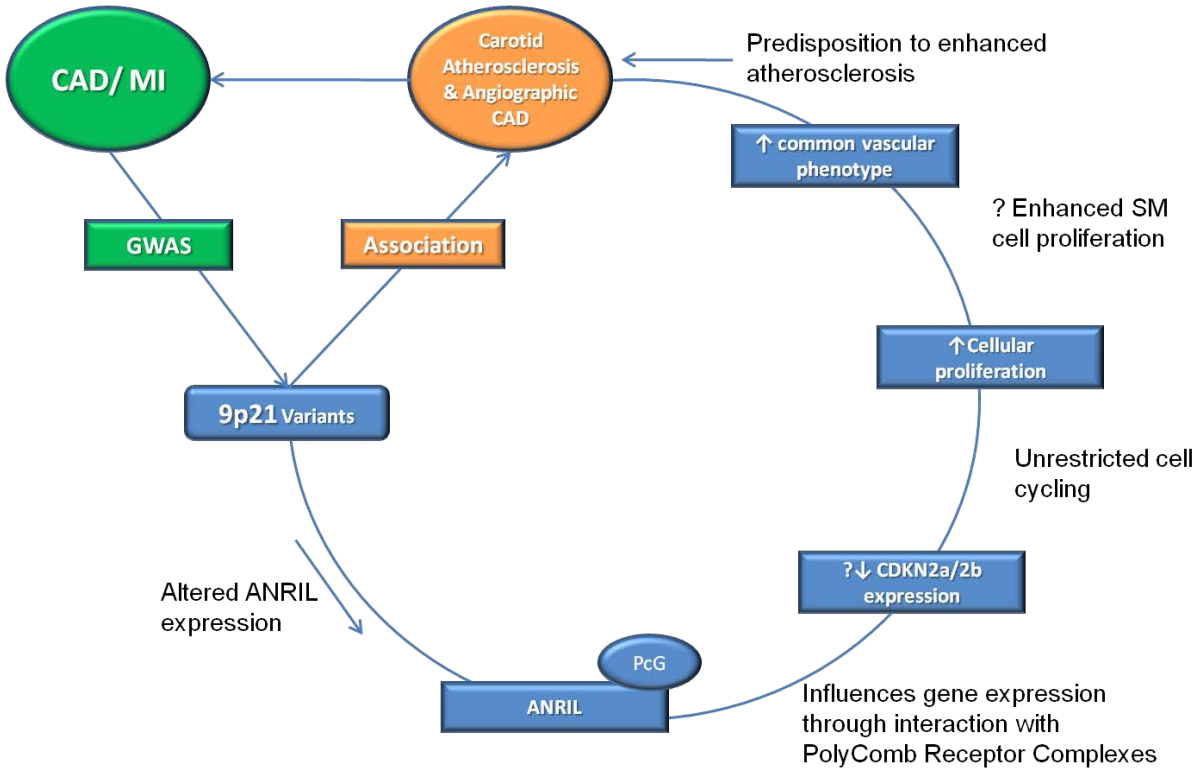
While one approach to this problem is to directly perform functional studies using, for example, knockout models of murine chromosomal analogues, another is to first refine the phenotype in order to focus functional studies towards a particular pathway.(Patel et al. 2010; Visel et al. 2010) GWAS performed to date have primarily examined CHD as a composite phenotype. However, as described in chapter 1, CHD encompasses a spectrum of phenotypes, each with very unique pathophysiological processes. Our understanding of these newly discovered variants has been incomplete due to phenotype heterogeneity, and refining the true association may assist in our efforts to understand how risk for CHD is manifested and thus can be modulated.

In chapter 3, we described 2 studies where we sought to refine CHD characteristics by using a carefully phenotyped population undergoing coronary angiography. In the first of the two studies we demonstrated that 9p21 is conclusively associated with angiographic coronary disease severity and progression. At around the same time that this data was reported, a second group also published almost identical findings.(Dandona et al. 2010) Together with earlier reports of association with carotid

atherosclerosis, our work has now established that 9p21 confers risk by promoting an atherosclerotic phenotype rather than a thrombotic or plaque rupture mechanism.

Functional studies have since made several advances in understanding the mechanism of 9p21. Even though the causal variants within this locus have yet to be identified, the risk associated SNPs appear to cluster together within a 53 kb interval roughly 100 kb upstream to the nearest gene cluster of *CDKN2a/b* (*INK4/ARF*). These genes encode three cyclin dependent kinases: $p16^{INK4a}$, $p15^{INK4b}$ and ARF ($p14^{ARF}$ in humans, $p19^{ARF}$ in mice) and gene expression has been correlated with 9p21 risk variants. (Jarinova et al. 2009; Liu et al. 2009; Cunnington et al. 2010) This is of interest in the context of our study, given that these proteins regulate cell division, restraining aberrant proliferation, a process thought to be important in atherosclerosis and confirmed by animal studies.(Gizard et al. 2005; Gonzalez-Navarro et al. 2010) Emerging evidence now suggests 9p21 variants may influence ANRIL regulation, (Folkersen et al. 2009; Holdt et al. 2010; Holdt et al. 2011) which in turn may regulate the CDKN2a/b gene complex through polycomb (PcG) complexes which inhibit gene expression through transcription repression.(Bracken et al. 2007; Yap et al. 2010) Altered expression and function of these genes may lead to excessive cellular proliferation and a potential vascular phenotype compatible with findings such as ours, which could predispose to atherosclerosis, thereby providing a possible mechanistic pathway for increased CHD risk from 9p21. (Figure 26)

Figure 26: Overview of postulated molecular basis of risk for 9p21



The second study in chapter 3 sought to refine the CHD phenotype for all 8 variants without known function (4 were believed to modulate lipid levels). We demonstrated that by using coronary angiographic data it is possible to identify specific and differential associations with sub-phenotypes of CHD. Of the 8 variants tested, 5 associated with MI. We then confirmed association with 9p21 and CAD severity, but also showed that variants at 1q41 and 6p24 also associated with CAD or atherosclerotic phenotypes and like 9p21 they also confer risk of MI through enhanced atherosclerosis. In contrast, two other variants at 21q22 and 2q33 associated with a phenotype of MI in the presence of CAD. We had previously shown in a larger multicenter collaboration that the 12 GWAS discovered variants associating with CHD do not confer risk of MI precipitation when CAD has already been established. However, we later proposed, as in this chapter, that CAD is a time-dependent phenotype, and to avoid misclassification, younger subjects and older controls had to be used. Overall, the conclusion from both of these studies was that it is important to refine the broad CHD phenotype to permit

better understanding of mechanism of risk. Large scale GWAS necessarily need to mix phenotypes to achieve statistical power. However, follow up studies such as this could play a key role in replicating and refining the underlying associations.

In chapter 4, we conducted two studies that further refine the phenotype but this time using intermediate phenotypes potentially causal in the pathway to CAD or MI. In the first, we confirm findings from other studies that the 8 risk variants without known function do not associate with quantitative measures of lipid/glucose; obesity and inflammation or blood pressure and heart rate. We observed some trends towards association with metabolic syndrome components for the variant at 12q24. While this was not replicated in the larger CAD cohort, the multiple associations with related phenotype warrants further study. We concluded that these variants likely modulate novel mechanisms of risk outside of influencing traditional risk factors such as lipids or inflammation. This is a very important finding, as it suggests that in carriers of these risk variants we may not be able to modulate all of the risk by modifying traditional risk factors such as statins or diabetes. As such clinical use of these risk variants may not be as simple as first postulated.

We followed on from this study, having concluded that these variants do not associate with *traditional* intermediate phenotypes, to see if they would associate with *novel* intermediate phenotypes. This strategy has already been used for 9p21, with associations tested for endothelial progenitor cells, intracranial aneurysms, echocardiography parameters and more.(Patel and Ye 2011) In our study we sought to associate these variants with the phenotype of central aortic pressure augmentation. Altered central haemodynamics arise as a consequence of arterial stiffness and greater arterial wave reflections which lead to greater after-load and myocardial infarction. Importantly, it is potentially modifiable with existing medications. Our study suggested that one variant at 6p24, which also associates with atherosclerosis from the previous chapter, may modulate this phenotype. Importantly, an independent GWAS found a positive association for a variant which is in tight LD with 6p24 with the independent but related phenotype of pulse wave velocity, which measures arterial stiffness. It is therefore possible that this locus modulates some structural feature of the arterial wall which may predispose to arterial stiffening and atherosclerosis. This is plausible given that it resides in an intronic region of PHACTR1, a gene involved in calcium cycling which may be relevant for vascular tone and smooth muscle function.

Studies in chapters 3 and 4 have thus demonstrated the importance of refining the genotype-phenotype associations from GWAS using detailed and refined phenotypes. This approach has replicated existing findings and yielded important novel findings which are now being pursued with functional studies. Although these studies have not identified a definite molecular mechanism for any of these variants, they have enhanced our understanding of these risk variants incrementally. With these studies we therefore propose a model whereby large scale GWAS identify genotypic signals using broad phenotypes to achieve statistical power; these findings can then be followed up by smaller studies with deeper phenotyping to refine the underlying association.

Enhanced individual CHD risk prediction

As outlined in chapter 1, risk prediction using traditional risk factors and risk models have significant limitations in predicting risk at an individual level. Genetic risk prediction has been proposed as offering more precise estimates of personalized risk. Genotype based risk prediction has a number of advantages over conventional biomarkers as genotypes are (1) fixed from birth, allowing early risk prediction; (2) less susceptible to biological variation over life, such as during inter-current illness, etc; and (3) are easy to obtain with minimal measurement error. Previously, a number of studies attempted to utilize risk scores from individual candidate genes in the hope of improving predictive value, but failed to demonstrate clinical utility.(Morrison et al. 2007) With the recent advances in genomic research, hopes have been raised again of utilizing genetic data to improve risk prediction.

In chapter 5, we examined the predictive utility of 9p21 for incident events in a CAD population over a 2.7 year follow up but found no significant association between genotype and incident risk. This was surprising given the robust cross-sectional associations with CHD. However, since then several other groups have identified similar lack of association in subjects with CAD. This is in contrast to population studies that have repeatedly found association with 9p21 and outcomes. The discrepancy may to be due to treatment effects given that patients with CAD are aggressively treated with prognostically important medications.(Patel et al. 2011b) Importantly, even studies showing association with genotype and events generally failed to improve the c-statistic as a metric of added value above traditional risk factors. Others noted some improvement in alternative metrics such as risk reclassification, (Wang et al. 2006;

Pencina et al. 2008) but the general consensus is currently that 9p21 does not add to risk prediction and its role in this is currently unclear particularly for secondary risk prediction.

Given that the "common disease common variant" hypothesis postulates the role of multiple variants in genetic predisposition to CHD, the next question was whether combination of risk variants into a risk score could be of greater value in risk prediction. In the second study in chapter 4, we created a risk score consisting of 11 CAD/MI variants and demonstrated that subjects with the very highest quintile of risk score showed association with prevalent MI with onset before the age of 70 years, with an effect size which was greater for more premature MI. This risk score also improved the C-statistic in models of prevalent MI, and did so especially when only family history and gender were modeled, given that they are fixed variable known in early life. However when we factored angiographic CAD across cases and controls, this effect was attenuated confirming prior work that these variants likely promote MI risk through atherosclerosis rather than any thrombotic or plaque rupture mechanisms per se.(Reilly et al. 2011) Importantly we were unable to show significant association with incident risk of MI or death. As with the 9p21 and incident risk study, this may be due to unmeasured treatment effects or that the variants fail to confer greater risk of MI once CAD has manifested. Our study was the first to examine the risk score in a CAD population to examine secondary risk prediction. Others have also created risk scores to enhance prediction models to improve on single marker risk prediction.(Davies et al. 2010) Talmud et al for example proposed that a hypothetical model with 10 SNPs with similar risk effect sizes to 9p21 would enhance the c-statistic.(Talmud et al. 2008) The results from two other large studies utilizing risk scores in population based cohorts are conflicting. (Paynter et al. 2010; Ripatti et al. 2010) Even the study confirming association with risk score and incident events failed to improve the c-statistic over traditional models of risk.

These studies including ours highlight the problem that genotype based risk prediction is difficult and does not add more information to that currently available, especially for secondary risk prediciton. Successful models of genotype based prediction will likely require even more variants given their small effect sizes. Statistical models suggest that we may need >200 of these common variants with ORs similar to those being observed of around 1.1 to obtain an effect size similar to sibling relative risk for complex disease such as CHD or diabetes.(Kraft and Hunter 2009)

It could, however, be argued that 10-year risk prediction algorithms, which is the current metric against which genetic risk prediction is currently assessed, are inappropriate. First, these risk algorithms do not fully capture cardiovascular risk and many individuals with low scores succumb to disease.(Cooper et al. 2005) Importantly, genetic effects are likely to be continuous from birth or may be enhanced or suppressed in response to environmental influences and may simply require a longer time frame in which to manifest their effects. Furthermore, once disease is established, as in middle age, genetic risk prediction will be relatively less effective as the genetic burden may already have taken its toll. As such some commentators have suggested use of lifetime risk scores to better assess predictive utility of genotype data.(Johansen and Hegele 2009)

Perhaps, genetic risk scores may find their niche in very young subjects, in whom atherosclerosis will have begun even before some other risk factors such as hypertension and dyslipidaemia have developed or impacted. This is perhaps intuitive given that genetic effects are most apparent in the young, possibly explaining why one study of elderly subjects found no association between 9p21 and incident risk.(Dehghan et al. 2008) Indeed in our studies in chapter 4, we demonstrate that association of the risk score with prevalent and incident MI is greatest in younger subjects. Risk prediction in early life would rely on known risk factors at the time, limited to gender and possibly family history. As such, use of genotype data at this stage would be expected to add significantly to risk prediction models. In fact we confirmed this hypothesis and found that when genetic risk score was added to an AUC model containing only gender and family history, there was a significant improvement in the c-statistic.

An important question often posed is why not use family history (FH) of CHD instead of complex molecular medicine to assess genetic risk? After all it has virtually no cost, encompasses all common and rare genetic variation as well as potential gene-gene and gene-environment effects. Interestingly, 9p21 appears to improve risk reclassification in models with the Framingham risk score which does not include FH, while those using the Reynolds Risk Score (which does include family history) actually showed greater misclassification.(Paynter et al. 2009) The study by Paynter et al also showed that FH was significantly associated with incident risk, while Talmud et al demonstrated that FH improved risk reclassification as much as if not more so than 9p21 alone. In contrast, the association of a genetic risk score with events by Ripatti et

al. found it was independent of FH and the association of 9p21 with prevalent MI also appears to be independent of FH.(Ripatti et al. 2010) We confirmed this finding in our analysis, demonstrating that the mean genetic risk score was not significantly different in patients with and without a positive FH. It is therefore unclear at present, given the known difficulties and variation in acquiring accurate FH data, whether genotype data adds more to risk prediction than FH alone. However, advantages of genotype data over FH include (1) providing an alternative when parental history is unknown; (2) earlier availability – at birth when parents may be young and have not developed the disease; and (3) avoidance of difficulties in assessing FH and relying on patient reported data.

Going further, even if genetic risk prediction comes to fruition, it is currently unclear how this risk could be modified. Given that some of the genetic risk is mediated by novel mechanisms and independent of known risk factors, simple modification of the latter would not be expected to reduce it associated risk. It may be argued that knowledge of increased susceptibility to CHD, for which protective lifestyle interventions exist, will motivate individuals to modify their life style. Yet as intuitively appealing as this contention may be, evidence to support it, particularly in the case of complex (non-Mendelian) disease, is currently scanty. A relevant study by Topol and colleagues recently demonstrated that in subjects undergoing a commercially available genetic test for a variety of diseases, there was no appreciable change in any behavioral, psychological or health screening measures at 6 months.(Bloss et al. 2011) The flip side, of course, is that patients who test negative may be falsely reassured and thus less motivated to comply with preventive recommendations.

In summary, it remains unclear whether genetic risk prediction using currently available data is likely to offer added value to current strategies and may be of more value in primary rather than secondary risk prediction. However, using genetic data in younger subjects at a time in life when traditional risk factors may not be evident is a strategy that requires further assessment. In addition, few novel risk factors appear to improve the C-statistic and so, use of alternative metrics to assess added value of novel biomarkers may be needed with longer time frames to fully appreciate genetic risk.

Novel therapeutic strategies and personalized medicine

Finally, the third promise of genomics is that it will lead to novel therapies which may be used to treat patients based on their genotype. Given that mechanisms for GWAS findings so far have not been fully elucidated, we are still some way from developing new therapies using this information. In contrast one of the most promising findings from genome wide linkage scans identified leukotriene gene variants as associating with MI risk. Furthermore, as added evidence of a link, risk carriers were noted to have greater levels of the product of the leukotriene pathway, LTB4 a potent inflammatory mediator.

In chapter 5 we therefore sought to determine whether targeting the leukotriene synthesis pathway, in subjects carrying the risk haplotypes could improve surrogate markers of risk. We demonstrated a clear relationship between drug related decreases in LTB4 levels and improved endothelial function. There were also trends towards improved oxidative stress biomarkers. The study showed that genetic findings can promote new therapies, as in this case where the pathway was only previously considered in asthma, but following genetic discovery focus shifted on leukotriene therapies for cardiovascular risk. While the study was unable to determine if therapy benefits are related to genotype status, it did confirm that it is feasible to identify patients carrying risk genotypes and then to target them with specific therapy.

Therapeutic advances are the ultimate aim of genomic research and will surely arise from the wealth of data so far generated. Intriguingly one of the variants discovered through genome wide association for lipid traits was found to be in the HMGCR gene.(Kathiresan et al. 2008; Willer et al. 2008; Reilly et al. 2011) Had this pathway not been known previously it is not inconceivable that functional studies would have identified its role and led to development of statins? Similarly, the role of the 1p13 variant is being actively elucidated as a key regulator of the sortillin protein in lipid metabolism and therapeutic strategies to modulate this protein are being actively pursued.

While genomic advances may lead to development of new therapies, it is likely that these new drugs would be used in a non targeted manner in the first instance. Although we have shown in chapter 5 that it is possible to identify and treat patients based on genotype, there are multiple challenges to initiating personalized or genotype based therapy in current clinical scenarios and healthcare settings. For example, recent

candidate gene and GWAS studies have shown significant progress in identifying variants which predict drug metabolism and side effects (e.g. CYP2C19 for clopidogrel; VKORC1 for warfarin; SLCO1B1 for statin myopathy).(Roden et al. 2011) For example, platelet reactivity studies suggest that as much as 70% in variability of clopidogrel dosing is heritable with the CYP2C19 common variant contributing approximately 15% of this, while common SNPs in VKORC1 are thought to account for 25% of variability in warfarin response.(Rieder et al. 2005; Shuldiner et al. 2009) In fact the drug label for clopidogrel now even describes variable response with genotype and a commercial test (Roch AMPLiChip for CYP2C19) has gained FDA approval. Despite this, it remains unclear how best to incorporate these findings into clinical practice and prospective studies are ongoing to determine if genotyping before prescribing can provide cost effectiveness and clinical benefit in guiding dosing or drug choice. If they do, then we will be faced with the practicalities of obtaining genotype data prior to prescribing and IT systems to support this new approach to prescribing.

Thus, genome wide discoveries are showing enormous potential in stimulating the search for novel therapies along with potential to personalize medical therapy. While novel drugs arising from GWAS discoveries are some way off, studies such as ours have shown that genetic studies can help to identify new therapeutic options for CV disease and that it is feasible, albeit challenging, to identify subjects on the basis of genotype to target therapy.

Conclusions

In summary, the main conclusions that can be drawn from this body of work include the following

1) The 9p21 MI risk locus is associated with angiographically defined coronary artery disease presence, burden and progression, supporting the growing body of evidence that it confers an atherosclerotic phenotype.
2) In addition to 9p21, the 1q41 and 6p24 loci are also associated with atherosclerotic phenotypes while 21q22 and 2q33 loci appear to confer risk of MI in the presence of established CAD suggesting they at least may mediate a plaque rupture or thrombotic phenotype
3) Of the 8 variants whose mechanistic basis is unknown, none associate with traditional and quantitative intermediate phenotypes of lipid and glucose, blood

pressure or obesity and inflammation parameters. This suggests that their mechanisms of risk are mediated through non-traditional pathways.

4) The 6p24 variant associates significantly with non invasively derived central pressure augmentation indices suggesting its phenotype may be related to systemic arterial stiffness and/or vasomotor tone leading to adverse arterial wave reflections and cardiovascular morbidity and risk.
5) The 9p21 risk locus does not associate with incident risk of MI or death in a population with CAD during a follow up period of 2.7 years. This is in contrast to prospective population studies showing association with incident events, but is in keeping with other prospectively studied CAD populations.
6) Similarly a multi-locus genetic risk score composed of 11 novel GWAS variants also associates with prevalent MI but not incident risk of MI or death in a population with CAD over a short follow up period. These findings suggest there may be differences in clinical utility for primary and secondary risk prediction.
7) Treatment of subjects carrying leukotriene risk haplotypes with a leukotriene synthesis blocker for 4 weeks reduces LTB4 levels and in parallel improves their endothelial function as assessed by brachial flow mediated dilatation. This has important implications for the novel use of existing leukotriene agents to treat CV risk as well as the concept of targeting drug therapy based on genotype.

Future work

These studies have highlighted that genome wide discovery is only the first step towards realizing the potential of genomics to cardiovascular medicine. Nonetheless, at the time of writing two studies of unprecedented size have been reported and identified many more novel variants for CHD.(C4D Consortium 2011; Schunkert et al. 2011) Thus in total, SNPs at over 30 chromosomal loci are today convincingly associated with CHD. (Table 37) It is apparent from Figure 27 that these common variants only confer modest risk ranging from 5-20% and while larger studies will be required to detect variants with still smaller effect sizes, greater effort identifying rarer variants may yield greater effect sizes and molecular insights into disease. The systematic characterization of rare <1% variants is beyond the means of studies that currently rely on LD patterns such as GWAS and it is likely that these approaches will be very shortly completely replaced by new high-throughput sequencing technologies to examine all variation in entire genomes. In the meantime until technology and cost is feasible for large scale

sequencing, studies are exploring intermediate frequency variants (The 1000 Genomes Project is comprehensively identifying variants with a frequency of >1%) as well as protein coding sequences (exomics uses sequencing of protein coding regions). The "1000 dollar genome" however is not far off, and may be available sooner than anticipated.

Ultimately greater focus needs to be directed towards functional studies to identify new molecular mechanisms underlying genetic risk. Work with 9p21 and 1p13 has shown significant promise and similar efforts need to be pursued for the remaining 30 or so variants now reported for CHD (Table 37). Further follow up studies to refine phenotypes such as those in this thesis may help identify mechanistic clues to these newer variants to guide functional enquiry.

In relation to this thesis, replication studies need to be conducted to validate our finding of association between CAD and MI for the variants described in chapter 3 and for the association between 6p24 and central haemodynamics as this may be amenable to therapy.

Genotype based risk prediction remains difficult and uncertain. However, we have shown that it is possible to create a risk score from published data and that this score is able to predict incident events. Scores now need to be constructed for all 30 or so CHD variants and tested in both primary and secondary prevention cohorts to establish their clinical validity and clinical utility. Prospective studies for the latter are especially important to determine if genotype based risk prediction and lifestyle interventions are effective and cost efficient. Other studies also need to try and demonstrate clinical utility in younger individuals, for a longer follow up and with metrics other than the c-statistic to evaluate their value.

Finally, our finding of leukotriene synthesis blockade and improved endothelial function needs further assessment given the substantial size of benefit observed. We would propose a randomized clinical trial of zileuton therapy with a genotype based selection arm to demonstrate if there is firstly benefit on surrogate markers of risk over placebo and whether there is a genotype interaction. This can then be followed with a clinical trial if results are promising.

Table 37: Significant GWAS findings for CHD as of 2011

			Allelic Effect Sizes from Large Scale GWAS (Phenotype)						
Locus	**Gene(s) region**	**Risk Allele Freq***	**A (CHD)**	**B[#] (CHD)**	**C (MI)**	**D (CHD)**	**E (*a*CAD)**	**F (CHD)**	**Refs for F**
1p13.3	CELSR2-PSRC1-SORT1	0.78	1.11	1.14	1.19	1.13	1.33		
1p32.2	PPAP2B	0.91	1.17						
1p32.3	PCSK9	0.82	1.08	1.05	1.15		1.03		
1q41	MIA3	0.74	1.14	1.09	1.14	1.10	1.09		
2q33.1	WDR12	0.15	1.14	1.11	1.17		1.28		
3q22.3	MRAS	0.18	1.12	1.08			1.15	1.15	(Erdmann et al. 2009)
6p21.31	ANKS1A	0.75	1.07						
6p24.1	PHACTR1	0.67	1.10	1.11	1.12		1.17		
6p24.1	C6orf105	0.07[+]						1.51	(Wang et al. 2011b)
6q23.2	TCF21	0.62	1.08						
6q25.3	LPA	0.02	1.51	-				1.92	(Tregouet et al. 2009)
7q22	BCAP29 + others	0.80		1.08					
7q32.2	ZC3HC1	0.62	1.09						
9p21.3	CDKN2A/B, ANRIL	0.46 - 0.56	1.29	1.20	1.29	1.20	1.30	1.20 - 1.33	(Helgadottir et al. 2007; McPherson et al. 2007; Samani et al. 2007; 2007)
9q33	DAB2IP	0.30						1.18	(Gretarsdottir et al. 2010)
9q34.2	ABO	0.37					1.21		(Reilly et al. 2011)
9q34.2	ABO	0.21	1.10						
10q11.21	CXCL12	0.87	1.09	1.06	1.17	1.11	1.08		
10p11.23	KIAA1462	0.38		1.07				1.15	(Erdmann et al. 2011)

10q23	LIPA	0.42		1.09					
10q24.32	CYP17A1,CNNM2,NT5C2	0.89	1.12						
11q22	PDGFD	0.32		1.07					
11q23.3	ZNF259, APOA5-A4-C3-A1	0.13	1.13						
12q24.12	SH2B3	0.44	1.07	1.05				1.13	(Gudbjartsson et al. 2009)
13q34	COL4A1,COL4A2	0.44	1.07						
14q32.2	HHIPLI	0.43	1.07						
15q25.1	ADAMTS7	0.57	1.08			1.19			
15q25	ADAMTS7-MORF4LI	0.65		1.07					
17p13.3	SMG6,SRR	0.37	1.07						
17p11.2	RASD1,SMCR3,PEMT	0.56	1.07						
17q21.32	UBE2Z,GIP,ATP5G1,SNF8	0.53	1.06						
19p13.2	LDLR	0.77	1.14	1.09	1.15	1.18			
21q22.11	SLC5A3-MRPS6-KCNE2	0.15	1.18	1.09	1.20	1.18			

Summary of major GWAS associations for CHD phenotypes, with effect sizes given from the largest published GWAS: A= CARDIoGRAM(Schunkert et al. 2011); B= C4D(2011); C= MIGEN(Kathiresan et al. 2009a); D= CAD Consortium(Samani et al. 2009); E= Reilly et al(Reilly et al. 2011); F= Individual GWAS

**Risk allele frequencies taken from the largest GWAS studies; #Pooled results for Caucasian and South Asian Populations; +Chinese Han population;*

CHD= Coronary Heart Disease; MI = Myocardial Infarction; aCAD = Angiographic CAD v Angiographic Controls;

Figure 27: Effect sizes of all GWAS CHD variants discovered by 2011

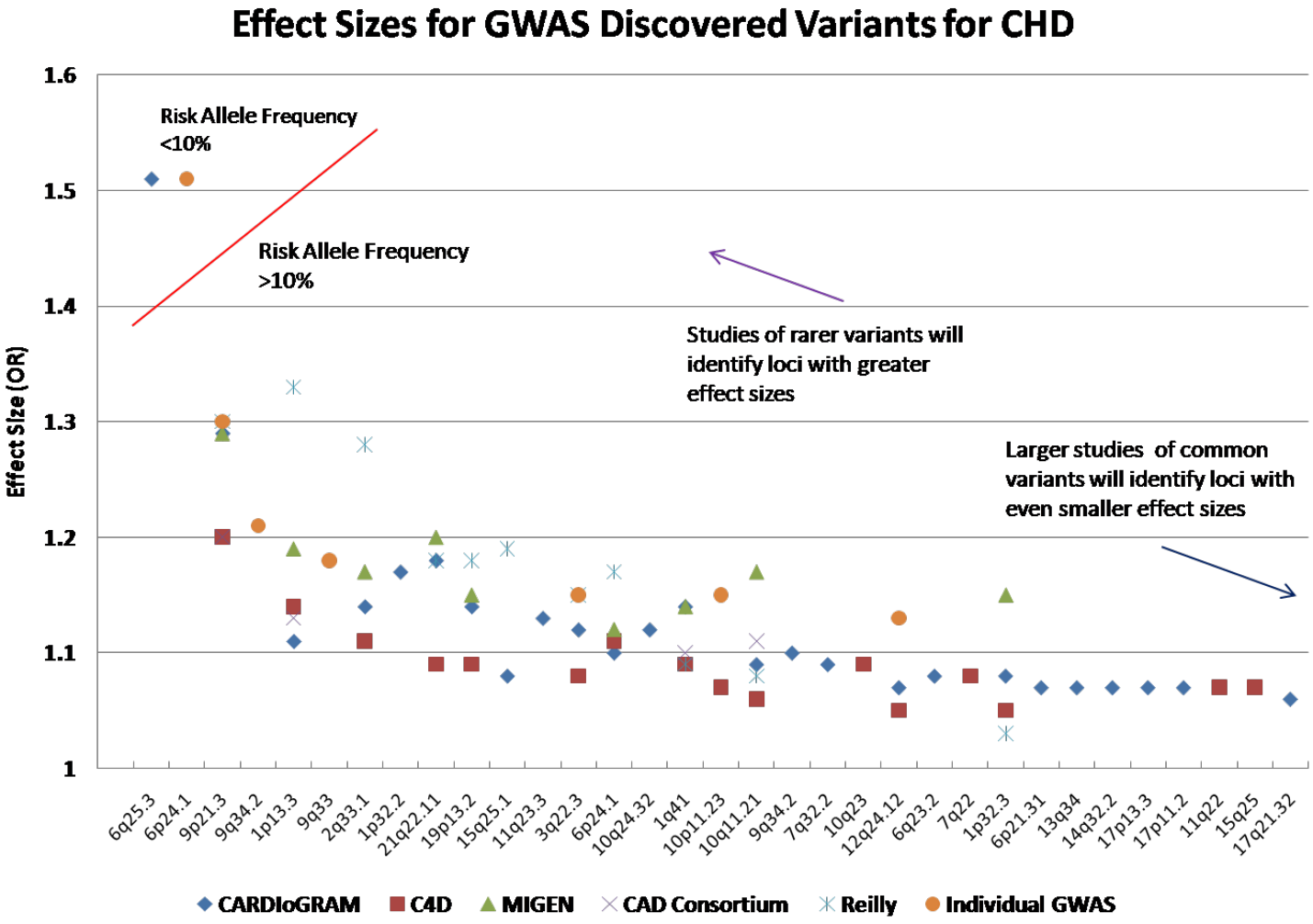


Figure 27 illustrating the effect sizes of the common variants identified for CHD. The largest studies of CARDIoGRAM and C4D have identified the smallest effect size variants below 1.10 and further larger GWAS consortia will identify variants with still smaller effect sizes. However it is likely that larger effect sizes will be identified through study of rarer variants through sequencing approaches rather than traditional GWAS. Unsurprisingly the two SNPs with the highest effect sizes have frequencies of <10%.

The Future

In summary, enormous strides have been made to understand the hereditary basis of CHD in recent years. As we move beyond GWAS and into the realms of next generation sequencing, functional studies are rapidly progressing in parallel to mine this wealth of new data and to enhance our understanding of this complex disease and of genomic regulatory mechanisms, with the ultimate hope of developing new therapies. Meanwhile, prospective clinical studies will help answer the question of clinical utility of genotype based testing for CHD and determine if the pipe dream of personalized medicine will materialize or remain just a vision. Today, while we still have some way to go, the evidence suggests that we should remain quietly optimistic that the genomic revolution will soon transform our approach to CHD and healthcare in general.

Chapter 8: References

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Zileuton (Zyflo) package insert.

Appendices

I. Genebank Questionnaire

II. Chart Abstraction form

III. Blood sample processing protocol

IV. Follow Up Form

V. Angiographic scoring rules

VI. Example PLINK files

VII. Zileuton Product Insert

Appendix I

Genebank Questionnaire

EMORY HEALTHCARE

Patient Study ID / Label

The Emory Cardiology Bio-Bank

Patient Questionnaire

Confidential

Please do your best to complete this questionnaire during your admission. If it cannot be completed during your stay, we may need to contact you by telephone to fill in any missing information. *If you do not wish to be contacted again for this or for other purposes, then please inform the fellow/researcher.*

Thank you for participating in this study.

EMORY UNIVERSITY

PATIENT IDENTIFICATION

Today's date (date of enrollment) mm/dd/yyyy _ _ / _ _ / _ _ _ _

First Name _____ Last Name _____

Date of Birth _ _ / _ _/ _ _ _ _

Full Address _____

City _____ State _ _ ZIP code_ _ _ _ _

Home phone (_ _ _) _ _ _ - _ _ _ _ Work phone (_ _ _) _ _ _ - _ _ _ _

Cell phone (_ _ _) _ _ _ - _ _ _ _ e-mail _____

RELATIVE CONTACT INFORMATION

Relationship: _____

First Name _____ Last Name _____

Address _____ State _ _ ZIP code_ _ _ _ _

Contact Phone (_ _ _) _ _ _ - _ _ _ _

BASELINE INTERVIEW STATUS & DEMOGRAPHICS

Are you completing this form **BEFORE** or **AFTER** your procedure? (Please circle)

HEIGHT & WEIGHT? Height : _____ Weight : _____

AGE? _____

RACE/ETHNICITY? (Please circle)

1. Caucasian (White)
2. African American (Black)
3. Hispanic
4. Asian
5. Native American
6. Pacific Islander
7. Other _____

MARITAL STATUS? (Please circle)

1. Married
2. Widowed
3. Divorced
4. Separated
5. Never Married

GENDER - Male or Female? (Please circle)

EMPLOYMENT STATUS? (Please circle)

1. Employed Full Time (more than 35 hrs./week)
2. Employed Part Time (less than 35 hrs./week)
3. Unemployed
4. Retired
5. Unable to work due to disability

HIGHEST LEVEL OF EDUCATION? (Please circle)

1. Elementary or Middle School
2. Some High School
3. High School Graduate
4. Some College
5. College Graduate
6. Graduate Education or Degree

PATIENT HEALTH HISTORY (Must be answered by patient alone)

Please indicate if a doctor has ever diagnosed you with one or more of the following conditions:

Has a Doctor ever diagnosed you with:	***If Yes – please check the box (☑)***	***If Yes - Age at first diagnosis***
a) Heart Attack (s)	☐	
b) Angina (chest Pain)	☐	
c) Blocked or narrowed arteries in the heart (coronary disease	☐	
d) Heart Failure	☐	
e) Stroke or TIA	☐	
f) Blocked or narrowed arteries in the neck	☐	
g) Blood clot (thrombosis) in the legs, arms or lungs	☐	
h) Blocked or narrowed arteries in your legs (Peripheral arterial disease)	☐	
i) Arrhythmia (abnormal or irregular heart rhythm)	☐	
j) Heart Valve disease	☐	
k) Aortic Aneurysm	☐	
l) High Blood Pressure	☐	
m) High Cholesterol	☐	
n) Diabetes	☐	
o) Asthma	☐	
p) Emphysema/COPD	☐	
q) Depression	☐	
r) Sleep apnea	☐	
s) Cancer	☐	
t) Rheumatoid arthritis	☐	
u) Any other conditions? (please state which)		

HISTORY OF SURGERY, PCI, OR DEVICES

Have you ever had one or more of the following procedures? (please circle yes or no)

1. **Coronary artery bypass surgery** (CABG)? **(please circle)** **Y / N**
   If yes, How many surgeries/procedures? _____
   Year of most recent surgery? _____
2. C**oronary angioplasty** (includes stent/balloon/ PTCA/PCI)? **(please circle)** **Y / N**
   If yes, How many angioplasty procedures? _____
   Year of most recent angioplasty? _____
3. **Heart transplant**? **(please circle)** **Y / N**
   If yes, When was the surgery? _____
4. P**ermanent pacemaker**? **(please circle)** **Y / N**
   If yes, When was the first procedure? _____

MOOD (This section must be answered by patient alone)

Over the last 2 weeks, how often have you been bothered by any of the following problems?

	Not at all	Several Days	More than half the days	Nearly every day
1) Little interest or pleasure in doing things	☐	☐	☐	☐
2) Feeling down, depressed, or hopeless	☐	☐	☐	☐
3) Trouble falling or staying asleep, or sleeping too much	☐	☐	☐	☐
4) Feeling tired or having little energy	☐	☐	☐	☐
5) Poor appetite or overeating	☐	☐	☐	☐
6) Feeling bad about yourself, or that you are a failure or have let yourself or your family down	☐	☐	☐	☐
7) Trouble concentrating on things, such as reading the newspaper or watching television	☐	☐	☐	☐
8) Moving or speaking so slowly that other people could have noticed. Or the opposite— being so fidgety or restless that you have been moving around a lot more than usual	☐	☐	☐	☐
9) Thoughts that you would be better off dead or of hurting yourself in some way	☐	☐	☐	☐

10) Are you currently taking medications or receiving counseling for depression? (circle)**Y / N**

STRESS: **For the following question, stress is defined as feeling irritable or filled with anxiety, or as having sleeping difficulties as a result of conditions at work or at home. (Please respond as you would have prior to having this procedure/admission)**

1) **How often have you felt stress at work the past year?** please circle or tick here if not working ☐

Never Experienced Stress *Some Period of Stress* *Several Periods of Stress* *Permanent Stress*

2) **How often have you felt stress at home in the past year?** (please circle)

Never Experienced Stress *Some Period of Stress* *Several Periods of Stress* *Permanent Stress*

3) **What level of financial stress do you feel?** (please circle)

Little/none *Moderate* *High/Severe*

4) **How much autonomy do you have in organizing the events of your work day?** (please circle)

None *Little* *Moderate* *Substantial* *Complete*
Not working

SLEEP QUESTIONNAIRE

1) **How often during the night do you snore in any way? (**please circle one)

Never *Rare* *Sometimes* *Often* *Almost Always*

2) **How often during the day or evening you get so sleepy you have to take a nap?**(please circle)

Never *Rare* *Sometimes* *Often* *Almost Always*

3) **Do you have unpleasant feeling in your legs - for example, creepy-crawling or tingly feelings when you lie down at night that make you feel restless and keep you from getting a good sleep?** (please circle one)

Never *Rare* *Sometimes* *Often* *Almost Always*

4) **How many hours of sleep do you usually get each night (or when you usually sleep)?____**

SLEEPINESS SCALE

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired? This refers to your usual way of life in recent times. Even if you have not done some of these things recently try to work out how they would have affected you.

	Chance of "dozing off" or falling asleep			
SITUATION	No chance	Slight chance	Moderate chance	High chance
Sitting and reading	☐	☐	☐	☐
Watching TV	☐	☐	☐	☐
Sitting inactive in a public place (e.g a theater or a meeting)	☐	☐	☐	☐
As a passenger in a car for an hour without a break	☐	☐	☐	☐
Lying down to rest in the afternoon when circumstances permit	☐	☐	☐	☐
Sitting and talking to someone	☐	☐	☐	☐
Sitting quietly after a lunch without alcohol	☐	☐	☐	☐
In a car, while stopped for a few minutes in traffic	☐	☐	☐	

FAMILY HISTORY(must be answered by patient alone, or by patient with assistance from interviewer)

Please fill out the table below about the health history of your parents and brothers/sisters. If you are unsure of an answer, please leave blank.

Relative (blood relatives only)	Living or Dead (L/D)	Age now or at Death	Heart Attack (y/n) If Y, then age at first heart attack.	Angioplasty (balloon/stent) or Bypass Surgery (CABG) If Y, then age at first procedure.	Stroke (y/n/dk) If Y, then age at first stroke	Sudden or unexplained death (y/n/dk) - experienced a sudden unexpected collapse when his/her heart stopped beating If Y then age at death
Example	*D*	*89*	*Y, 72*	*Y, surgery, 73*	*Y, 78*	*N*
Father						
Mother						
Brother(s)						
Sister(s)						
Son(s)						
Daughter(s)						

BEHAVIORAL (must be answered by patient alone or by patient with assistance from interviewer)

Smoking:

1. Are you a : (please circle one)

   Current smoker **Ex-smoker (quit)** **Lifelong Non-smoker**

2. **How many cigarettes** a day do you/did you smoke? _____
3. For **how many years** in your life have you smoked? _____
4. If you quit smoking, how many years have you not smoked? _____
5. Are you currently smoking **cigars or pipes**? (please circle) Y / N

Alcohol:

1. Do you currently consume any alcohol? (Please circle) Y/ N
2. During an average week, how many **cans or bottles of beer** do you drink? _____
3. During an average week, how many **glasses of wine, sangria or champagne** do you drink? _____
4. During an average week, how many drinks of **hard liquor such as tequila, gin, vodka, scotch, rum, whiskey** do you drink (either as shots or mixed drinks)? _____
5. Considering all types of alcoholic beverages, how many times in an average 30-day period do you drink 5 or more drinks on a single occasion? _____
6. Was there a time in the past that you drank significantly more alcohol than you drink now? Y/ N

Activity:

7. Prior to the onset of your symptoms, **how many hours in a typical week did you regularly engage in strenuous physical activity** (examples: running/jogging, rapid walking, aerobics, tennis, basketball, weight lifting, etc.). ____ Hours.
8. **Prior to the onset of symptoms, how active were you at work and during your leisure time?**
   a) **At work** (check one):
      - ☐ Mainly sedentary
      - ☐ Predominately walking on one level, no heavy lifting
      - ☐ Mainly walking, including climbing stairs, or walking uphill or lifting heavy objects
      - ☐ Heavy physical labour
      - ☐ Subject does not work

   b) **During your leisure time** (check one):
      - ☐ Mainly sedentary (sitting e.g. reading, watching television)
      - ☐ Mild exercise (minimal effort eg. yoga, archery, sport fishing, easy walking)
      - ☐ Moderate exercise (e.g. walking, bicycle riding, or light gardening at least 4 hours/week)
      - ☐ Strenuous exercise (heart beats rapidly e.g. running/jogging, football, vigorous swimming)
9. **Do you play sports or exercises during your leisure time?** Y / N
   How many hours per week do you do this level of activity? _____ hours/week
   How many months per year do you do this level of activity? _____ months/year

ANGINA (Must be answered by patient alone)

Over the <u>past 4 weeks</u>, on average, how many times have you had chest pain, chest tightness or angina?

4 or more times per day	1-3 times per day	3 or more times per week but not every day	1-2 times per week	Less than once a week	None over the past 4 weeks**
☐	☐	☐	☐	☐	☐

If answered "None over the past 4 weeks" to the question above, please skip to **Page 9** (Shortness of Breath/SRH questions)

The following is a list of activities that people often do during the week. Although for some people with several medical problems it is difficult to determine what it is that limits them, **please go over the activities listed below and indicate how much limitation you have had due to chest pain, chest tightness or angina <u>over the past 4 weeks</u>**.

	Extremely Limited	Quite a bit Limited	Moderately Limited	Slightly Limited	Not at all Limited	Limited for other reasons or did not do the activity
Dressing yourself	☐	☐	☐	☐	☐	☐
Walking indoors on level ground	☐	☐	☐	☐	☐	☐
Showering	☐	☐	☐	☐	☐	☐
Climbing a hill or a flight of stairs without stopping	☐	☐	☐	☐	☐	☐
Gardening, vacuuming or carrying groceries	☐	☐	☐	☐	☐	☐
Walking more than a block at a brisk pace	☐	☐	☐	☐	☐	☐
Running or jogging	☐	☐	☐	☐	☐	☐
Lifting or moving heavy objects (e.g. furniture, children)	☐	☐	☐	☐	☐	☐
Participating in strenuous sports (e.g. swimming, tennis)	☐	☐	☐	☐	☐	☐

ANGINA (Continued)

Over the <u>past 4 weeks</u>, on average, how many times have you had to take nitroglycerin (tablets or spray) for your chest pain, tightness or angina?

4 or more times per day	1-3 times per day	3 or more times per week but not every day	1-2 times per week	Less than once a week	None over the past 4 weeks
☐	☐	☐	☐	☐	☐

Over the <u>past 4 weeks</u> how much has your chest pain, chest tightness or angina limited your enjoyment of life?

It has extremely limited my enjoyment of life	It has limited my enjoyment of life quite a bit	It has moderately limited my enjoyment of life	It has slightly limited my enjoyment of life	It has not limited my enjoyment of life at all
☐	☐	☐	☐	☐

If you had to spend the rest of your life with your chest pain, chest tightness or angina the way it is right now, how would you feel about this?

Not satisfied at all	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

SHORTNESS OF BREATH/SRH

1) Over the past 4 weeks, have you experienced shortness of breath while…..

	Yes	No
…hurrying on level ground or walking up a slight hill?	☐	☐
…walking with people your own age on level ground?	☐	☐
…walking at your own pace on level ground?	☐	☐
…washing or dressing?	☐	☐
…just resting or sitting?	☐	☐

2) How often do you think or worry that you may have a heart attack or die suddenly?

I can't stop thinking or worrying about it	I often think or worry about it	I occasionally think or worry about it	I rarely think or worry about it	I never think or worry about it
☐	☐	☐	☐	☐

3) In General would you say your health is:

Excellent	Very good	Good	Fair	Poor
☐	☐	☐	☐	☐

FEMALE PATIENTS ONLY: Please answer the following questions, to the best of your ability, regarding your gynecological history. If exact dates are unknown, give the best approximation.

1. Have you had any menstrual periods during the past 2 years? (please circle) Y / N
2. When was your last menstrual period? Month: _____ Year: _____
3. What was your typical menstrual cycle when you were between the ages of 18 and 22 years (excluding time around pregnancies or when using oral contraceptives)?

   ☐No periods ☐Very regular (started within 3 days of expected date)
   ☐Usually regular ☐Usually irregular ☐Always irregular ☐Can't remember
4. Have you reached menopause? (please circle) Y / N
5. If yes, At what age do you think you became post-menopausal? _____
6. Was your menopause natural or the result of surgery or radiation?
   ☐Natural ☐Radiation ☐Unknown ☐Surgical
7. If surgery did you have your ovaries removed? (please circle) Y/ N
8. If yes, was it: ☐one ovary ☐both ovaries ☐don't know.
9. Have you had a hysterectomy? Y / N If yes, when? _____
10. Do you have fibroids? Y / N
11. Do you now or have you in the past ever had difficulty getting pregnant? Y / N
12. How many times have you been pregnant? _____
13. How many live births have you had? _____
14. Have you ever been diagnosed with Polycystic Ovary Syndrome (PCOS) Y / N
15. If yes, did you ever have an ultrasound that confirmed multiple cysts on your ovaries? Y/ N
16. Have you ever taken any of the following female hormone preparations?

	Have you ever used them?	If yes, are you currently using them
Birth control pills?	☐	☐
Post-menopausal hormones?	☐	☐
If yes, which type:		
Pills	☐	☐
Shots?	☐	☐
Implants?	☐	☐
Transdermal	☐	☐

17. Did you also have to take a progesterone supplement in addition to estrogen (see examples below)? Y / N

Some examples of both estrogen and progesterone pills/patches are Activell, Combipatch, Depo-Provera, Estratest, estrogen + medroxyprogesterone, estrogen+metheytestosterone, FemHRT, medroxyprogesterone, Ortho-Prefest, Premphase, Prempro, Provera, Prometrium.

Appendix II

Chart Abstraction Form

Genebank ID :	Enroll Date:	EMPI

DEMOGRAPHICS & VITALS

Age	Gender (circle): M / F	Race/Ethnicity	
Ht (m)	Wt (kg)	resting BP	resting HR

ADMISSION DETAILS

Elective or Emergent procedure (circle)

Admission Symptoms (eg CP, dyspnea)

Procedure:

Admission Diagnosis:

Peak Troponin (pre procedure)

PAST MEDICAL HISTORY (Circle & give details - age diagnosed; etc)

- MI (age, type, territory)
- CAD (age, procedures)
- Diabetes (age, type)
- Hypertension
- Hyper/Dyslipidemia
- Heart Failure (EF, NYHA, type)
- Arrhythmia (type, treatment, age)
- PAD (territory)
- Stroke (bleed/ischemic)

Other conditions – please list

Family History of CAD (details)

PAST SURGICAL HISTORY (Circle; give details - age diagnosed; etc)

- CABG (age, no. vessels)
- PCI (age first proc, no. PCIs, no. stents, types)
- Valve replacement/repair (valve, age, valve type)
- PPM/ICD/BiV (circle, age, indication)
- Other major cardiac or non cardiac surgery

SOCIAL HISTORY

Smoker? (circle)

Yes No Former

Pack Years

Alcohol (circle)

Yes No Former

Units

Other?

Other Investigations (brief details)

- CXR
- ECHO
- EKG
- Stress
- Other

Admit Medications (circle)

Aspirin	Plavix	Coumadin
Statin	Ezetemibe	Fibrate
Niacin	BB	CA antag
ACE	ARB	HCTZ
Furosemide	Nitrate	Metformin

Others

Comments

Blood Results

Lipids	TC	TG	HDL	LDL	VLDL	A1C
Chemistry	Na	K	BUN	Cr	Alb	Gluc
CBC	WCC	Hb	MCV	Plt	RDW	
C Enzymes	CK-MB Pre		CK MB post		Trop Pre	Trop post

Cardiac Cath Findings and Interventions – Native vessels

Cath or PCI	Date	Comment	LM	P LAD before 1st branch	P LAD after 1st branch	D1	Septal	D2	M LAD	D LAD	Ramus	P LCX	OM1	OM2	OM3	M LCX	D LCX	P RCA	M RCA	D RCA	D RCA post PDA	PDA	LPDA from circ	Dominance	EF	Outcome
Cath	*1/1/2010*	*Example*							*75*			*30*				*20*		*60*				*20*		*L*	*50*	*PCI*
PCI		*Example*							*DES*																	

Cardiac Cath Findings and Interventions – Grafts

	Date	Comment	LAD	D1	D2	Ramus	LCX	OM1	OM2	RCA	PDA	Other	Other	Other	Outcome
Type of graft	*1/1980*	*Example*	*LIMA*				*SVG*			*SVG*					
Cath	*1/1/2010*	*Example*	*75*				*20*			*30*					*PCI*
PCI	*1/1/2010*	*Example*	*BMS*												

Appendix III

Genebank Sample Processing Details

Atlanta Clinical & Translational Science Institute
Core Laboratory

SPECIMEN PROCESSING PROTOCOL

Protocol# 5103_GENEBANK

Cardiology Genebank

PI: Arshed Quyyumi, MD

Research Coordinator:
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Visits
There is one visit type, a lab only protocol. These studies are conducted in the cath lab and samples will be transported by tube. Coordinator/Cath lab will call the lab prior to sending samples.

Materials
Collection tubes and labels are provided by the investigator.
Storage tubes, labels, and boxes are provided by the ACTSI.

Notification:
Participants will be scheduled using CR-Assist but will not show on the schedule.

Comments:
This study is in CR-Assist and LIMS. An Intake Form has been created and the study team has been trained.

Store aliquots in -80C, top shelf of Scooby.

You will receive:
1. One (1) 2.5mL PAX tube
2. One (1) 10.0mL Red top tube
3. Two(2) 10.0mL Lav top tubes
4. One (1) 4.0mL Lav top tube

Atlanta Clinical & Translational Science Institute
Core Laboratory

SPECIMEN PROCESSING PROTOCOL

Protocol# 5103_GENEBANK

Cardiology Genebank

Sample Processing

PAX RNA TUBE (1 X 2.5-mL)

1. DO NOT SPIN.
2. Store tube in next available cells in PAX Tube Storage Box for this study.

RED-TOP TUBE (1 X 10-mL)

1. Allow blood to clot at room temperature for 30-60 minutes post-collection before centrifugation.
2. Spin tube at 3000 RPM x 10 minutes at 4^{o}C.
3. Divide serum into 3 equal aliquots (~1.0 mL each). Use standard 2.0-mL cryovials with red caps.
4. Store aliquots in next available cells in Serum Storage Box for this study.

LAV-TOP TUBE (2 X 10-mL)
NOTE: Primary purpose of study is for DNA. Leave a 2-3 mm thick layer of plasma (~thickness of a pen) above the buffy coat.

1. First lav top tube (labeled PLASMA+DNA and has DECODE label provided by PI):
    a. Prior to spinning, aliquot 0.7mL of whole blood into DNA cryovial (BLD-BLD-EDT – CMG).
    b. Recap tube and spin tube at 3000 RPM x 10 minutes at 4^{o}C.
    c. Divide serum into 3 equal aliquots (~1.0 mL each). Use standard 2.0-mL cryovials with lavender caps.
    d. Store aliquots in next available cells in Plasma Storage Box for this study.
    e. Store remaining blood in next available cell in DECODE Storage Box.
2. Second lav top tube (labeled PLASMA):
    a. Spin tube at 3000 RPM x 10 minutes at 4^{o}C.
    b. Divide serum into 3 equal aliquots (~1.0 mL each). Use standard 2.0-mL cryovials with lavender caps.
    c. Store aliquots in next available cells in Plasma Storage Box for this study.
    d. Store tube in next available cell in Cardiology Storage Box.

LAV-TOP TUBE (1 X 4-mL)

1. DO NOT SPIN.
2. Place in a biohazard specimen transport bag.
3. Call Qunna in the Waller lab (7-3086) before sending through the tube (#601).

Appendix IV

Genebank Follow-up Form

GENEBANK FOLLOW-UP FORM

SSN: (Y/N) _____
DOB: (Y/N) / /
Gender M / F
Name
Address
Home phone
Current MD
MD phone
Future contact Y / N (If no, see comments)

MRN Height in
UNIQUE ID Weight lbs
Date of Enrollment

Type of Follow-up (circle one):
One year -1
Five year -5
Specific Study (Study #)
Study name
PI
IRB number
Pt.authorization waived Y / N
Pt.authorization obtained Y / N

Contact Date (or last date known alive) / /
Status A / D **DOD** / /

Cause of death (circle only one field):
l) immediate cardiac death; within one hour after onset of symptoms
s) sudden cardiac death; within 24 hours after onset of symptoms
c) cardiac death; not sudden or immediate
v) vascular; non-cardiac, but vascular related death
o) non-cardiovascular death
b) suicide
u) unknown cause of death

Associated events relating to patient's death (circle all that apply):
a) acute MI
c) CHF
r) arrhythmia
v) CVA (cerebrovascular accident (stroke)
m) malignancy
t) trauma
h) operative; during OHS
b) procedural - during PCI
d) procedural - during cath
o) post OHS (during same admission)
p) post PCI (during same admission)
s) cardiogenic shock

(Confirm the patient's hospitalization / procedure history from the data sheet before proceeding.)

1. Other than the ones we have discussed, have you had any other hospitalizations or procedures? Y / N / U

2. If yes, how many? _____

Please complete the following information concerning the hospitalizations or procedures: ***(most recent first)***

# 1: Date: / /		**# 2: Date:** / /		**# 3: Date:** / /	
Hospitalized:	Y / N	Hospitalized:	Y / N	Hospitalized:	Y / N
Hosp. Name:		Hosp. Name:		Hosp. Name:	
Outpt or office visit:	Y / N	Outpt or office visit:	Y / N	Outpt or office visit:	Y / N
Chest pain/angina	**R** Y / N / U	Chest pain/angina	**R** Y / N / U	Chest pain/angina	**R** Y / N / U
MI	**R** Y / N / U	MI	**R** Y / N / U	MI	**R** Y / N / U
Heart failure (CHF)	**R** Y / N / U	Heart failure (CHF)	**R** Y / N / U	Heart failure (CHF)	**R** Y / N / U
Cardiac Cath	**R** Y / N / U	Cardiac Cath	**R** Y / N / U	Cardiac Cath	**R** Y / N / U
PCI	**R** Y / N / U	PCI	**R** Y / N / U	PCI	**R** Y / N / U
PTA	**R** Y / N / U	PTA	**R** Y / N / U	PTA	**R** Y / N / U
CABG	**R** Y / N / U	CABG	**R** Y / N / U	CABG	**R** Y / N / U
Valve surgery	**R** Y / N / U	Valve surgery	**R** Y / N / U	Valve surgery	**R** Y / N / U
Heart transplant	**R** Y / N / U	Heart transplant	**R** Y / N / U	Heart transplant	**R** Y / N / U
Arrhythmia	**R** Y / N / U	Arrhythmia	**R** Y / N / U	Arrhythmia	**R** Y / N / U
Cardioversion	**R** Y / N / U	Cardioversion	**R** Y / N / U	Cardioversion	**R** Y / N / U
Pacemaker	**R** Y / N / U	Pacemaker	**R** Y / N / U	Pacemaker	**R** Y / N / U
Defibrillator	**R** Y / N / U	Defibrillator	**R** Y / N / U	Defibrillator	**R** Y / N / U
Stress test	**R** Y / N / U	Stress test	**R** Y / N / U	Stress test	**R** Y / N / U
Vascular	**R** Y / N / U	Vascular	**R** Y / N / U	Vascular	**R** Y / N / U
Stroke	**R** Y / N / U	Stroke	**R** Y / N / U	Stroke	**R** Y / N / U
TIA	**R** Y / N / U	TIA	**R** Y / N / U	TIA	**R** Y / N / U
Other	**R** Y / N / U	Other	**R** Y / N / U	Other	**R** Y / N / U
Unknown reason	**R** Y / N / U	Unknown reason	**R** Y / N / U	Unknown reason	**R** Y / N / U

# 4: Date: / /		# 5: Date: / /		# 6: Date: / /	
Hospitalized:	Y / N	Hospitalized:	Y / N	Hospitalized:	Y / N
Hosp. Name:		Hosp. Name:		Hosp. Name:	
Outpt or office visit:	Y / N	Outpt or office visit:	Y / N	Outpt or office visit:	Y / N
Chest pain/angina	**R** Y / N / U	Chest pain/angina	**R** Y / N / U	Chest pain/angina	**R** Y / N / U
MI	**R** Y / N / U	MI	**R** Y / N / U	MI	**R** Y / N / U
Heart failure (CHF)	**R** Y / N / U	Heart failure (CHF)	**R** Y / N / U	Heart failure (CHF)	**R** Y / N / U
Cardiac Cath	**R** Y / N / U	Cardiac Cath	**R** Y / N / U	Cardiac Cath	**R** Y / N / U
PCI	**R** Y / N / U	PCI	**R** Y / N / U	PCI	**R** Y / N / U
PTA	**R** Y / N / U	PTA	**R** Y / N / U	PTA	**R** Y / N / U
CABG	**R** Y / N / U	CABG	**R** Y / N / U	CABG	**R** Y / N / U
Valve surgery	**R** Y / N / U	Valve surgery	**R** Y / N / U	Valve surgery	**R** Y / N / U
Heart transplant	**R** Y / N / U	Heart transplant	**R** Y / N / U	Heart transplant	**R** Y / N / U
Arrhythmia	**R** Y / N / U	Arrhythmia	**R** Y / N / U	Arrhythmia	**R** Y / N / U
Cardioversion	**R** Y / N / U	Cardioversion	**R** Y / N / U	Cardioversion	**R** Y / N / U
Pacemaker	**R** Y / N / U	Pacemaker	**R** Y / N / U	Pacemaker	**R** Y / N / U
Defibrillator	**R** Y / N / U	Defibrillator	**R** Y / N / U	Defibrillator	**R** Y / N / U
Stress test	**R** Y / N / U	Stress test	**R** Y / N / U	Stress test	**R** Y / N / U
Vascular	**R** Y / N / U	Vascular	**R** Y / N / U	Vascular	**R** Y / N / U
Stroke	**R** Y / N / U	Stroke	**R** Y / N / U	Stroke	**R** Y / N / U
TIA	**R** Y / N / U	TIA	**R** Y / N / U	TIA	**R** Y / N / U
Other	**R** Y / N / U	Other	**R** Y / N / U	Other	**R** Y / N / U
Unknown reason	**R** Y / N / U	Unknown reason	**R** Y / N / U	Unknown reason	**R** Y / N / U

2a. Was there a "same-day PCI" – i.e.: angioplasty/stent on the day of enrollment? Y / N

2b. Since enrollment (cath date), have you been diagnosed by a doctor with any of the following:

Condition	Y/N/U	Date of diagnosis	Type	Treatment
Hypertension	Y/N/U			None / Meds
Diabetes	Y/N/U		Type 1 / Type 2	Insulin / Meds / Diet
Arrhythmia	Y/N/U		Atrial Fibrillation Atrial flutter SVT VT Other Unknown	Medications DC Cardioversion EP RF ablation Other
Valve disease	Y/N/U		Aortic stenosis Aortic insufficiency Mitral Stenosis Mitral insufficiency Tricupsid insufficiency Other	None Surgery Valvuloplasty
Obstructive Sleep Apnea (OSA)	Y/N/U			CPAP Y/N
Cancer	Y/N/U			None Chemotherapy Radiotherapy Surgery Other

MEDICATIONS

3. Please list all your medications:

CDB Code	Name of medication	CDB Code	Name of medication	CDB Code	Name of medication

4. Have you had any chest pain, chest tightness, or angina within the past month? Y / N / U

(IF NO CHEST PAIN, CHEST TIGHTNESS OR ANGINA, SKIP TO # 16)

5. If yes, angina class: (circle the appropriate number)

Class	Description of symptoms
0	**No angina** with any level of activity.
1	Ordinary physical activity, such as walking and climbing stairs, does not cause angina. **Angina present with strenuous or rapid or prolonged exertion at work or recreation.**
2	**Slight limitation of ordinary activity**. Walking or climbing stairs rapidly, walking uphill, walking or stair climbing after meals, in cold, or when under emotional stress or only during the few hours after awakening. Walking more than two blocks on the level and climbing more than one flight of stairs at a normal pace and in normal conditions.
3	**Marked limitation of ordinary physical activity.** Walking one to two blocks on the level and climbing more than one flight of stairs in normal conditions.
4	**Inability to carry on any physical activity without discomfort;** anginal symptoms may be present at rest.

6. The following is a list of activities that people often do during the week. Please indicate how much you have been limited by the following activities due to chest pain, chest tightness, or angina over the past four weeks.

	Extremely Limited	Quite a bit Limited	Moderately Limited	Slightly Limited	Not at all Limited	Limited for other reasons or did not do the activity
Dressing yourself	☐	☐	☐	☐	☐	☐
Walking indoors on level ground	☐	☐	☐	☐	☐	☐
Showering	☐	☐	☐	☐	☐	☐
Climbing a hill or a flight of stairs without stopping	☐	☐	☐	☐	☐	☐
Gardening, vacuuming or carrying groceries	☐	☐	☐	☐	☐	☐
Walking more than a block at a brisk pace	☐	☐	☐	☐	☐	☐
Running or jogging	☐	☐	☐	☐	☐	☐
Lifting or moving heavy objects (e.g. furniture, children)	☐	☐	☐	☐	☐	☐
Participating in strenuous sports (e.g. swimming, tennis)	☐	☐	☐	☐	☐	☐

6.

7. <u>Compared with 4 weeks ago</u>, how often do you have chest pain, chest tightness or angina when doing <u>your</u> most strenuous activities?

Much more often	Slightly more often	About the same	Slightly less often	Much less often	I have had no chest pain over the last 4 weeks
☐	☐	☐	☐	☐	☐

8. Over the <u>past 4 weeks</u>, on average, how many times have you had chest pain, chest tightness or angina?

4 or more times per day	1-3 times per day	3 or more times per week but not every day	1-2 times per week	Less than once a week	None over the past 4 weeks
☐	☐	☐	☐	☐	☐

9. Over the <u>past 4 weeks</u>, on average, how many times have you had to take nitroglycerin (nitroglycerin tablets or spray) for your chest pain, chest tightness or angina?

4 or more times per day	1-3 times per day	3 or more times per week but not every day	1-2 times per week	Less than once a week	None over the past 4 weeks
☐	☐	☐	☐	☐	☐

10. How bothersome is it for you to take your pills for chest pain, chest tightness or angina as prescribed?

Extremely bothersome	Quite a bit bothersome	Moderately bothersome	Slightly bothersome	Not bothersome at all	My doctor has not prescribed pills
☐	☐	☐	☐	☐	☐

11. How satisfied are you that everything possible is being done to treat your chest pain, chest tightness or angina?

Not satisfied at all	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

12. How satisfied are you with the explanations your doctor has given you about your chest pain, chest tightness or angina?

Not satisfied at all	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

. **13. Overall, how satisfied are you with the current treatment of your chest pain, chest tightness or angina?**

Not satisfied at all	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

14. Over the <u>past 4 weeks</u>, how much has your chest pain, chest tightness or angina limited your enjoyment of life?

It has extremely limited my enjoyment of life	It has limited my enjoyment of life quite a bit	It has moderately limited my enjoyment of life	It has slightly limited my enjoyment of life	It has not limited my enjoyment of life at all
☐	☐	☐	☐	☐

15. If you had to spend the rest of your life with your chest pain, chest tightness or angina the way it is now, how would you feel about this?

Not satisfied at all	Mostly dissatisfied	Somewhat satisfied	Mostly satisfied	Completely satisfied
☐	☐	☐	☐	☐

16. How often do you think or worry that you may have a heart attack or die suddenly?

I can't stop thinking or worrying about it	I often think or worry about it	I occasionally think or worry about it	I rarely think or worry about it	I never think or worry about it
☐	☐	☐	☐	☐

17. Have you experienced shortness of breath over the past four weeks Y/ N / U

18. Over the past 4 weeks, have you experienced shortness of breath under the following circumstances:

…hurrying on level ground or walking up a slight hill? Y / N

…walking with people your own age on level ground? Y / N

…walking at your own pace on level ground? Y / N

…washing or dressing? Y / N

….while just sitting or at rest? Y / N

19. Since your cath have you been diagnosed with congestive heart failure? Y / N / U

20. If yes, CHF class within the past 4 weeks: (*circle the appropriate number*)

Class	Description of symptoms
1	No SOB with ordinary activity; no limitations
2	SOB with ordinary activity
3	SOB with less than ordinary activity
4	SOB at rest
0	Heart Transplant / Date:

21. In general, would you say your health is:

22. Excellent ☐ Very Good ☐ Good ☐ Fair ☐ Poor ☐

Over the <u>last 2 weeks</u>, how often have you been bothered by any of the following problems?

	Not at all	Several Days	More than half the days	Nearly every day
Little interest or pleasure in doing things	☐	☐	☐	☐
Feeling down, depressed, or hopeless	☐	☐	☐	☐
Trouble falling or staying asleep, or sleeping too much	☐	☐	☐	☐
Feeling tired or having little energy	☐	☐	☐	☐
Poor appetite or overeating	☐	☐	☐	☐
Feeling bad about yourself, or that you are a failure or have let yourself or your family down	☐	☐	☐	☐
Trouble concentrating on things, such as reading the newspaper or watching television	☐	☐	☐	☐
Moving or speaking so slowly that other people could have noticed. Or the opposite—being so fidgety or restless that you have been moving around a lot more than usual	☐	☐	☐	☐

23. Are you currently taking medications or receiving counseling for depression? Y / N

24. Do you have any health insurance coverage that helps pay for your prescription medications?

Y / N

25. Overall, how difficult is it for you to get medical care when needed?

Extremely Difficult	Moderately Difficult	Somewhat Difficult	Not very Difficult	No Problem at all
☐	☐	☐	☐	☐

26. If you feel it is difficult getting medical care (even a little), what would you say the major reason is for your difficulty?

Cost, paying for care	Finding a doctor	Lack of insurance	Travel time	Other
☐	☐	☐	☐	☐

LIFESTYLE

27. How often did you have a drink containing alcohol within the past year? (Consider a "drink" to be a can or bottle of beer, a glass of wine, a wine cooler, or one cocktail or a shot of hard liquor (like scotch, gin, or vodka).

Never	Monthly or less	2-4 times a month	2-3 times a week	4-5 times a week	6 or more times a week
☐	☐	☐	☐	☐	☐

28. Which of the following best describes your cigarette smoking status?

I have never smoked, not even a puff	I have smoked in the past but less than 100 cigarettes total	I stopped smoking more than 1 year ago	I stopped smoking between 1 month and 1 year ago	I have smoked (even a puff) in the past 30 days
☐	☐	☐	☐	☐

If you have smoked cigarettes at all in the past 30 days,

How many cigarettes did you smoke each day, on average? ____

How old were you when you began smoking regularly? _____

29. What is the highest level of education you have completed?

☐ 1	☐ 4	☐ 7	☐ 10	☐ Some college or vocational school
☐ 2	☐ 5	☐ 8	☐ 11	☐ Graduated from college
☐ 3	☐ 6	☐ 9	☐ 12	☐ Post-graduate degree

30. Do you currently work for pay?

Yes, I work full-time for pay	Yes, I work part-time for pay	No, I don't currently work for pay	I am on a leave of absence
☐	☐	☐	☐

31. (6 month follow-up only) If yes, when did you return to work after your initial hospitalization?

Immediately	Within 1 week	Within 2 weeks	Within 4 weeks	Within 3 months	Within 6 months	Still out
☐	☐	☐	☐	☐	☐	☐

32. If you are not working for pay, which of the following best describes you?

Full-time Homemaker	Retired	Disabled	I work as an unpaid volunteer	Unemployed but looking for work	Not working and not looking for work	Student
☐	☐	☐	☐	☐	☐	☐

33. Marital Status?

Married	Widowed	Divorced	Separated	Never Married
☐	☐	☐	☐	☐

FORM COMPLETED BY: _____ (Interviewer's code number)

COMMENTS:

Appendix V

Angiogram Scoring Forms

Angiographic scoring systems for Coronary Disease Severity, 9/2008

Name: MRN# Cath date:

Angiographic report:

Diag16	Diag27	LAD2	LAD3	LAD4	LAD5	Septal	LCX10	LCX11	LCX12

OM113	OM214	OM315	LeftMain1

Ramus9	LPDA16	RCA17	RCA18	RCA19	RCA20	RV21	RPDA22	LVbr23

1. GENSINI		
Lesion severity (% stenosis both concentric and eccentric)	Segment Functional Significance	
25-50 = 1	pRCA x1	
50-75 = 2	mRCA x1	
75-90 = 4	dRCA x1	
90 = 8	PDA (rdom) x1	
99 = 16	LM x5	
100 = 32	pLAD x2.5	
	D1 x1	
	D2 x5	
	mLAD x1.5	
	apical LAD x1	
	pLCx x2.5 (x3.5 lft dom)	
	dLCx x1 (x2 lft dom)	
	PDA (x1 lft dom)	
	PL x5	
	OM x1	

Lesion score	Multiplier	Subtotal
		Total score:

2. CASS (number of diseased vessels, 50% cutoff)				
Number of vessels with >= 50% stenosis, Left main is considered 2 vessel disease				
LAD	LCx	RCA	Left Main	Total score

3. Modified CASS 50

Left Main	LAD	D1	LCx	OM1	OM2	RCA	PDA	Ramus	Total Score

4. CASS (number of diseased vessels, 70% cutoff)				
Number of vessels with >= 70% stenosis, Left main is considered 2 vessel disease				
LAD	LCx	RCA	Left Main	Total score

5. Modified CASS 70

Left Main	LAD	D1	LCx	OM1	OM2	RCA	PDA	Ramus	Total Score

6. Duke Angiographic Jeopardy Score, max of 12 points, >70% cutoff

Does lesion (>70%) affect the following?	Points awarded
LAD	2
Diagonal	2
Septal Perforator	2
Left Circumflex	2
Obtuse Marginal	2
PDA	2
Total score:	

7. Friesinger Score (lesion severity and number in same vessel; 3 major epicardial vessels only)

0: No arteriographic abnormalities seen.
1: Trivial irregularity(ies) in luminal diameter (up to and including 50%)
2: Localized narrowing estimated to be greater than 50% but less than 90% of the luminal cross section area.
3: Multiple narrowings in the same vessel, estimated to be greater than 50% and less than 90%.
4: Narrowinig or narrowings estimated to be greater than 90% of the lulminal cross section area.
5: Total obstruction of a vessel without any filling of the distal segment from the proximal portion.

Modified = addition of Left Main into calculation

LAD	LCx	RCA	(Left Main)	Total	Modified Total

8. Sullivan Score (3 scores)

a. Vessel Score

LAD	LCx	RCA	Left Main	Total

b. Stenosis Score
1= 1-49%, 2= 50-74%, 3= 75-99%, 4= total occlusion, total possible 32

Segment Involved	Score
Left Main	
LAD	
Diagonal	
1st septal perforator	
LCx	
OM or PL branch	
RCA	
PDA	
	Total score:

c. Extent Score (total percentage of coronary tree involved, max 100)
LM = 5, LAD = 20, Diag = 10, 1st septal perf = 5, LCx = 20, OM or PL = 10, RCA = 20, PDA = 10

Segment Involved	Score
Left Main	
LAD	
Diagonal	
1st septal perforator	
LCx	
OM or PL branch	
RCA	
PDA	
	Total score:

9. Jenkins score

<50%=1, 50-74%=2, 75-99%-3, 100%-4

Segment Involved	Score
Left Main	
LAD to D2	
Proximal third of major septal branch	
Proximal third of major diagonal branch	
LCx to OM2	
Proximal third of OM1	
RCA to PDA	
PDA	
	Total score:

10. Duke CAD Severity Index (lesion severity and location)

Extent of CAD	Prognostic Weight
No CAD >= 50%	0
1V 50-74%	19
>=1VD 50-74%	23
1V 75%	23
1V >= 95%	32
2V	37
2V both >=95%	42
1V >=95% proximal LAD	48
2V 95% LAD	48
2V >=95% proximal LAD	56
3VD	56
3VD >=95% at least one	63
3VD proximal LAD	67
3VD >=95% proximal LAD	74
Left Main 75%	82
Left Main >=95%	100

11. Coronary Extent Score (number of 5 to 75% lesions in a 15 segment coding system

Segment Involved	Score
1 RCA proximal	
2 RCA mid	
3 RCA distal	
4 Posterior descending artery	
5 Left Main	
6 LAD proximal	
7 LAD mid	
8 LAD apical	
9 First diagonal	
10 Second diagonal	
11 Proximal circumflex artery	
12 Obtuse marginal	
13 Distal circumflex artery	
14 Posterolateral branch (left)	
15 Posterior descending (left)	
	Total score:

12. Hamsten Score

Plaque Number (a)	Score
Normal vessel wall	0
1-2 plaques	1
>2 plaques located in one or several groups with intervening normal vessel wall portions	2
>2 plaques producing continuous vessel wall irregularities	3
Plaque size (b)	
Slight indentation (<10% reduction of the vessel diameter)	1
Intermediate size indentation (10%-25% reduction of the vessel diameter)	2
Large plaque (>25% reduction of the vessel diameter)	3

Segment Involved	Plaque Number (a)	Plaque Size (b)	Segment score (axb)
1 RCA proximal			
2 RCA mid			
3 RCA distal			
4 Posterior descending artery			
5 Left Main			
6 LAD proximal			
7 LAD mid			
8 LAD apical			
9 First diagonal			
10 Second diagonal			
11 Proximal circumflex artery			
12 Obtuse marginal			
13 Distal circumflex artery			
14 Posterolateral branch (left)			
15 Posterior descending (left)			
			Subtotal (x):
			# Segments analyzed (y):
			Total (x/y):

13. APPROACH (based on Green Lane, modified by pathologic data; LV regions, >=70% cutoff, >50% for LM, all summed for max of 100)

LAD septum = 29%	LAD, 2/3 for mLAD, 1/3 for dLAD
LAD anterolateral = 4%	LAD
Diagonal anterolateral = 13%	LAD
Obtuse marginal = 18%	LCx (only OM2 for mLCx)
Posterolateral branch = 13%	LCx (dLCx in lft dom and codom)
PDA posterolateral = 13%	RCA (LCx in lft dom)
PDA septum = 13%	RCA (LCx in lft dom)
Apex = 5%	LAD

Total score:

14. GREEN LANE (Numerical value for lesion severity (50% cutoff) and takes account of the amount of myocardium supplied by an affected vessel

Grade

a = 100%
b = 90-99%
c = 75-89%
d = 50-74%
e = less than 50%

Myocardial Value (total 15 units) for basic anatomy

Septum = 7 (5 and 2 for LAD, PDA respectively or 2.5 each if meet at apex)
OM and inferior areas = 3 each
Diagonal = 2
Total Myocardial Score (max total = 15)

Table 1 - Grade of severity of arterial **obstruction**

Grade % cross-sectional area loss
a 100
b 90-99
c 75- 89
d 50-74
e Less than 50

Table 2 - **Myocardial score**

Grade Myocardial value

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A	1	2	3	4	5	6	7	8	9	10
B	0.8	1.6	2.4	3.2	4	4.8	5.6	6.4	7.2	8.0
C	0.6	1.2	1.8	2.4	3	3.6	4.2	4.8	5.4	6
D	0.4	0.8	1.2	1.6	2	2.4	2.8	3.2	3.6	4
E	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2

Artery	Grade	Myocardial value (15)	Myocardial Score
LAD			
LCx			
RCA			
			Total:

Appendix VI

Example PLINK files

Examples of PLINK analysis

```
@----------------------------------------------------------@
|      PLINK!     |    v1.07    |   10/Aug/2009    |
|----------------------------------------------------------|
| (C) 2009 Shaun Purcell, GNU General Public License, v2  |
|----------------------------------------------------------|
| For documentation, citation & bug-report instructions:  |
|      http://pngu.mgh.harvard.edu/purcell/plink/       |
@----------------------------------------------------------@

Web-based version check ( --noweb to skip )
Recent cached web-check found... OK, v1.07 is current

Writing this text to log file [ plink.log ]
Analysis started: Thu Jul 28 21:37:27 2011

Options in effect:
        --ped pmrmeta_whites.ped
        --map pmrmetagb_whites.map
        --compound-genotypes
        --missing-genotype N
        --pheno pmrmetagbphenoall3.txt
        --missing-phenotype -999
        --all-pheno
        --reference-allele SNPS.txt
        --assoc



@----------------------------------------------------------@
|      PLINK!     |    v1.06    |   24/Apr/2009    |
|----------------------------------------------------------|
| (C) 2009 Shaun Purcell, GNU General Public License, v2  |
|----------------------------------------------------------|
| For documentation, citation & bug-report instructions:  |
|      http://pngu.mgh.harvard.edu/purcell/plink/       |
@----------------------------------------------------------@

Web-based version check ( --noweb to skip )
Recent cached web-check found... OK, v1.06 is current

Writing this text to log file [ plink.log ]
Analysis started: Tue Jun 01 12:59:41 2010

Options in effect:
        --ped gb_whites_score.ped
        --map gb_whites_score.map
        --compound-genotypes
        --missing-genotype N
        --score 10SNPscore.raw.txt
```

Appendix VII

Zyflo© (Zileuton) Product Details

TEAR AT PERFORATION

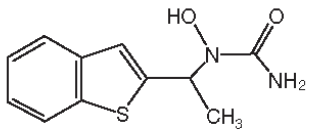
7001338Z

LN42000 Rev 01, Nov. 2005

ZYFLO®
(zileuton tablets)
Tear at perforation to dispense patient information.
℞ only

ZYFLO®
(zileuton tablets)

DESCRIPTION
Zileuton is an orally active inhibitor of 5-lipoxygenase, the enzyme that catalyzes the formation of leukotrienes from arachidonic acid. Zileuton has the chemical name (±)-1-(1-Benzo[b]thien-2-ylethyl)-1-hydroxyurea and the following chemical structure:



Zileuton has the molecular formula $C_{11}H_{12}N_2O_2S$ and a molecular weight of 236.29. It is a racemic mixture (50:50) of R(+) and S(-) enantiomers. Zileuton is a practically odorless, white, crystalline powder that is soluble in methanol and ethanol, slightly soluble in acetonitrile, and practically insoluble in water and hexane. The melting point ranges from 144.2°C to 145.2°C. ZYFLO tablets for oral administration are supplied in one dosage strength containing 600 mg of zileuton.

Inactive Ingredients: crospovidone, hydroxypropyl cellulose, hypromellose, magnesium stearate, microcrystalline cellulose, pregelatinized starch, propylene glycol, sodium starch glycolate, talc, and titanium dioxide.

CLINICAL PHARMACOLOGY
Mechanism of Action:
Zileuton is a specific inhibitor of 5-lipoxygenase and thus inhibits leukotriene (LTB_4, LTC_4, LTD_4, and LTE_4) formation. Both the R(+) and S(-) enantiomers are pharmacologically active as 5-lipoxygenase inhibitors in *in vitro* systems. Leukotrienes are substances that induce numerous biological effects including augmentation of neutrophil and eosinophil migration, neutrophil and monocyte aggregation, leukocyte adhesion, increased capillary permeability, and smooth muscle contraction. These effects contribute to inflammation, edema, mucus secretion, and bronchoconstriction in the airways of asthmatic patients. Sulfido-peptide leukotrienes (LTC_4, LTD_4, LTE_4, also known as the slow-releasing substances of anaphylaxis) and LTB_4, a chemoattractant for neutrophils and eosinophils, can be measured in a number of biological fluids including bronchoalveolar lavage fluid (BALF) from asthmatic patients.

Zileuton is an orally active inhibitor of *ex vivo* LTB_4 formation in several species, including dogs, monkeys, rats, sheep, and rabbits. Zileuton inhibits arachidonic acid-induced ear edema in mice, neutrophil migration in mice in response to polyacrylamide gel, and eosinophil migration into the lungs of antigen-challenged sheep.

Zileuton inhibits leukotriene-dependent smooth muscle contractions *in vitro* in guinea pig and human airways. The compound inhibits leukotriene-dependent bronchospasm in antigen and arachidonic acid-challenged guinea pigs. In antigen-challenged sheep, zileuton inhibits late-phase bronchoconstriction and airway hyperreactivity. In humans, pretreatment with zileuton attenuated bronchoconstriction caused by cold air challenge in patients with asthma.

PHARMACOKINETICS
Zileuton is rapidly absorbed upon oral administration with a mean time to peak plasma concentration (T_{max}) of 1.7 hours and a mean peak level (C_{max}) of 4.98 µg/mL. The absolute bioavailability of ZYFLO is unknown. Systemic exposure (mean AUC) following 600 mg ZYFLO administration is 19.2 µg·hr/mL. Plasma concentrations of zileuton are proportional to dose, and steady-state levels are predictable from single-dose pharmacokinetic data. Administration of ZYFLO with food resulted in a small but statistically significant increase (27%) in zileuton C_{max} without significant changes in the extent of absorption (AUC) or T_{max}. Therefore, ZYFLO can be administered with or without food (see **DOSAGE AND ADMINISTRATION**).

The apparent volume of distribution (V/F) of zileuton is approximately 1.2 L/kg. Zileuton is 93% bound to plasma proteins, primarily to albumin, with minor binding to α1-acid glycoprotein.

Elimination of zileuton is predominantly via metabolism with a mean terminal half-life of 2.5 hours. Apparent oral clearance of zileuton is 7.0 mL/min/kg. ZYFLO activity is primarily due to the parent drug. Studies with radiolabeled drug demonstrated that orally administered zileuton is well absorbed into the systemic circulation with 94.5% and 2.2% of the radiolabeled dose recovered in urine and feces, respectively. Several zileuton metabolites have been identified in human plasma and urine. These include two diastereomeric O-glucuronide conjugates (major metabolites) and an N-dehydroxylated metabolite of zileuton. The urinary excretion of the inactive N-dehydroxylated metabolite and unchanged zileuton each accounted for less than 0.5% of the dose. *In vitro* studies utilizing human liver microsomes have shown that zileuton and its N-dehydroxylated metabolite can be oxidatively metabolized by the cytochrome P450 isoenzymes 1A2, 2C9 and 3A4 (CYP1A2, CYP2C9 and CYP3A4).

Special populations:
Effect of age: The pharmacokinetics of zileuton were investigated in healthy elderly volunteers (ages 65 to 81 years, 9 males, 9 females) and healthy young volunteers (ages 20 to 40 years, 5 males and 4 females) after single and multiple oral doses of 600 mg every 6 hours of zileuton. Zileuton pharmacokinetics were similar in healthy elderly subjects (≥65 years) compared to healthy younger adults (18 to 40 years).

Effect of gender: Across several studies, no significant gender effects were observed on the pharmacokinetics of zileuton.

Renal insufficiency: The pharmacokinetics of zileuton were similar in healthy subjects and in subjects with mild, moderate, and severe renal insufficiency. In subjects with renal failure requiring hemodialysis, zileuton pharmacokinetics were not altered by hemodialysis and a very small percentage of the administered zileuton dose (<0.5%) was removed by hemodialysis. Hence, dosing adjustment in patients with renal dysfunction or undergoing hemodialysis is not necessary.

Hepatic insufficiency: ZYFLO is contraindicated in patients with active liver disease (see **CONTRAINDICATIONS** and **PRECAUTIONS, Hepatic**).

CLINICAL STUDIES
Two double-blind, parallel, placebo-controlled, multi-center studies have established the efficacy of ZYFLO in the treatment of asthma. Three hundred seventy-three (373) patients were enrolled in the 6-month, double-blind phase of Study 1, and 401 patients were enrolled in the 3-month double-blind phase of Study 2. In these studies, the patients were mild-to-moderate asthmatics who had a mean baseline FEV_1 of approximately 2.3 liters and who used inhaled beta-agonists as needed, the mean being approximately 6 puffs of albuterol per day from a metered-dose inhaler. In each study, patients were randomized to receive either ZYFLO 400 mg four times daily, ZYFLO 600 mg four times daily, or placebo. Only the ZYFLO 600 mg four times daily dosage regimen was shown to be efficacious by demonstrating statistically significant improvement across several parameters.

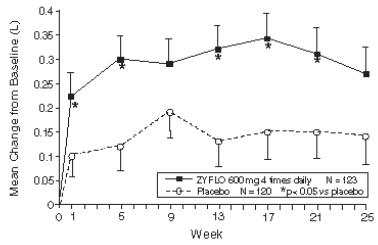
Efficacy endpoints measured in Study 1 are shown in Table 1 below as mean change from baseline to the end of the study (six months). Statistically significant differences from placebo at the $p<0.05$ level are indicated by an asterisk(*). Similar results were observed after three months in Study 2.

Table 1
MEAN CHANGE FROM BASELINE TO END OF STUDY
(Six-Month Study)

Efficacy Endpoint	ZYFLO 600 mg 4 times/day	Placebo
Trough FEV_1 (L)	0.27	0.14
AM PEFR (L/min)	30.60*	5.04
PM PEFR (L/min)	24.59*	7.98
β-Agonist Use (puffs/day)	-1.77*	-0.22
Daily Symptom Score (0-3 Scale)	-0.49*	-0.28
Nocturnal Symptom Score (0-3 Scale)	-0.29*	-0.04

Figure 1 shows the mean effect of ZYFLO versus placebo for the primary efficacy variable, trough FEV_1, over the course of Study 1.

Figure 1
Mean Change From Baseline in Trough FEV_1 (L)



Of all the patients in Study 1 and Study 2, 7.0% of those administered ZYFLO 600 mg four times daily required systemic corticosteroid therapy for exacerbation of asthma, whereas 18.7% of the placebo group required corticosteroid treatment. This difference was statistically significant.

In these trials, there was a statistically significant improvement from baseline in FEV_1, which occurred 2 hours after initial administration of ZYFLO. This mean increase was approximately 0.10 L greater than that in placebo-treated patients.

These studies evaluated patients receiving as-needed inhaled beta-agonist as their only asthma therapy. In this patient population, post-hoc analyses suggested that individuals with lower FEV_1 values at baseline showed a greater improvement.

The role of ZYFLO in the management of patients with more severe asthma, patients receiving anti-asthma therapy other than as-needed, inhaled beta-agonists, or patients receiving it as an oral or inhaled corticosteroid-sparing agent remains to be fully characterized.

INDICATIONS AND USAGE
ZYFLO is indicated for the prophylaxis and chronic treatment of asthma in adults and children 12 years of age and older.

CONTRAINDICATIONS
ZYFLO tablets are contraindicated in patients with:
- Active liver disease or transaminase elevations greater than or equal to three times the upper limit of normal (≥3xULN) (see **PRECAUTIONS, Hepatic**).
- Hypersensitivity to zileuton or any of its inactive ingredients.

WARNINGS
ZYFLO is not indicated for use in the reversal of bronchospasm in acute asthma attacks, including status asthmaticus. Therapy with ZYFLO can be continued during acute exacerbations of asthma.

Co-administration of ZYFLO and theophylline results in, on average, an approximate doubling of serum theophylline concentrations. Theophylline dosage in these patients should be reduced and serum theophylline concentrations monitored closely (see **PRECAUTIONS, Drug Interactions**).

Co-administration of ZYFLO and warfarin results in a clinically significant increase in prothrombin time (PT). Patients on oral warfarin therapy and ZYFLO should have their prothrombin times monitored closely and anticoagulant dose adjusted accordingly (see **PRECAUTIONS, Drug Interactions**).

Co-administration of ZYFLO and propranolol results in doubling of propranolol AUC and consequent increased beta-blocker activity. Patients on ZYFLO and propranolol should be closely monitored and the dose of the propranolol reduced as necessary (see **PRECAUTIONS, Drug Interactions**).

PRECAUTIONS
Hepatic: Elevations of one or more liver function tests may occur during ZYFLO therapy. These laboratory abnormalities may progress, remain unchanged, or resolve with continued therapy. In a few cases, initial transaminase elevations were first noted after discontinuing treatment, usually within 2 weeks. The ALT (SGPT) test is considered the most sensitive indicator of liver injury. In placebo-controlled clinical trials, the frequency of ALT elevations greater than or equal to three times the upper limit of normal (3xULN) was 1.9% for ZYFLO-treated patients, compared with 0.2% for placebo-treated patients.

In a long-term safety surveillance study, 2458 patients received ZYFLO in addition to their usual asthma care and 489 received their usual asthma care. In patients treated for up to 12 months with ZYFLO in addition to their usual asthma care, 4.6% developed an ALT of at least 3xULN, compared with 1.1% of patients receiving only their usual asthma care. Sixty-one percent of these elevations occurred during the first two months of ZYFLO therapy. After two months of treatment, the rate of new ALT elevations ≥3xULN stabilized at a mean of 0.30% per month for patients receiving ZYFLO-plus-usual-asthma care compared with 0.11% per month for patients receiving usual asthma care alone. Of the 61 ZYFLO-plus-usual-asthma-care patients with ALT elevations between 3 to 5xULN, 32 patients (52%) had ALT values decrease to below 2xULN while continuing ZYFLO therapy. Twenty-one of the 61 patients (34%) had further increases in ALT levels to ≥5xULN and were withdrawn from the study in accordance with the study protocol. In patients who discontinued ZYFLO, elevated ALT levels returned to <2xULN in an average of 32 days (range 1-111 days).

In controlled and uncontrolled clinical trials involving more than 5000 patients treated with ZYFLO, the overall rate of ALT elevation ≥3xULN was 3.2%. In these trials, one patient developed symptomatic hepatitis with jaundice, which resolved upon discontinuation of therapy. An additional 3 patients with transaminase elevations developed mild hyperbilirubinemia that was less than three times the upper limit of normal. There was no evidence of hypersensitivity or other alternative etiologies for these findings. In subset analyses, females over the age of 65 appeared to be at an increased risk for ALT elevations. Patients with pre-existing transaminase elevations may also be at an increased risk for ALT elevations (see **CONTRAINDICATIONS**).

It is recommended that hepatic transaminases be evaluated at initiation of, and during therapy with, ZYFLO. Serum ALT should be monitored before treatment begins, once-a-month for the first 3 months, every two to three months for the remainder of the first year, and periodically thereafter for patients receiving long-term ZYFLO therapy. If clinical signs and/or symptoms of liver dysfunction (e.g., right upper quadrant pain, nausea, fatigue, lethargy, pruritus, jaundice, or "flu-like" symptoms) develop or transaminase elevations greater than 5 times the ULN occur, ZYFLO should be discontinued and transaminase levels followed until normal.

Since treatment with ZYFLO may result in increased hepatic transaminases, ZYFLO should be used with caution in patients who consume substantial quantities of alcohol and/or have a past history of liver disease.

Information for Patients: Patients should be told that:
- ZYFLO is indicated for the chronic treatment of asthma and should be taken regularly as prescribed, even during symptom-free periods.
- ZYFLO is not a bronchodilator and should not be used to treat acute episodes of asthma.
- When taking ZYFLO, they should not decrease the dose or stop taking any other anti-asthma medications unless instructed by a physician.
- While using ZYFLO, medical attention should be sought if short-acting bronchodilators are needed more often than usual, or if more than the maximum number of inhalations of short-acting bronchodilator treatment prescribed for a 24-hour period are needed.
- The most serious side effect of ZYFLO is elevation of liver enzyme tests and that, while taking ZYFLO, they must return for liver enzyme test monitoring on a regular basis.
- If they experience signs and/or symptoms of liver dysfunction (e.g., right upper quadrant pain, nausea, fatigue, lethargy, pruritus, jaundice, or "flu-like" symptoms), they should contact their physician immediately.
- ZYFLO can interact with other drugs and that, while taking ZYFLO, they should consult their doctor before starting or stopping any prescription or non-prescription medicines.

A patient leaflet is included with the tablets.

Drug Interactions: In a drug-interaction study in 16 healthy volunteers, co-administration of multiple doses of zileuton (800 mg every 12 hours) and theophylline (200 mg every 6 hours) for 5 days resulted in a significant decrease (approximately 50%) in steady-state clearance of theophylline, an approximate doubling of theophylline AUC, and an increase in theophylline C_{max} (by 73%). The elimination half-life of theophylline was increased by 24%. Also, during co-administration, theophylline-related adverse events were observed more frequently than after theophylline alone. Upon initiation of ZYFLO in patients receiving theophylline, the theophylline dosage should be reduced by approximately one-

half and plasma theophylline concentrations monitored. Similarly, when initiating therapy with theophylline in a patient receiving ZYFLO, the maintenance dose and/or dosing interval of theophylline should be adjusted accordingly and guided by serum theophylline determinations (see **WARNINGS**).

Concomitant administration of multiple doses of ZYFLO (600 mg every 6 hours) and warfarin (fixed daily dose obtained by titration in each subject) to 30 healthy male volunteers resulted in a 15% decrease in R-warfarin clearance and an increase in AUC of 22%. The pharmacokinetics of S-warfarin were not affected. These pharmacokinetic changes were accompanied by a clinically significant increase in prothrombin times. Monitoring of prothrombin time, or other suitable coagulation tests, with the appropriate dose titration of warfarin is recommended in patients receiving concomitant ZYFLO and warfarin therapy (see **WARNINGS**).

Co-administration of ZYFLO and propranolol results in a significant increase in propranolol concentrations. Administration of a single 80-mg dose of propranolol in 16 healthy male volunteers who received ZYFLO 600 mg every 6 hours for 5 days resulted in a 42% decrease in propranolol clearance. This resulted in an increase in propranolol C_{max}, AUC, and elimination half-life by 52%, 104%, and 25%, respectively. There was an increase in β-blockade and decrease in heart rate associated with the co-administration of these drugs. Patients on ZYFLO and propranolol should be closely monitored and the dose of propranolol reduced as necessary (see **WARNINGS**). No formal drug-drug interaction studies between ZYFLO and other beta-adrenergic blocking agents (i.e., β-blockers) have been conducted. It is reasonable to employ appropriate clinical monitoring when these drugs are co-administered with ZYFLO.

In a drug interaction study in 16 healthy volunteers, co-administration of multiple doses of terfenadine (60 mg every 12 hours) and ZYFLO (600 mg every 6 hours) for 7 days resulted in a decrease in clearance of terfenadine by 22% leading to a statistically significant increase in mean AUC and C_{max} of terfenadine of approximately 35%. This increase in terfenadine plasma concentration in the presence of ZYFLO was not associated with a significant prolongation of the QTc interval. Although there was no cardiac effect in this small number of healthy volunteers, given the high inter-individual pharmacokinetic variability of terfenadine, co-administration of ZYFLO and terfenadine is not recommended.

Drug-drug interaction studies conducted in healthy volunteers between ZYFLO and prednisone and ethinyl estradiol (oral contraceptive), drugs known to be metabolized by the P450 3A4 (CYP3A4) isoenzyme, have shown no significant interaction. However, no formal drug-drug interaction studies between ZYFLO and dihydropyridine, calcium channel blockers, cyclosporine, cisapride, and astemizole, also metabolized by CYP3A4, have been conducted. It is reasonable to employ appropriate clinical monitoring when these drugs are co-administered with ZYFLO.

Drug-drug interaction studies in healthy volunteers have been conducted with ZYFLO and digoxin, phenytoin, sulfasalazine, and naproxen. There was no significant interaction between ZYFLO and any of these drugs.

Carcinogenesis, Mutagenesis, Impairment of Fertility: In 2-year carcinogenicity studies, increases in the incidence of liver, kidney, and vascular tumors in female mice and a trend towards an increase in the incidence of liver tumors in male mice were observed at 450 mg/kg/day (providing approximately 4 times [females] or 7 times [males] the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). No increase in the incidence of tumors was observed at 150 mg/kg/day (providing approximately 2 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). In rats, an increase in the incidence of kidney tumors was observed in both sexes at 170 mg/kg/day (providing approximately 6 times [males] or 14 times [females] the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). No increased incidence of kidney tumors was seen at 80 mg/kg/day (providing approximately 4 times [males] or 6 times [females] the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). Although a dose-related increased incidence of benign Leydig cell tumors was observed, Leydig cell tumorigenesis was prevented by supplementing male rats with testosterone.

Zileuton was negative in genotoxicity studies including bacterial reverse mutation (Ames) using *S. typhimurium* and *E. coli*, chromosome aberration in human lymphocytes, *in vitro* unscheduled DNA synthesis (UDS), in rat hepatocytes with or without zileuton pretreatment and in mouse and rat kidney cells with zileuton pretreatment, and mouse micronucleus assays. However, a dose-related increase in DNA adduct formation was reported in kidneys and livers of female mice treated with zileuton. Although some evidence of DNA damage was observed in a UDS assay in hepatocytes isolated from Aroclor-1254 treated rats, no such finding was noticed in hepatocytes isolated from monkeys, where the metabolic profile of zileuton is more similar to that of humans.

In reproductive performance/fertility studies, zileuton produced no effects on fertility in rats at oral doses up to 300 mg/kg/day (providing approximately 8 times [male rats] and 18 times [female rats] the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). Comparative systemic exposure (AUC) is based on measurements in male rats or nonpregnant female rats at similar dosages. However, reduction in fetal implants was observed at oral doses of 150 mg/kg/day and higher (providing approximately 9 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). Increases in gestation length, prolongation of estrous cycle, and increases in stillbirths were observed at oral doses of 70 mg/kg/day and higher (providing approximately 4 times the systemic exposure (AUC) achieved at the maximum recommended human daily oral dose). In a perinatal/postnatal study in rats, reduced pup survival and growth were noted at an oral dose of 300 mg/kg/day (providing approximately 18 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose).

Pregnancy: Pregnancy Category C: Developmental studies indicated adverse effects (reduced body weight and increased skeletal variations) in rats at an oral dose of 300 mg/kg/day (providing approximately 18 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose). Comparative systemic exposure [AUC] is based on measurements in nonpregnant female rats at a similar dosage. Zileuton and/or its metabolites cross the placental barrier of rats. Three of 118 (2.5%) rabbit fetuses had cleft palates at an oral dose of 150 mg/kg/day (equivalent to the maximum recommended human daily oral dose on a mg/m² basis). There are no adequate and well-controlled studies in pregnant women. ZYFLO should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: Zileuton and/or its metabolites are excreted in rat milk. It is not known if zileuton is excreted in human milk. Because many drugs are excreted in human milk, and because of the potential for tumorigenicity shown for ZYFLO in animal studies, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use: The safety and effectiveness of ZYFLO in pediatric patients under 12 years of age have not been established.

Geriatric Use: In subset analyses, females over the age of 65 appeared to be at an increased risk for ALT elevations. Zileuton pharmacokinetics were similar in healthy elderly subjects (≥65 years) compared to healthy younger adults (18 to 40 years) (see **PHARMACOKINETICS** - ***Special populations:*** Effect of age).

ADVERSE REACTIONS

Clinical Studies: A total of 5542 patients have been exposed to zileuton in clinical trials, 2252 of them for greater than 6 months and 742 for greater than 1 year.

Adverse events most frequently occurring (frequency ≥3%) in ZYFLO-treated patients and at a frequency greater than placebo-treated patients are summarized in Table 2.

TABLE 2
Proportion of Patients Experiencing Adverse Events in Placebo-Controlled Studies in Asthma

BODY SYSTEM/Event	ZYFLO 600 mg 4 times daily % Occurrence (N – 475)	Placebo % Occurrence (N – 491)
BODY AS A WHOLE		
Headache	24.6	24.0
Pain (unspecified)	7.8	5.3
Abdominal Pain	4.6	2.4
Asthenia	3.8	2.4
Accidental Injury	3.4	2.0
DIGESTIVE SYSTEM		
Dyspepsia	8.2*	2.9
Nausea	5.5	3.7
MUSCULOSKELETAL		
Myalgia	3.2	2.9

* $p \leq 0.05$ vs placebo

Less common adverse events occurring at a frequency of greater than 1% and more commonly in ZYFLO-treated patients included: arthralgia, chest pain, conjunctivitis, constipation, dizziness, fever, flatulence, hypertonia, insomnia, lymphadenopathy, malaise, neck pain/rigidity, nervousness, pruritus, somnolence, urinary tract infection, vaginitis, and vomiting.

The frequency of discontinuation from the asthma clinical studies due to any adverse event was comparable between ZYFLO (9.7%) and placebo-treated (8.4%) groups.

In placebo-controlled clinical trials, the frequency of ALT elevations ≥3xULN was 1.9% for ZYFLO-treated patients, compared with 0.2% for placebo-treated patients. In controlled and uncontrolled trials, one patient developed symptomatic hepatitis with jaundice, which resolved upon discontinuation of therapy. An additional 3 patients with transaminase elevations developed mild hyperbilirubinemia that was less than three times the upper limit of normal. There was no evidence of hypersensitivity or other alternative etiologies for these findings. ZYFLO is contraindicated in patients with active liver disease or transaminase elevations greater than or equal to 3xULN (see **CONTRAINDICATIONS**). It is recommended that hepatic transaminases be evaluated at initiation of and during therapy with ZYFLO (see **PRECAUTIONS, Hepatic**).

Occurrences of low white blood cell count ($\leq 2.8 \times 10^9$/L) were observed in 1.0% of 1,678 patients taking ZYFLO and 0.6% of 1,056 patients taking placebo in placebo-controlled studies. These findings were transient and the majority of cases returned toward normal or baseline with continued ZYFLO dosing. All remaining cases returned toward normal or baseline after discontinuation of ZYFLO. Similar findings were also noted in a long-term safety surveillance study of 2458 patients treated with ZYFLO plus usual asthma care versus 489 patients treated only with usual asthma care for up to one year. The clinical significance of these observations is not known.

In the long-term safety surveillance trial of ZYFLO plus usual asthma care versus usual asthma care alone, a similar adverse event profile was seen as in other clinical trials.

Post-Marketing Experience: Rash and urticaria have been reported with ZYFLO.

OVERDOSAGE

Human experience of acute overdose with zileuton is limited. A patient in a clinical trial took between 6.6 and 9.0 grams of zileuton in a single dose. Vomiting was induced and the patient recovered without sequelae. Zileuton is not removed by dialysis. Should an overdose occur, the patient should be treated symptomatically and supportive measures instituted as required. If indicated, elimination of unabsorbed drug should be achieved by emesis or gastric lavage; usual precautions should be observed to maintain the airway. A Certified Poison Control Center should be consulted for up-to-date information on management of overdose with ZYFLO.

The oral minimum lethal doses in mice and rats were 500-4000 and 300-1000 mg/kg in various preparations, respectively (providing greater than 3 and 9 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose, respectively). No deaths occurred, but nephritis was reported in dogs at an oral dose of 1000 mg/kg (providing in excess of 12 times the systemic exposure [AUC] achieved at the maximum recommended human daily oral dose).

DOSAGE AND ADMINISTRATION

The recommended dosage of ZYFLO for the symptomatic treatment of patients with asthma is one 600-mg tablet four times a day for a total daily dose of 2400 mg. For ease of administration, ZYFLO may be taken with meals and at bedtime. Hepatic transaminases should be evaluated prior to initiation of ZYFLO and periodically during treatment (see **PRECAUTIONS, Hepatic**).

HOW SUPPLIED

ZYFLO Tablets are available as 1 dosage strength: 600-mg white to off white, ovaloid, film coated tablets debossed "CT 1" on one side and bisect on the other side.

High-density polyethylene bottles of:

120 Tablets.....NDC 68734-700-10

Recommended storage: Store tablets at controlled room temperature between 20°-25°C, (68°-77°F). See USP. Protect from light.

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