Using Genomic Data to Understand Novel Pathways in Abdominal Aortic Aneurysm

By

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To Mas, Ciaran & Cillian

The following manuscripts have been published in peer reviewed journals as a result of research described in this thesis;-

Association of a sequence variant in *DAB2IP* with coronary heart disease.

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Interleukin-6 Receptor Pathways in Abdominal Aortic Aneurysm.

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Harrison SC, Smith AJ, Jones GT, Swerdlow DI, Rampuri R, Bown MJ; on behalf of the Aneurysm Consortium, Folkersen L, Baas AF, de Borst GJ, Blankensteijn JD, Price JF, van der Graaf Y, McLachlan S, Agu O, Hofman A, Uitterlinden AG, Franco-Cereceda A, Ruigrok YM, Van't Hof FN, Powell JT, van Rij AM, Casas JP, Eriksson P, Holmes MV, Asselbergs FW, Hingorani AD, Humphries SE.

A gene-centric study of common carotid artery remodeling

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High-density lipoprotein cholesterol and abdominal aortic aneurysm – a Mendelian Randomisation study.

British Journal of Surgery Supplement, 2013, April, 100(S2):5-6

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Declaration

I, Seamus C Harrison, declare that this thesis titled, 'Using genomic data to understand novel pathways in Abdominal Aortic Aneurysm' and the workpresented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given
- With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made   clear exactly what others did and what I have contributed myself.

Signed:

Date:

Contribution of others to this work

The analysis of the CHD cohorts in Chapter 3 was carried out in conjunction with Dr Jackie Cooper, statistician in the centre for cardiovascular genetics.

In chapter 4, Dr Michael V Holmes and Riaz Rampuri aided in the design and completion of the literature search. Dr Andrew Smith carried out the *in vitro* analyses.

In chapter 5, Michael V Holmes aided in the design of the literature search.

I did not have access to individual participant data for some of the studies, as described in Chapter 2. Under these circumstances I designed the analysis, which was then carried out by individuals with full data access.

Other individuals prepared the quality controlled genome wide datasets, as described in Chapter 2

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Abstract

In this thesis, following development of extensive research collaborations, I use genetic epidemiology methods to understand disease pathways in abdominal aortic aneurysm (AAA).

In the first replication study of a variant in *DAB2IP* found to be associated with AAA by genome wide association study I found that rs7025486 was consistently associated with higher risk of both AAA and coronary heart disease in newly genotyped studies, but was not associated with a panel of emerging cardiovascular biomarkers and did not interact with the 9p21 cardiovascular disease locus.

Using a Mendelian Randomisation (MR) approach I provide strong statistical evidence that signaling through the interleukin-6 receptor is likely to be a causal pathway in AAA and may therefore represent a valid therapeutic target. Extending the MR paradigm, by utilizing multiple genetic variants combined into a score I provide evidence that HDL-C mediated pathways may also be causal in AAA, in support of a meta-analysis I performed that shows strong association between HDL-C concentration and AAA.

Finally, by using a novel quantitative trait genomics approach I performed a gene centric scan of carotid artery remodelling and found variants on Chromosome 1 in *DNM3-PIGC* to be associated with both carotid artery size *and* risk of AAA, providing compelling evidence that AAA is a focal manifestation of a systemic dilating disease.

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AAA – Abdominal Aortic Aneurysm

AC – Aneurysm Consortium

AP – Anterior-posterior

AROC – Area under the receiver operating curve

BMI – Body Mass Index

LDL-C – Low-density Lipoprotein Cholesterol

HDL-C – High-density Lipoprotein Cholesterol

IL-6R – Interleukin 6 receptor

IL-6 – Interleukin 6

SIL-6R – Soluble Interleukin 6 Receptor

TG – Triglycerides

TC – Total Cholesterol

GWAS – Genome Wide Association Study

MR – Mendelian Randomisation

ICCAD – Interadventitial Common Carotid Artery Diameter

CIMT – Carotid Intima Media Thickness

CRP – C-reactive Protein

IRAD – Infrarenal aortic diameter

BP – Blood Pressure

T2DM – Type 2 Diabetes Mellitus

CHD – Coronary Heart Disease

CVD – Cerebrovascular Disease

PVD – Peripheral Vascular Disease

WHII – Whitehall II

ECG – Electrocardiogram

USS – Ultrasound scan

FH – Familial Hypercholesterolaemia

EVAR – Endovascular Aneurysm Repair

IFN-γ - Interferon-γ

VTE – Venous Thromboembolism

NPSHII – Northwick Park Heart Study II

CETP - Cholesterylester transport protein

TCZ – Tocilizumab

GRS – Genetic Risk Score

SMD – Standardised Mean Difference

SD – Standard Deviation

SE – Standard Error

WMD – Weighted Mean Difference

CI – Confidence Interval

QC – Quality Control

OR – Odds Ratio

HR – Hazard Ratio

GLGC – Global Lipids Genetics Consortium

WTCCC – Wellcome Trust Case Control Consortium

MDS – Multi-dimensional Scaling

1 Introduction

1.1 Abdominal Aortic Aneurysms

An aneurysm is defined as a permanent, focal dilatation of an artery or chamber to twice its normal diameter (1). Aneurysms occur throughout the arterial system but certain anatomical locations are more prone to developing aneurysms than others;- namely the infra-renal aorta, the thoracic aorta (both ascending and descending), the circle of Willis, the common iliac arteries and the popliteal arteries. Aneurysmal degeneration is, however very uncommon in other vascular beds such as the common carotid arteries or the external iliac system (2, 3). The natural history of aneurysms is occult expansion over time followed by rupture, which in many cases is a catastrophic event. An abdominal aortic aneurysm (AAA) refers to an aneurysm of the aorta below the level of the diaphragm, most of which are infra-renal and extend to the aortic bifurcation   (Figure 1). The commonly used threshold for diagnosis of AAA is an infra-renal aortic diameter (IRAD) greater than three cm in anterior-posterior diameter (1).
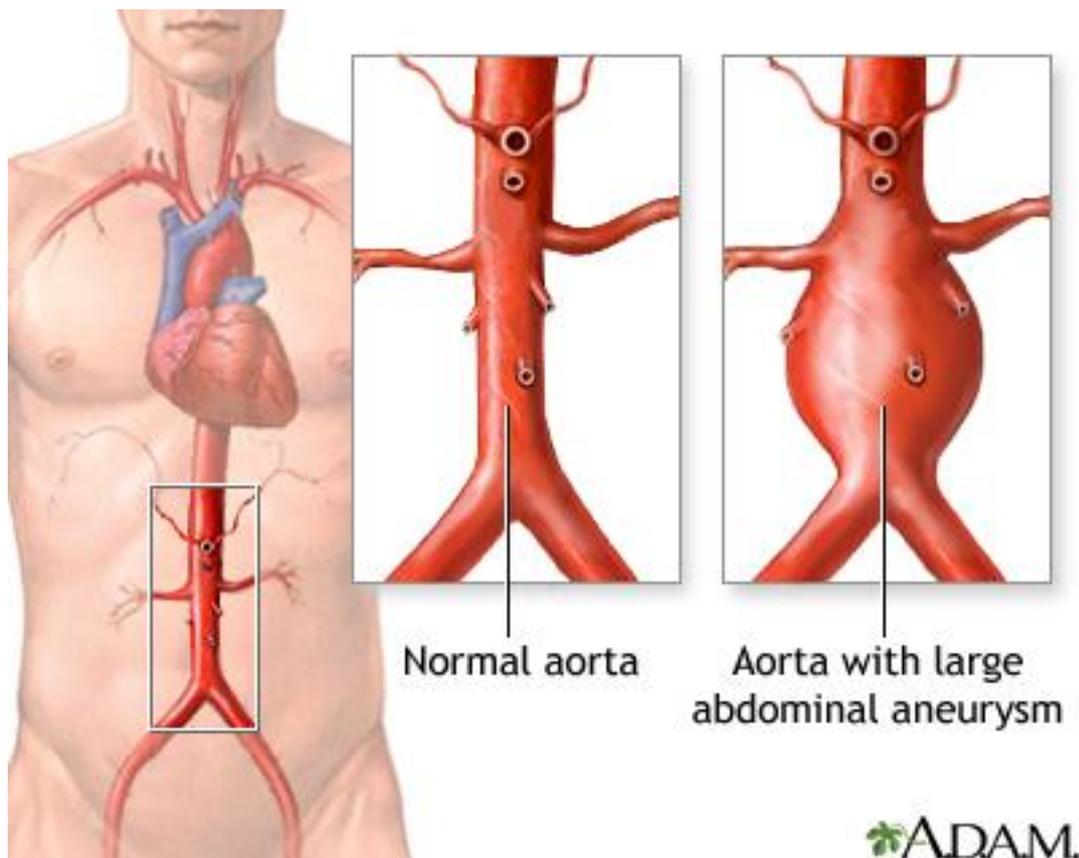
Figure 1 - An Aneurysm of the infrarenal aorta. Image is from http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001215/

The risk of AAA rupture is closely correlated with maximal aneurysm diameter (4)(Table 1). Approximately 80% of patients with a ruptured AAA that reach hospital die and half of those who undergo emergency repair do not survive (5, 6). Other factors that appear to increase rupture risk include hypertension, current cigarette smoking and female gender (7). The annual rate of aneurysm growth-rate of a small AAA is estimated to be approximately 2.21 mm/year (7) but there is considerable inter-individual heterogeneity (8) and inter-cohort heterogeneity with regard to how growth rates are actually measured (9).

Table 1 – Annual Risk of AAA rupture by diameter. Adapted from (10)

AAA Diameter (cm)	Estimated Annual Rupture Risk (%)
<4	0
4-5	0.5 - 5
5-6	3 - 15
6-7	10 - 20
7-8	20 - 40
>8	30 - 50

1.1.1   Abdominal Aortic Aneurysms – Epidemiology

It has been estimated that approximately 6,800 deaths are caused each year by AAA in England and Wales (11). A major resource for understanding the epidemiology of AAA has been large-scale cross-sectional screening studies. There have been four large randomized controlled trials of screening for AAA in males aged > 65 years that have reported a AAA prevalence ranging from 4 – 7.7% in this subgroup (11-14). In the Aneurysm Detection and Management (ADAM) study over 100,000 male US veterans were screened and the prevalence of AAA was noted to be 4.2 % (15). In females, there have been fewer studies but the only randomized controlled trial that included females (aged 65-80) noted a prevalence of 1.3% (16). The incidence of new AAA diagnoses is approximately 0.5% per year in Western populations (12, 17).

There is however, a growing body of evidence to suggest the epidemiology of AAA has changed in the last decade. Screening programs have reported a 3-4 fold lower than expected prevalence of AAA in Sweden and the UK (18, 19), and it

has been reported that hospitalizations for AAA are falling in Australia (20). In the UK there has been a fall in the number of hospital admissions for ruptured AAA, though the number of admissions for planned repair appears to be stable (21). The reasons for the changing epidemiology of the disease are not entirely clear, but it is possible that a reduction in the prevalence of smoking and an improved cardiovascular risk factor profile are key drivers of the change (22).

1.1.2 Risk Factors for AAA

1.1.2.1 Gender

Males are approximately five-fold more likely to develop AAA than females (23). The reasons for the gender disparity are not fully elucidated at present but suggested explanations include a protective effect of female hormones and a lower proportion of risk behavior such as smoking in females compared to males. It has recently been shown that genetic variation on the Y-chromosome shows a strong association with CHD (24), but whether or not this locus plays a role on the development of AAA is not clear. Studies that have looked at genetic variation in sex hormones in AAA have not produced convincing results (25). It has also been postulated that at least some of the difference in prevalence between males and females can be explained by disparities in the definition of AAA. Females have smaller aortas than males, and using an absolute threshold of 3cm does not take this into account, and an IRAD of 3cm in females represents a considerably larger *relative* aortic diameter than in males (26). The prospective Tromso study demonstrated that adjusting for baseline IRAD abolished the gender specific

differences in risk of incident AAA (using the 3cm definition)(27), prompting the authors to suggest that the reason for the prevalence disparity may be due simply to the size threshold used to define AAA. In-keeping with this view is evidence that females also have an increased diameter-adjusted growth rate and rupture risk compared to males(7), and studies have demonstrated an increased hospitalization rate of AAA in females (28).

1.1.2.2   Family History

A family history of AAA is an established risk factor for AAA. Following initial observations that brothers of probands had a high risk of developing AAA (29) a number of studies have studied the familial tendency towards AAA development. In analysis of 126,196 individuals (5,214 cases) from the Veterans Affairs Cooperative Study group a family history of AAA was associated with a doubling of risk of AAA (15). A large-scale cross-sectional analysis of over 30,000 AAA cases and 3 million controls identified by Lifeline screening© in the United States reported that a family history of AAA increased the risk of disease by approximately fourfold(30). In the Swedish Twin registry of over 172,000 twins (including 265 with diagnosed AAA) reported odds ratios of 71 and 7.6 respectively for monozygotic and dizygotic twins of probands and they estimated the heritability of AAA to be approximately 70%. Overall, there is strong evidence that family history of AAA is a major risk factor for the condition.

1.1.2.3   Smoking

The most important modifiable risk factor for the development of AAA is cigarette smoking. In the prospective Tromso study, current heavy cigarette smoking (>20 cigarettes per day) was associated with a thirteen fold increased risk for incident AAA (17). A range of large-scale cross sectional studies (15, 30) have also shown that cigarette smoking is a major risk factor for AAA. The precise mechanism by which smoking increases the risk of AAA has not been fully elucidated but evidence points to a pro-inflammatory effect, altered expression of proteolytic proteins and an altered response to oxidative stress (23). Furthermore, it has been suggested that a reduction in the prevalence of cigarette smoking is a key factor driving changes in the epidemiology of AAA (22).

1.1.2.4   Hypertension

A diagnosis of hypertension has been associated with a modest increase in risk of both prevalent and incident AAA (15, 17). While there has been evidence that hypertension is associated with rapid AAA growth in a rat model (31) this has not been demonstrated in humans to date (7).

1.1.2.5   Lipids

The role of dyslipidaemia in AAA risk is controversial. The largest cross-sectional studies indicate that "hypercholesterolaemia", defined in binary terms, is independently associated with an increased risk of AAA, but these studies do not report the contribution of each of the different lipid fractions (15, 30). Unlike

coronary heart disease (CHD), in which large trials of statin therapy have unequivocally demonstrated a causal role for LDL-C, the role of LDL-C in AAA formation and progression is less clear. In sub-group analyses from the Heart Protection Study (32) statin use was not associated with a lower number of AAA repairs. Two studies have unexpectedly reported that statin use is associated with an *increased* risk of AAA (17, 33). The numbers of patients included in these analyses is, however, small and the results are thought to represent confounding by other cardiovascular risk factors, even after adjusting for these in the statistical model. Retrospective studies have reported a reduction in the growth rate of small AAA with statin therapy, but a recently published study that examined individual patient data (IPD) from over 15000 cases of small AAA found no evidence that statins alter AAA growth (7), and meta-analysis of studies reporting the effect of statins on AAA growth did not demonstrate a significant association (34). There have been no prospective randomized trials of statins that have examined AAA growth as an end-point but studies that have used surrogate markers such as inflammation in the AAA wall show a beneficial effect of statin therapy (35) but whether or not this is clinically meaningful is yet to be demonstrated. Despite this controversy, it is clear that patients with a dilated aorta have an excess risk of other cardiovascular disease events (CHD, CVA & PVD)(36-38) so it is likely that statin therapy in all patients with small AAA is indicated and is recommended by the Society for Vascular Surgery (39). Furthermore, there are data to suggest that statins improve perioperative outcomes in patients with large AAA (40), which supports universal prescription in AAA. Low circulating HDL-C has been

associated with an increased risk of AAA in prospective and cross-sectional studies and this is discussed in more detail in Chapter 5 of this thesis.

Lp(a) is a lipoprotein subclass that has shown a consistent association with risk of atherosclerotic disease in epidemiological studies. Meta-analysis of published literature has demonstrated that Lp(a) levels are associated with an increased risk of AAA (41), and recent genetic studies have demonstrated that genetic variants in *LPA* that increase Lp(a) concentrations are also associated with increased risk of AAA (42), suggesting that Lp(a) is likely to be a causal factor in AAA and therefore novel treatment target.

Elevated concentrations of triglycerides are also associated with an increased risk of CHD and large-scale MR analyses suggest that this may be a causal relationship(43). There are, however, few data reporting the association between TG concentrations and AAA and no preclinical data therefore the association between TG and AAA is currently poorly defined.

1.1.3   Diabetes

One of the most surprising observations from the observational epidemiological data of AAA is that of an inverse association with Type II Diabetes Mellitus (T2DM), which is a major risk factor for occlusive atherosclerotic disease. Furthermore, there seems to be an association between diabetes and slower AAA growth (7)., but the precise mechanism and therefore therapeutic potential of these observations has not yet been realized. Possible biologic reasons for this surprising observation could be a result of diabetic medications or an effect of

chronic hyperglycaemia on the vascular tree. For example, hyperglycaemia reduces progression of aneurysms in an animal model (44) and rosiglitazone (historically used to treat T2DM) protects against AAA in the AngII infusion model(45). An alternative explanation is that the observed association is simply due to some form of systematic bias in the studies, for example survival bias (ie people with T2DM may have poorer overall survival and therefore not live long enough to develop AAA complications). In my view, however, this latter hypothesis is unlikely to be the case given the repeated and consistent observation made across many different studies.

1.1.3.1 Other Risk Factors

Central obesity has been associated with a modest increase in risk of AAA in screening and cross-sectional studies (30, 46), as has alcohol consumption, but the mechanism underlying these associations is unclear(47).

1.1.4 AAA and Atherosclerosis

An important question to consider is whether or not AAA is simply a manifestation of atherosclerosis or whether it represents a distinct pathological entity. While it is clear that patients with AAA have a heavy burden of atherosclerotic disease, the current view is that AAA and atherosclerosis are likely to represent overlapping but distinct disease processes(23). Although the risk factor profile of the diseases is similar, there are important distinctions (Figure 2) and there does not appear to be a dose-response relationship between the burden

of atherosclerosis (as measured by intima-media thickness) and risk of aneurysmal

disease (48). It is therefore, important to understand both where the diseases share

pathobiological pathways and where they differ.

Atherosclerosis   **AAA**

Clinical Risk Factors
•Type 2 DM
•M:F – 2-3:1
Biomarkers
•LDL-C (strong Causal Relationship)
Heritability
•~50%
Histology
•Intima-media thickening & plaque formation

Clinical Risk Factors
•Age
•Smoking
•Obesity
Biomarkers
•CRP
•Fibrinogen
•HDL
•IL-6

Clinical Risk Factors
•?Type 2 DM protective
•M:F – 5:1
Biomarkers
•Relationship with LDL-C unclear
•PIIINP*
Heritability
•~70%
Histology
•Transmural chronic inflammation

* Procollagen III-N-terminal-propeptide – biomarker of collagen synthesis

Figure 2 - Overlap between occlusive atherosclerotic disease and AAA – Factors that suggest AAA and atherosclerosis are distinct disease entities include a stronger male predisposition in AAA, a greater heritability, lack of association with LDL-C, an apparently negative association with Type 2 DM and distinct histopathological differences. Procollagen III-N-terminal-propeptide (PIIINP) is a biomarker of collagen synthesis that has been associated with AAA

1.1.5   Screening for AAA

In many cases AAA remains occult until rupture and has been dubbed as a "silent killer" in the lay-press. As the diagnosis of AAA can be made reliably and safely with non-invasive imaging of the abdominal aorta, this prompted speculation that population-based screening may be an effective means to reduce the burden of AAA rupture. Following promising reports from early pilot screening programs a number of randomized controlled trials of aneurysm screening have been reported in the literature (11, 14, 49, 50)(Table 2). Meta-analysis of the results of these has reported a consistent reduction in AAA-related death in male populations that are screened (51), and as a result, population screening is currently being rolled out in the UK (http://aaa.screening.nhs.uk/), for men only.

Table 2 - Randomized controlled trials reporting the utility of population screening of men for AAA using ultrasound scan. RR – Relative Risk for aneurysm related death.

Trial Name	Invited/Not Invited	Attendance Rate (%)	Age	RR (95% CI)
MASS	33,839/33,961	80	65-74	0.52 (0.45-0.74)
Viborg	6,339/6,391	69	65-73	0.31 (0.13-0.79)
Western Australia	19,352/19,352	63	65-83	0.72 (0.39-1.32)
Chichester	4,682/4,660	65	65-80	0.57 (0.27-1.29)

1.1.6   Management of AAA

The management of AAA is primarily determined on size criteria. Operative repair is offered to patients whose AAA is greater than 5.5cm in maximum

diameter. This threshold is largely based upon data from randomized controlled trials of surveillance versus open surgical repair in patients with small AAA that have failed to demonstrated any benefit for early intervention (52, 53). Patients with AAA less than 5.5cm are entered into ultrasound surveillance programs and the frequency of repeat scans is largely determined by the size of the AAA, as the growth rate of AAA is correlated to maximal aneurysm diameter (8). In the UK, the recommendation is that individuals with a AAA < 4.5cm are followed up annually, and those over that threshold are offered three monthly scans(54)(54)[54](54)(54)(54)(54)(54)(54)(54)(54)(54).

Most AAA discovered by screening are small and do not warrant immediate surgical intervention (11). Given the inherent risks of surgical repair, a non-surgical/pharmacologic treatment to attenuate growth of small AAA is a potentially attractive alternative to the surveillance and surgery paradigm. Given the average age of onset and average growth rate of small AAA, a treatment that effectively reduces expansion by 50% will delay the need for operative repair by 10 years, which in many cases will be longer than the patients' lifespan (55). Despite this, there is no strong evidence base to support use of any particular pharmacological agent specifically to attenuate the growth of small AAA in humans. Non-randomised observational studies in the literature have reported associations between a variety of cardiovascular preventative medicines and AAA growth rates, including statins, anti-platelet medications and anti-hypertensives

and the results have been consistently inconsistent (56). Beta-receptor blockade with propranolol has been subjected to randomized controlled trials in AAA, but two large studies failed to show any clear benefit with regard to AAA growth rates (57, 58). In both studies, the intervention was poorly tolerated and whether or not newer formulations would perform better is unclear. There is also evidence from a large RCT that perioperative beta-blockade may be of use in preventing myocardial infarction in patients with AAA(59). Doxycycline, an antibiotic that has anti-metalloproteinase activity, has also been evaluated in a small RCT that reported a non-statistically significant reduction in AAA growth rates. As a result, two larger trials are underway and are expected to report in 2014(56). There are also trials of ACE inhibition and mast cell stabilization underway and these are expected to report in coming years also. Given the lack of strong evidence for treatments to attenuate AAA growth, current medical management of patients with small AAA is primarily aimed at reducing *overall* cardiovascular risk and targets risk factors such as LDL-C, blood pressure and thrombotic risk(39).

1.1.7 Intervention for AAA

There are two surgical interventions that are routinely used to prevent rupture of large AAA. Open surgical repair involves a laparotomy (either longitudinal or transverse) and replacement of the aneurysmal segment with a surgical graft that is sewn into the aorta. An alternative, minimally invasive technique, known as endovascular aneurysm repair (EVAR), has been around for more than 20 years. This technique involves placement of a self-expanding stent-graft into the aorta

through the femoral arteries. The stent-graft forms a "seal" above and below the aneurysm and this prevents pressurized flow into the aneurysm sac. There have been many studies that have compared the efficacy of the two methods. The largest of these is the EVAR-1 study that compared outcomes in patients randomized to receive either open surgical repair or endovascular repair. It was shown that EVAR had superior outcomes in the early post operative period, but the survival benefit is not maintained in the longer term, where no difference is observed (Figure 3). That said, both treatment modalities are largely successful in preventing the aneurysm from rupturing and in the UK the decision on whether a patient has open surgical repair or EVAR is dependent upon a number of factors including patients demographics, patient choice, anatomical configuration of the AAA and expertise at the treating institution. Unless treated surgically (with open or endovascular techniques), ruptured AAA is usually fatal. A trial to compare the modalities is currently underway in the UK, but ruptured AAA has a poor outcome whatever modality is used. A recent meta-analysis demonstrated that the mortality for operated on ruptured AAA is still in the region of 40%, despite overall improvements in outcomes over time (~3.5% improvement in mortality per decade)(60).
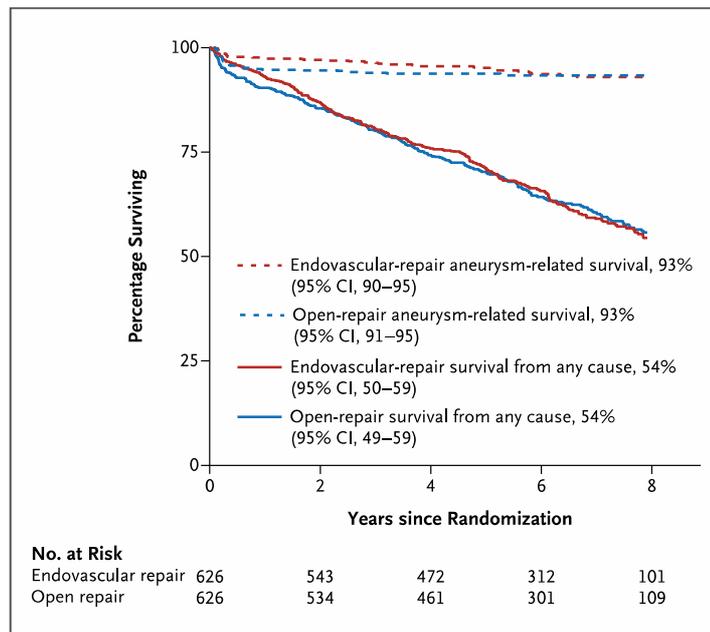
Figure 3 – Kaplan-meier survival curves from the EVAR-1 study, demonstrating equal survival following open and endovascular repair of AAA.

1.1.8 Understanding the Pathophysiology of AAA

Aneurysmal degeneration of the aortic wall is likely to be a complex remodeling process mediated by inflammatory pathways that promote proteolysis within the arterial wall (23). Attempts to understand these processes and define novel therapeutic targets have largely come from experimental animal models of AAA and studies examining tissue harvested at the time of aneurysm repair.

1.1.8.1 Animal Models of AAA formation

Aortic aneurysms can be induced in small animals by a variety of means that can be broadly characterized as chemical, genetic or surgical (Table 3). Although there are many differences in anatomy and physiology between small animal models and humans there have been some notable discoveries using these models

that have translational potential. For example, following observations that
doxycycline had anti-metalloproteinase effect in mice aortae (61) and a protective
effect against aneurysm formation (62) in experimentally induced aneurysms,
trials in human AAA(63) were initiated and are ongoing. Other findings from
experimental models that have the potential for translational studies are
summarized in Table 4.

Table 3 - Animal Models of Arterial Aneurysm

Model	Method	Animal	Reference
Chemical	Elastase Perfusion	Rat	(31)
	AngII Infusion Model, hyperlipidaemic mice	Mouse	(64)
	Calcium Chloride Infusion	Multiple	(65)
Genetic KO	*Fbln4* knockout	Mouse	
	Lrp1 knockout	Mouse	(66)

Surgical	Xenograft	Rat	(67)
	Arterial Ligation	Mouse	(68)

Table 4 - Summary of some of the major findings from studies using small animal models of aortic aneurysm

Reference	Experimental Model	Finding
(69)	AngII Infusion and CaCl$_2$	JNK Inhibitors both prevented aneurysm formation and induced regression of established aneurysms
(70)	Elastase	Mast cell stabilisation prevented aneurysm formation
(45)	AngII	Rosiglitazone prevented aneurysm formation and rupture
(71)	AngII & Elastase	MicroRNA 21 prevented aneurysm formation in both models

1.1.9 Tissue Studies of AAA

Histological studies of tissue harvested at the time of AAA repair have reported

the presence of trans-mural inflammation (72). On the basis of these a number of

studies have been published examining the expression levels of various cytokines,

with an overarching hypothesis that pharmacological manipulation of pathways related to these cytokines may be a useful strategy on non-surgical management of AAA. A systematic review of studies investigating cytokine expression in AAA tissue reported that there appears to be consistent upregulation of tumour necrosis factors alpha and interferon-gamma in aneurysm tissue (73). The review did, however, identify methodological flaws such as poor matching of cases and controls and small sample sizes that limits confidence in the validity and clinical application of the results. Perhaps the greatest limitation of these studies is, however, that the tissue inevitably comes from large AAA, which by definition represents an end-stage of the disease and so the observed changes may be the result of the advanced pathology rather than truly causal.

It has been proposed that a key factor in the development of AAA is destruction of elastin in the medial layer of the aorta, while loss of collagen is a key driver of aneurysm enlargement and eventual rupture (72), but the data prompting this hypothesis have come largely from the study of a single animal model of aneurysm, and has not been validated in humans. Indeed, it is not clear whether or not aneurysm formation, growth and rupture represent distinct processes that are driven by separate pathways, or whether they are all manifestations of a common pathological pathway. Evidence to support the former hypothesis come from the animal models of AAA that have shown more aggressive aneurysm phenotypes by manipulating pathways such as TGF-beta signaling (74), whereas support for

the latter comes from studies that demonstrate that the major risk factors for developing an aneurysm also appear to be associated with growth rates and rupture (for example smoking, hypertension and presence of diabetes)(7).

1.2 The genetics of complex disease

1.2.1 Complex diseases

A "complex disease" is one that results from a combination of genetic, environmental and lifestyle factors in which no single exposure in isolation is necessary or sufficient to cause the disease. This is in contrast to "Mendelian" disorders that are caused by single gene mutations and follow established inheritance patterns such as autosomal dominant or autosomal recessive. Most common diseases such as AAA, CHD, Alzheimer's disease, Parkinson's disease and schizophrenia are considered to be complex diseases. This network of environmental and genetic risk factors makes deciphering the precise causal mechanisms extremely difficult and a commonly used analogy is that of trying to build a jigsaw puzzle without having available all the pieces. Furthermore, there may be "interaction" between genetic and environmental factors that adds a layer of complexity to the challenge of understanding such diseases.

1.2.2 Determining the environmental and genetic risk factors that predispose to complex diseases

1.2.2.1 Case-control studies

In this method, risk factor(s) are compared between a selected group of individuals with a specific diagnosis (cases) and a group of individuals that do not show evidence of that disease (controls). There are many benefits from this approach; recruitment from medical centers treating the condition is straightforward, follow-up time is short and in general it is relatively inexpensive. The major advantage lies in diseases that are not very common, as population-based studies are prohibitively large in the study of rare disease. There are, however, a number of possible biases in case-control designs that can limit the generalizability of the results to the general population (Figure 4).

1.2.2.2 Prospective Cohort Studies

In this method, a cohort of individuals is followed up over a period of time, and the incidence of disease events is carefully recorded. This is considered the gold-standard method in epidemiology, as precise quantification of exposures preceding the development of a disease can be made. The caveat to this is that study of diseases that are rare in the population can be prohibitively expensive, as cohort sizes need to be extremely large to achieve adequate statistical power.

- Prevalence incidence bias – selection of a current case may select out a subgroup of cases with less aggressive disease, as those in the population who have already died from the disease are not includable. This is relevant in AAA as rupture in many cases is fatal.
- Diagnosis bias – individuals with known exposures (e.g. smoking) may be more likely to be referred for diagnostic studies than others.
- Surveillance bias – individuals under frequent medical surveillance may be more likely to be diagnosed. For example, people with CHD may be more likely to have a AAA detected than the healthy population.
- Selection bias – controls are not representative of the cases
- Recall Bias – questions about previous exposures may be inaccurately recalled and focus on suspected putative causative factors (e.g. smoking).
- Family information bias – cases with a family history of a disease may be more likely to be diagnosed and/or likely to participate in such studies

Figure 4 - potential biases in cross sectional studies

1.2.3 Genetics of Complex diseases

By definition, complex diseases do not follow simple inheritance patterns and it is believed that the heritable component of such diseases is likely to be explained by the co-inheritance of multiple genetic variants, each with a modest effect on disease risk. This theory is termed the "common-variant common disease" hypothesis and underpins many of the methodologies that are currently used to determine the genetic factors that underpin complex diseases. In a candidate-gene approach, the frequency of variants in genes thought, by the investigators, to be important in a disease process is compared between cases and controls. Although

this approach yielded many important insights in many complex diseases including AAA (75), many published studies were underpowered and the results were in some cases inconsistent. A major problem with the candidate gene approach was that small studies with nominally significant results were more likely to be published than larger studies with more statistical power, which reported negative results (so called publication bias). Furthermore, under a candidate gene approach there is an inherent selection bias in the variants chosen to study, which are based upon an investigators belief of what is important in any particular disease.

Many of these problems have been overcome by the development of genome wide association studies (Figure 5). In this approach a large number of single nucleotide polymorphisms (0.5 – 1 million SNPs) are simultaneously genotyped. The frequency of each of these variants can then be compared in groups of cases and controls. Although there are considerably more than 1 million known SNPs in the genome, knowledge of linkage disequilibrium (LD – the non-random association of alleles at two or more loci) means that only a fraction of all known variants need to be genotyped, and information on missing genotypes can be imputed, such that up to 90% of common genetic variants (i.e. minor allele frequency (MAF) > 5%) can be captured by a single chip. This approach is said to be "hypothesis free" as no single area of the genome is given a higher priority than any other. The major challenge with GWAS is, however, statistical power, as there are a high number of independent tests and therefore a high chance of false

positive associations. To account for this, a Bonferonni correction is used and

consequently, the p-value thresholds used to define genome wide significance is

$p<5x10^{-8}$. This fact and the need to replicate discovery findings necessitates very

large samples sizes before a GWAS for a common complex disease can be

undertaken.
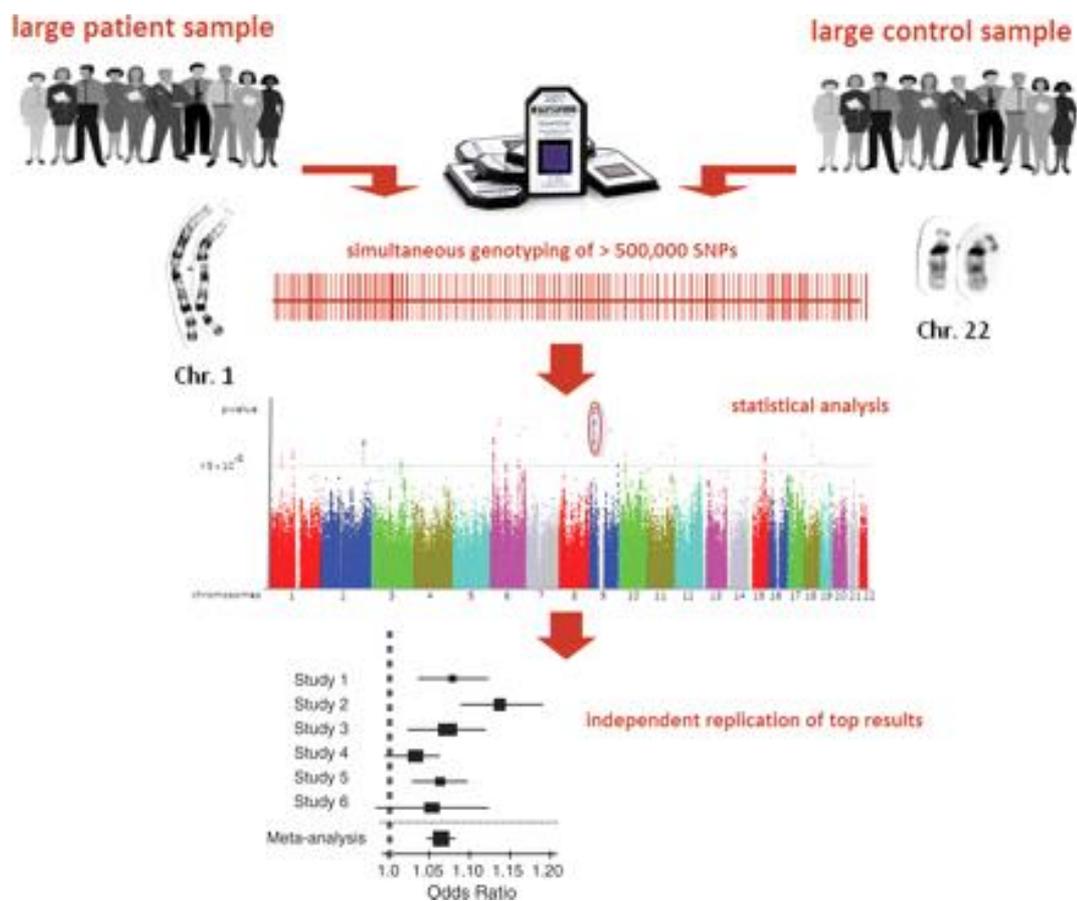
Figure 5 - Genome Wide association studies, from(76). A large case control cohort is simultaneously genotyped (for 0.5 – 1 million SNPs) and allele frequencies are compared.

In 2007, the field of cardiovascular genomic research was ignited by simultaneous

publication of three GWAS of cardiovascular disease (77-79). Each of the studies

demonstrated a strong association between common SNPs on the short arm of

chromosome 9 at position 21 and a higher risk of myocardial infarction. These data exemplified the power of GWAS, as this locus would not have been given priority using a candidate gene approach. The limitations were, however also highlighted, as the functional significance of this locus was unclear and it has taken a further 3-4 years to understand the biological consequences of variants at this locus at a cellular level.

Over 900 GWAS have now been performed in a diverse range of common complex disorders (http://www.genome.gov/26525384#1) (Figure 6). Many novel and previously unsuspected pathways for common disease have been uncovered, such as the role of autophagy in Crohn's disease, while many of the "expected" pathways have been confirmed, such as genes that influence both LDL-cholesterol metabolism and coronary heart disease (CHD)(80).
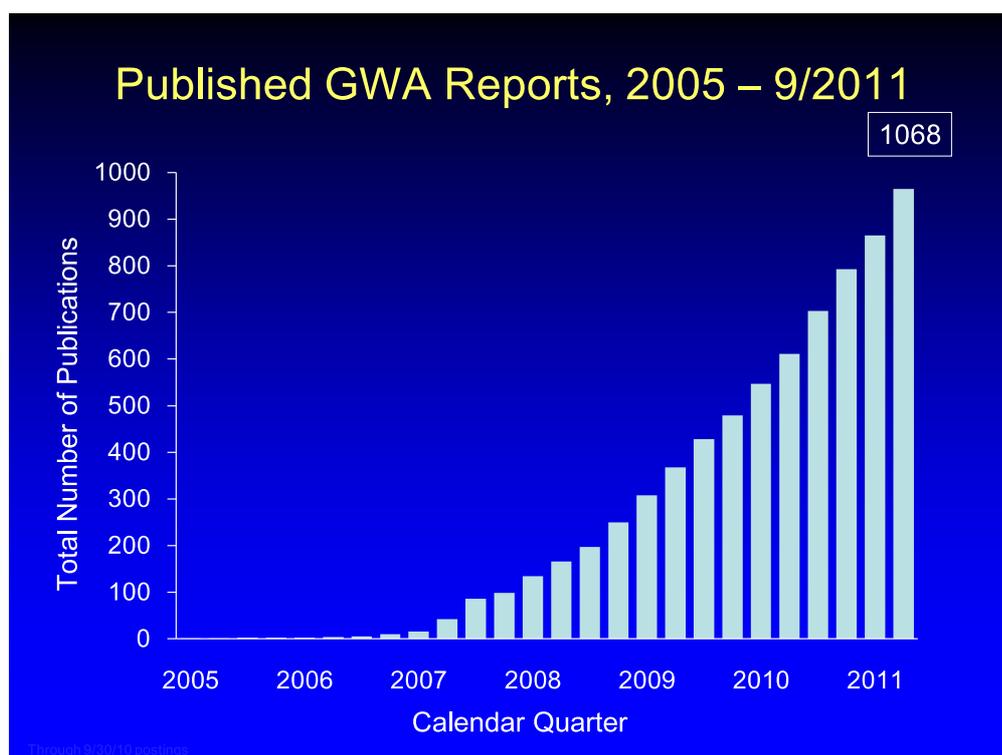
Figure 6 – Exponential increase in the number of published GWAS in recent years (source www.genome.gov/multimedia/illustrations/Published_GWA_Reports_2005-June2011.pdf)

A number of themes have emerged from the first wave of GWAS published in the last 3-4 years (Figure 7). Although many variants have been reliably identified by GWAS, the variants individually or in combination explain only a fraction of observed disease heritability. This heritability-gap, and more precisely, the reason for this, is perhaps the most hotly debated of these points in the literature (81). Some argue that GWAS has proved the common-variant hypothesis, and therefore increasing sample sizes will be the best method to explain the missing heritability. Others argue that in some ways, GWAS findings have argued against the common

variant hypothesis, and that the heritability gap will be explained by rare variants of large effect that are not adequately covered by GWAS. An alternative hypothesis poses that it is in the interaction of genes with each other (epistasis) or with environmental exposures (gene environment interaction) where much of the genetic "dark matter" lies. Perhaps the most pragmatic view, and the one that I take, is that a combination of the above factors will be at play. Finally, it is possible that heritability estimates, which in many cases come from relatively small studies, are inflated and chasing the heritability will be a futile endeavor.

Small effect sizes – under the common variant hypothesis it is expected that variants have a modest effect, the observed effect sizes have been smaller than expected. A typical variant identified by GWAS increased the risk of a dichotomous trait by ~ 5-20% per risk allele carried. Furthermore, it is considered that the early discoveries had the largest relative effects (the "low hanging fruit") and identifying variants with ever-decreasing effect sizes will be extremely difficult.

Missing heritability – for both continuous and dichotomous traits, the SNPs identified by GWAS explain a small proportion of the expected heritability if those diseases.

Pleiotropic effects & GWAS Hotspots – Some of the identified loci appear to associate with a number of distinct diseases, not previously thought share common pathological features.

The elusive "causal" variant(s) – the variants identified are often in non-coding areas of the genome and are not in LD with coding variants. As a result, for any given variant it is often not immediately clear how this variant increases risk of disease, or indeed which variant is actually causal.

Personalized medicine may be more diffic lt than anticipated - Variants identified by GWAS, either in isolation or combination do not accurately predict the onset of diseases accurately enough to advocate routine usage in clinical practice.

Figure 7 – Themes emerging from the first wave of GWAS.

1.3 Genetics of AAA

1.3.1 Monogenic diseases of the Aorta

There are no known monogenic diseases that are characterized by development of infra-renal aortic aneurysms. This is not, however, the case for aneurysms of the thoracic aorta where more progress has been made in understanding the genetic architecture of the disease. Aneurysms of the thoracic aorta often occur in families and tend to show autosomal dominant inheritance (82). The importance of the TGF-beta signaling superfamily has been demonstrated in both familial and sporadic aneurysms of the thoracic aorta. Marfan syndrome, characterized by tall stature and development of aortic dissections and aneurysms, is caused primarily by mutations in the gene encoding Fibrillin-1 (*FBN1*)(83), a member of the TGF-beta family. Interestingly, a recent GWAS of sporadic thoracic aortic aneurysms identified common variants in *FBN1* to be associated with sporadic forms of TAAA (84). Furthermore, two recent linkage studies have provided definitive evidence that loss of function mutations in *TGFB2* cause familial forms of thoracic aortic aneurysms (85, 86). Given the overwhelming evidence that alterations on TGF-beta signaling causes thoracic aortic disease, there has been considerable interest in whether or not the same pathway plays a role AAA. A number of candidate gene studies have been performed in AAA, but no definitive evidence of an association has yet been provided (Table 5).

Table 5 - candidate gene studies investigating SNPs in genes of the TGF-beta superfamily and AAA

Reference	Genes	Cases/Controls	Findings
(87)	26 SNPs in *LTBP1, LTBP3, TGFB1, TGFB3*	1890/3875	No association with AAA, weak association between *LTBP1* haplotype and AAA growth rates
(88)	58 SNPs in *TGFBR1, TGFBR2, TGFB1*	1003/1711	No association with AAA
(89)	28 SNPs in *TGFBR1 & TGFBR2*	736/1002	1 SNP in *TGFBR1* and 2 in *TGFBR2* associate with risk of AAA, but finding not replicated in other cohorts

1.3.2 Familial Studies

There have been a number of segregation studies of AAA to determine the mode of inheritance. A summary of segregation studies is provided in Table 6. Overall there is strong evidence for a genetic component to AAA but no single mode of inheritance has been identified, suggesting that multiple genetic and environmental exposures are the likely cause of the disease.

Table 6 - Summary of familial/segregation in AAA

Reference	Methods & Findings
(90)	• 50 families with clustering of 2 or more 1st degree relatives with AAA • If a single gene - autosomal, multigenic inheritance cannot be excluded
(91)	• Family members of 60 AAA patients studied • 70% Heritability

(92)	• Families of 91 probands examined • Many autosomal recessive inheritance likely
(93)	• 276 Patients with a FH of AAA • Single gene locus, dominant, allele frequency of 1:250
(94)	• 233 Families with 2 affected members • Autosomal recessive fits 75%, autosomal dominant 25%, lack of consistency suggestive of multifactorial

1.3.3 Candidate gene studies of AAA

The "common-disease common variant" hypothesis poses that common complex diseases arise from the co-inheritance of multiple genetic variants, each with a modest effect on risk (low penetrance), plus the influence of one or more environmental risk factors (95, 96). This hypothesis has underpinned genetic association studies, whereby the frequency of common single nucleotide polymorphisms (SNPs) in candidate genes are compared between cases and controls. A candidate gene is one that is selected by the investigator (s), for reasons such as biological function of the gene(s) and or previous studies that have suggested a role in AAA. A number of candidate gene studies have been published for AAA, examining genes involved in tissue remodeling, inflammation, lipid metabolism and extra-cellular matrix biology. In 2008, Thompson et al performed a systematic review of published candidate gene studies in AAA(75). Many of the studies identified were underpowered and replication in independent cohorts was not usually attempted, which reduces confidence in many of the findings. It was, however, reported that variants in

three candidate genes (*ACE, MTHFR & MMP9*)(Table 7) showed replicated

association with AAA. Since the 2008 review a number of other candidate gene

studies have been published in the literature, but few have shown convincing

associations that have been replicated in independent cohorts (25, 46, 87, 97-101).

Table 7 - SNPs in candidate genes with replicated association with AAA

Gene/Polymorphism	Number of Studies (total Cases/Controls)	Effect Size (OR & 95% CI)
Angiotensin Type 1 Receptor/A116C (rs5186)	3 (1226/1712)	1.39 (1.2-1.60)
Angiotensin Converting Enzyme I/D (rs4646994)	4 (1657/2238)	1.24 (1.12-1.36)
Methlyenetetrahydrofolate reductase +677C>T	5 (1086/895)	1.23 (1.02-1.50)
MMP9 (1562 C>T)	3 (848/802)	1.09 (1.01-1.18)

1.3.4   Genome Wide Association Studies   (GWAS)

Following the landmark discovery of the 9p21 locus association with myocardial

infarction, it was reported that SNPs at this locus were also strongly associated

with the presence of AAA (102), an association that has been replicated in a

number of well-powered case-control studies (Table 8)(103-105). Approximately

20% of the population (of European ancestry) carry two risk alleles at this locus and have an approximately 70% increased risk of AAA compared to the 25% of the population who carry zero risk alleles.

Table 8 - Studies reporting asssociation between 9p21 variants and AAA.

Study	Controls/Cases	SNP	Per allele odds ratio & p-value
Helgadottir[29]	16,732/2,836	rs10757278	1.31, p = 1.2 x 10^{-12}
Bown[33]	815/899	rs1333049	1.22, p=0.004
Thompson[34]	1366/741	rs10757278	1.38, p=0.03

The first GWAS specifically of AAA was published in 2009 and identified association of a SNP on Chr3p12.3 with AAA (OR 1.33, p=0.0028)(106). This did not meet conventional levels of genome wide significance and has not been replicated in independent sample sets(107). In 2010, a larger GWAS with greater statistical power reported a novel association with sequence variant in *DAB2IP* on Chr9q33(108). The discovery phase included 1,292 individuals with AAA (defined as an infrarenal aortic diameter >3cm) and 30,530 unscreened controls (a small proportion of whom are likely to harbor AAA), while follow-up replication studies included 3,297 cases and 7,451 controls (all cases and controls were of European ancestry). The variant conferred a per allele odds ratio for AAA of 1.21 (95% CI 1.14-1.29, P=4.6 x 10^{-10}), which translates to an approximately 20% increased risk of AAA for each copy of the risk allele carried. Interestingly, the investigators also found an association between this SNP and CHD, venous

thromboembolism and peripheral arterial disease, suggesting those diseases share at least some common pathobiological pathways.

The third GWAS of AAA was published in 2011, and I was one of the investigators in this study (109)(Appendix 2). The discovery phase included 1,866 cases of AAA and 5,423 unscreened controls. The replication phase included data from a further 4,362 cases and 43,734 controls. This identified a common variant (rs1466535) in the first intron of the low-density lipoprotein related protein 1 (*LRP1*) that was associated with risk of AAA (OR 1.15, 95% CI 1.10-1.21, P=4.5 x 10^{-10}). In contrast to the 9p21 and *DAB2IP* loci, this variant showed no association with increased risk of CHD.

LRP1 had been previously implicated as a potential candidate gene in AAA, as a murine model with *Lrp1* -/- vascular smooth muscle cells developed aortic aneurysms (66, 110). Surprisingly though, it was found that the AAA risk allele of rs1466535 was associated with *increased* expression of *LRP1* in aortic tissue, although this association was relatively weak (CC vs TT expression fold change = 1.19, 95%CI 1.04 – 1.36, P=0.026). It is thought that the risk variant alters an SREBP-1 binding site and therefore acts as an enhancer for *LRP1* transcription.

1.3.5   Novel insights into AAA pathology from GWAS findings

The functional significance of the 9p21 locus was not immediately obvious, as the lead SNP (or any in close LD with it) does not lie in a protein-coding gene. It has, however, been identified that this risk variant overlaps with the recently annotated

non-coding RNA (ncRNA), *ANRIL.* NcRNAs can alter expression of protein coding genes by mechanisms such as gene silencing, DNA methylation, chromatin remodeling and RNA interference (111). Functional studies of this locus have demonstrated that carriers of the risk variant have reduced expression of *ANRIL*, along with other nearby genes such as *CDKN2A* and *CDKN2B* (112)*, while Jarinova et al found that the risk locus has enhancer activity in primary human aortic smooth muscle cells and that pathways involved in cellular proliferation were upregulated in risk allele carriers (113). Visel et al recently demonstrated that targeted deletion of this region in a mouse model leads to increased expression of *CDKN2A* and *CDKN2B,* and that aortic smooth muscle cells from these animals displayed excessive proliferation and diminished senescence (114). More recently, it was shown that the region at 9p21 is densely packed with enhancer sites that are capable of altering expression of both neighboring and long- range genes by physical interaction. Specifically, variants associated with CHD (and AAA) disrupt binding of the transcription factor STAT1, which results in altered expression of *CDKN2A* and *CDKN2B, MTAP* and *IFNA21.* It was also demonstrated that the transcriptional control of the 9p21 enhancers was remodeled with interferon-γ, providing evidence that genetic variation determines the response to inflammatory stimuli within the vasculature (115).

The SNP in *DAB2IP* discovered by GWAS also associates with coronary artery disease, peripheral arterial disease, venous thrombo-embolism and pulmonary

embolism but shows no association with any classical CHD risk factors (108, 116). *DAB2IP,* located on Chromosome 9q33, is a GTPase activating protein known to play an important role in prostate cancer metastasis (117). A SNP in this gene has been associated with aggressive prostate cancer[49], while *in vitro* functional studies have demonstrated that loss of the protein leads to enhanced cell proliferation and reduced apoptosis, via the PI3-Akt pathway (118). *DAB2IP* expression is significantly reduced in AAA tissue compared to tissue from healthy controls (119), and this SNP did correlate with reduced expression of the protein in aortic tissue (though this was not reproduced in mammary artery tissue)(108). It is possible, therefore that this variant also promotes excessive VSMC proliferation, through reduced expression of *DAB2IP* in aortic tissue. *DAB2IP* expression is modulated by EZH2 (an enzyme) that has been proposed as a potential target in prostate cancer (120, 121) so if, at a molecular level the link between genetic variation at this locus, *DAB2IP* expression and vascular disease were uncovered, enzymes such as EZH2 could be potential novel targets in pharmacological therapies to attenuate AAA formation.

Interestingly, an animal model with knockout of VSMC low-density lipoprotein receptor-related protein 1 (*Lrp1)* develops both aortic aneurysms and occlusive disease, independent of circulating lipid levels, and VSMCs from these mice display a highly proliferative phenotype (66). It appears therefore that SNPs discovered for AAA by GWAS may converge upon a common disease pathway –

excessive VSMC cell proliferation. This suggests that accumulation of small

disturbances in different elements of the VSMC proliferation pathway combine to

increases the risk of both atherosclerosis and AAA (Figure 8), as suggested by the

common-variant hypothesis.
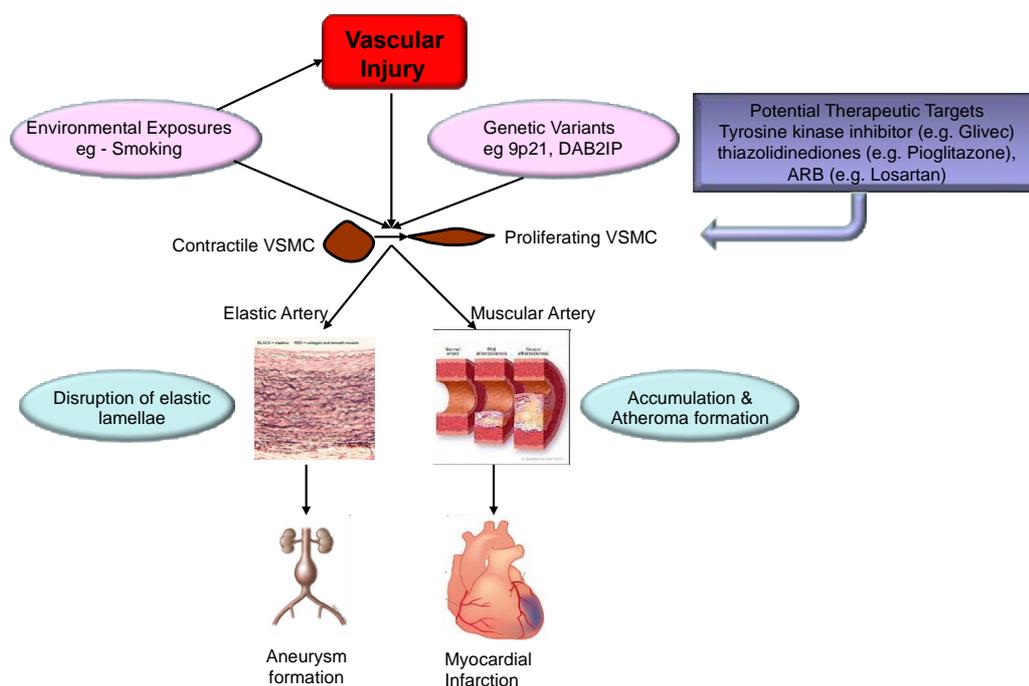
Figure 8 - Potential mechanism by which GWAS identified SNPs increase risk of AAA +/- CHD (122). VSMC – vascular smooth muscle cell.

The fact that SNPs discovered by GWAS have relatively small effect sizes,

explaining merely a fraction of observed heritability does not preclude potential

biological importance, as they may highlight important pathways in disease (123).

For example, genes highlighted by GWAS of type 2 diabetes mellitus (T2DM) are known targets for thiazolidinediones and sulphonylureas (124), drugs commonly used in this condition. For AAA, the genome wide data are pointing to pathways involved in promoting excessive VSMC proliferation. Cigarette smoking, a major environmental risk factor for both diseases leads to increased levels of proliferation in VSMCs (125, 126), whilst a role for excessive VSMC proliferation in aneurysm formation elsewhere in the arterial tree has been demonstrated - mutations in *ACTA2* and *TGFBR2* are all causal for thoracic aneurysmal disease (127, 128) and effect smooth muscle cell proliferation, while rare conditions such as Moyamoya disease and fibromuscular dysplasia are characeristised by arterial occlusion secondary to excessive VSMC proliferation (129).

1.4 Post Genome Wide Association Studies

1.4.1 Common diseases are quantitative traits

It has been suggested that the findings from the first wave of GWAS have supported the common-variant common disease hypothesis, as a large number of loci with relatively small effects are being identified. A 2008 article in *Nature Reviews in Genetics* argues that these findings should "lead us to think about disorders as the extremes of quantitative traits and, ultimately, to focus on quantitative traits rather than disorders" (130). Although the vast majority of GWAS employed a case-control design, many subsequent studies took the approach suggested by Plomin, and focused upon quantitative traits associated

with complex disease endpoints. Many examples of this approach have now been published in the literature (table 9) and this has proved to be a useful adjunct to the case-control discovery studies. This approach has not been used in AAA and this is something that I aimed to do in this thesis.

Table 9 – Genomic studies that have focused upon quantitative traits related to a dichotomous disease outcome. T2DM – Type 2 Diabetes Mellitus, IMT – Intima-media thickness, CAC – coronary artery calcification.

Continuous Trait	Disease	Reference	Finding
Obesity	T2DM	(131)	11 of 14 SNPs associated with Waist-Hip Ratio had concordant effects on risk of T2DM
Fasting Blood Glucose	T2DM	(3)	5 from 9 SNPs associated FBG traits were associated with T2DM
Blood Pressure	CHD	(132)	A genetic risk score of 16 variants was strongly associated with incident and prevalent CHD
Carotid IMT	CHD	(133)	2 of 3 loci associated with CIMT were also associated with CHD
Coronary Artery Calcium	CHD	(134)	2 SNPs associated with both CAC and CHD
Lipids	CHD	(135)	14 of 95 lipid loci show association with CHD.
Breast Density	Breast Cancer	(136)	Variant identified that associated with both higher mammographic density and breast cancer

1.5 Mendelian Randomization

1.5.1 Causal Relationships in biomedical research

Understanding causality is key in biomedical research in order to determine disease aetiology, assess the impact of public health interventions, to inform public policy, prioritize resource utility, develop treatment guidelines, and accurately advise upon the impact of lifestyle choices. The starting point for assessment of causal relationships tends to be observational epidemiological studies, whereby the statistical association between an exposure variable and outcome is assessed. Candidates from the strongest observational associations may then be followed up in randomized controlled trials of interventions that target the exposure of interest. If the intervention affects the outcome then it may be considered that the risk factor is causal. An example of this is the study of LDL-C, where following a wealth of studies reporting an association between higher LDL-C levels and risk of CHD randomized trials of statins then confirmed the causal relationship (32).

There have been, however, a number of risk factors that have shown robust association in the observational literature but then failed at RCT, for example HDL-C and CHD, Vitamin E in CHD and beta-carotene in solid cancers (137, 138). The reasons that an exposure may shown strong association with an outcome, without actually being causal are;-

1) Confounding – a confounding factor is a variable that is associated with both the exposure and the outcome variable (Figure 9).

2) Reverse Causation – this is when the exposure variable is altered by presence of the outcome variable. This relationship may be present at an subclinical phase of disease and therefore not accounted for even in prospective cohort studies (Figure 9).
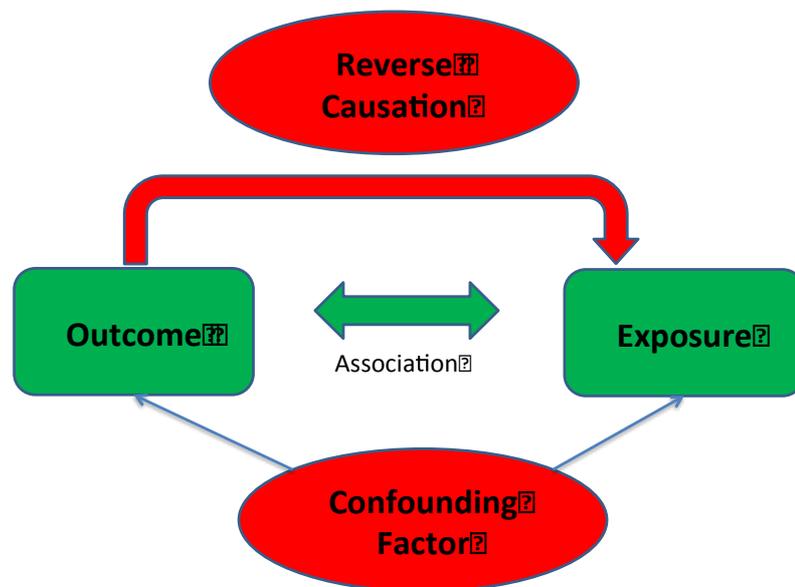
Figure 9 – Confounding and reverse causality are reasons that an observed association between a risk factor and disease may not reflect causality.

Given the fact that RCTs are expensive, time-consuming and potentially risky it is simply not feasible to perform one for every promising biomarker. Therefore an alternative means inferring causality from observation epidemiology is an attractive proposition.

1.5.2 Mendelian Randomization for causal inference

The principle of Mendelian randomization (MR) utilizes some of the fundamental properties of genetic variation to make causal inferences in complex disease. In epidemiological terms, genotype is a unique population 'exposure' for the following reasons:

1. Genotype at a given locus is allocated at random during the random segregation of alleles at gametogenesis. This minimizes potential confounding. This has drawn paradigms with the randomization process used in clinical trials.

2. A central dogma of molecular biology dictates that information flows in only one direction from a DNA sequence through messenger RNA (mRNA) and protein to a more complex phenotype. Therefore genotype is not prone to reverse causality.
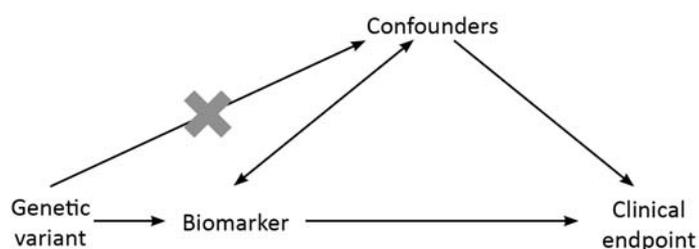
Figure 10 – The Mendelian randomization paradigm(139). Concordance of effect of the genetic variant and biomarker on disease risk supports a causal hypothesis for the biomarker.

Therefore genotype can be used as an instrument to examine potentially causal relationships between biomarkers and disease endpoints as shown in Figure 10. If a specific genotype is consistently associated with higher circulating biomarker concentration, and the biomarker is consistently associated with a disease end-point, then it is expected that the same genotype should be associated with the disease end-point, if that biomarker is indeed causal. Furthermore, the effect size should be broadly consistent with effect of the genotype on the biomarker. MR studies have addressed many classic risk factors in CHD (Table 10). The relevance of MR can be considered from a translation research viewpoint, as the results can be used to prioritise novel targets for drug development, guide the design of clinical trials by informing researchers which biomarkers are actually causal (Figure 11). In addition, the results of MR may help interpret the results of clinical trials that have failed to demonstrate the expected clinical benefit. Although there have been a number of MR studies in the field of CHD, none have specifically focused upon AAA.
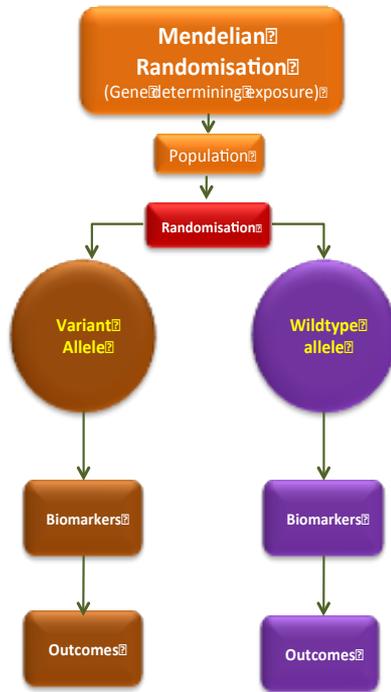
Figure 11 – Mendelian randomization – "Natures Randomised Trial" (140).
Random allocation of genotype is similar to the randomisation process used in
clinical trials

Table 10 – Summary of some major Mendelian Randomisation Studies of CHD

Biomarker	Finding	Reference
CRP	Genetically raised CRP does not appear to increase the risk of CHD, suggesting the association is not causal.	(141)
Fibrinogen	Genetically raised Fibrinogen does not appear to increase the risk of CHD, suggesting the association is not causal.	(142)
LDL-C	Genetic Variants that increase LDL-C increase the risk of CHD, suggesting a causal association. This is in-keeping with randomized trials of LDL-C lowering	(143)
Lp-PLA2	Genetically raised Lp-PLA2 does not appear to associate with an increased risk of CHD, suggesting the association is not causal.	(144)
HDL-C	Genetic variants that increase HDL-C do no effect risk of CHD, suggesting the association is not causal.	(143)

With an increased awareness of genetic association analyses, and development of large datasets it is likely the MR analyses will become more frequent in the published literature. Although MR is an attractive paradigm with which to address questions regarding causality, there are a number of considerations that need to be made when considering the validity of an MR analysis.

There are three main assumptions that are made in MR analysis;-

1) The genetic variant is independent of typical/known confounding factors.

2) The genetic variant is reliably associated with the exposure of interest.

3) The genetic variant only effects the outcome through the exposure of interest.

In practice it is often impossible to statistically exclude assumptions 1 & 3 as confounders and alternate pathways may be unobserved. It is important, therefore that the MR analyses must be justified by biological knowledge of the variant and/or associated biological pathways.

1.5.3   Problems & limitations with MR

1.5.3.1   Linkage disequilibrium

Genetic variants identified by GWAS tag an area of the genome that may contain a large number of genes. Therefore the *functional* variant that actually exerts the effect on the exposure may actually be in a different gene (Figure 12). Under this model the assumptions of MR are not necessarily violated, so long as the functional variant only exerts it's effects through the exposure variable of interest. Knowledge of the function of the variant and/or its gene can therefore be helpful in making this assessment (as is shown in Chapter 4 of this thesis).
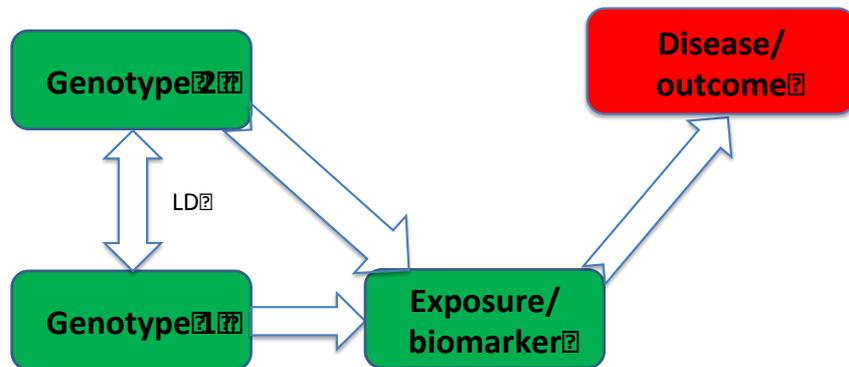
Figure 12 – Mendelian randomisation where the chosen genotype is in linkage disequilibrium with another causal variant. In this scenario the assumptions underlying MR are not violated if genotype 2 affects disease risk through the biomarker of interest.

1.5.3.2 Pleiotropy

One of the main themes of discoveries made by GWAS is that of pleiotropic effects, whereby genetic variants are associated with a number of traits that in some cases have not traditionally been thought to share common biology. For example variants at Chromosome 9p21 have been found to be associated with AAA, CHD, T2DM and thyroid malignancy(102). This is also the case for genetic variants associated with circulating biomarkers, whereby a single variant may exert effects on a range of biomarkers. This presents problems with the 3rd assumption of MR analysis and in practical terms it may not be possible to

statistically exclude all potential pleiotropic effects. There are however, steps that can be taken to address the problem;-

1) Knowledge of the biological function of the genetic variant allows researchers to assess the plausibility of their hypothesis.

2) Given the large number of publically available datasets, it is possible to "look-up" the effect of the genotype on a range of potential intermediate phenotypes by which the variant could exert its effect.

3) If the variant does have pleiotropic effects, it may be possible to adjust the regression analysis for presence of this variable, if this data is available.

1.5.3.3 Instrument strength and Sample Size

The "strength" of the genetic instrument is an important consideration. In general this is described by the F-statistic, generated by the regression of the risk variant on the intermediate phenotype. In general, an F-statistic less than 10 is considered weak, and this can lead to "weak instrument bias" in the direction of the observational confounded association (145, 146). This is a particularly important point given the modest observed effects of most genetic variants identified by GWAS.

In addition to weak instrument bias, the small effect of genotype on a biomarker often necessitates extremely large sample to achieve adequate statistical power to

confirm or refute the association. This often means combining analyses from a large number of studies in meta-analysis, which can also introduce biases as a result of inter-study heterogeneity.

It may be possible to combine multiple genetic variants into a single instrumental variable (known as a genetic risk score), to increase statistical power, and this is discussed in more detail in Chapter 5 of the thesis. The source of the F-statistic should also be considered as if it comes solely from the sample in which the MR analysis is performed this can also bias the results, and where possible it is optimal to use variants that have independently identified (ie another GWAS)(147).

1.5.3.4 Population Structure

One of the most attractive points of the MR concept is that genotype is randomly allocated at conception (Figure 11), analogous to the randomization procedure in a trial. This means that potential confounding factors are randomly distributed between cases and controls. It is possible, however, that differences in the genetic make-up of populations (whereby a certain genotype has different frequencies in different populations) used in the analysis can lead to false positive results. In general, with GWAS it is possible to detect population structure, and where present this should be adjusted for in the analysis.

1.5.3.5   Relevance of MR for clinical interventions

By definition, a (functional) genetic variant represents lifelong exposure to the particular cognate biomarker/risk factor it affects. Although this may predispose to a particular disease end-point there are some factors that need to be considered with regard to potential clinical relevance. First, it may be that an intervention based upon the action of a genetic variant may be futile because elements of the disease in question are irreversible (148). Second, the small lifelong effect of the genetic variant may not extrapolate to the effect of a short-term intervention that has a large effect on the intermediate phenotype of interest. Finally, it may be possible that individuals develop alternative physiological pathways to compensate for the effect of the genotype over their lifetime (known as canalization and seen in knockout animal models) and so the relevance of this for planning clinical interventions remains unclear (148).

1.6   Overall Aims & Hypotheses

In this thesis I intend to use genomic data and established techniques in genetic epidemiology to shed new light on the pathways that underpin pathways involved in AAA formation. To do this, I will focus upon quantitative traits and biomarkers that show association with AAA. Specifically I aim to;-

1. Assess the potential mechanism of the *DAB2IP* locus in the development of AAA by investigating its effect on a broad range of imaging and biochemical phenotypes i.e. a phenome scan.

2. Perform a Mendelian Randomisation Study of interleukin-6 signalling pathways in the development of AAA.

3. Perform a Mendelian Randomisation study of HDL-C and AAA

4. Investigate the genetic determinants of vascular remodeling as a continuous trait, and investigate their role in the development of AAA.

2   Study Populations used in this thesis

2.1   AAA

2.1.1   The Aneurysm Consortium

The Aneurysm Consortium genome wide association study of AAA recruited cases of AAA from centres in the UK, New Zealand and Western Australia. Cases were defined as an infra-renal aortic diameter $\geq$ 3cm, measured by USS of CT scan. Subjects that presented with AAA rupture (without prior imaging) were also included. Controls were taken from the WTCCC2 common control group (Table 11). I had access to individual level genotypes, gender, centre and MDS co-ordinates & aneurysm diameter (provided by Mr Matt Bown, - PI for the GWAS). Other phenotype/covariate data for the AC was extremely limited.

DNA samples were processed at the Wellcome Trust Sanger Institute. Genomic DNA was quantified by PicoGreen assay and quality control assured by both agarose gel electrophoresis and Sequenom iPLEX geno- typing of 29 SNPs and four gender markers. Genotyping for was performed with Illumina 1.2M (controls) or 670K (AAA) BeadChips. Raw intensity data were normalized with BeadStudio, and genotypes were called concurrently from the combined case control data set with the Illuminus algorithm.

Table 11 - The Aneurysm Consortium GWAS of AAA

	AAA		Controls	
Centre	N	%Male	N	%Male
Chichester,UK	58	100		
Leeds, UK	319	100		
Leicester, UK	778	100		
Otago, NZ	129	76		
St Georges, UK	70	87		
UKSAT	262	100		
Western Australia	250	100		
WTCCC2 1958 BC			4157	50
WTCCC2 NBS			1278	51

2.1.2   The SMART Study

The Secondary Manifestations of ARTerial disease (SMART) study is a prospective outpatient cohort study among patients aged 18-74 years newly referred to the University Medical Center Utrecht, The Netherlands, because of atherosclerotic vascular disease or for treatment of atherosclerotic risk factors. The objective of SMART is to determine the prevalence of concomitant asymptomatic arterial disease and risk factors in patients presenting with a manifestation of arterial disease or risk factor, and to study the incidence of future cardiovascular events and their predictors in these high-risk patients. DNA for wet-lab genotyping

was available in a total of 8,361 SMART participants. There are 631 incident and prevalent AAA cases (defined as an infra-renal aortic diameter $\geq$ 3cm) and 6342 controls included in the analyses. There were 222 incident AAA end-points seen during follow. AAA endpoints included death, open surgical repair and/or endovascular stenting. I did not have access to individual level data but simply summary level results. I drew up plans and Dr Folkert W Asselsbergs performed analyses within the SMART study. Individuals in the SMART study were genotyped using KASpar at KBiosciences, UK (www.kbioscience.co.uk).

2.1.3   The Utrecht Genetics AAA study

The AAA sample set from Utrecht was recruited in 2007-2009 from eight centres in The Netherlands, mainly when individuals visited their vascular surgeon in the polyclinic or, in rare cases, during hospital admission for elective or emergency AAA surgery. An AAA was defined as an infrarenal aorta $\geq$ 3 cm. The Dutch controls used in the AAA GWAS were recruited as part of the ERGO/Rotterdam Study. There are 862 cases of AAA and 1866 controls. Subjects were genotyped with the Illumina HumanHap370 or HumanHap610 SNP chips. I had access to only summary level data. Analyses were drawn up by me and performed by Dr Annette Baas.

2.1.4   The New Zealand Study

Individuals from New Zealand with AAA were recruited from the Otago-Southland region. The vast majority (>97%) were of Anglo-European ancestry.

Approximately 80% of these individuals had undergone surgical AAA repair (typically AAA's > 50 mm in diameter). The control group consisted of elderly individuals with no previous history of vascular disease from the same geographical region. An abdominal ultrasound scan excluded concurrent abdominal aortic aneurysm from the control group and Anglo-European ancestry was required for inclusion. Genotyping was with the TaqMan allelic discrimination method or with the Affymetrix 6.0 chip. Analyses were performed in conjunction with Dr Gregory T Jones.

2.1.5 The Iceland AAA Genome Wide Association Study

Icelandic individuals with AAA (defined as diameter of infarenal aorta of $\geq$ 3cm) were recruited from a registry of individuals who were admitted at Landspitali University Hospital, in Reykjavik, Iceland, 1980 – 2006. The 27,712 Icelandic controls used in the AAA GWAS were selected from among individuals who have participated in various GWA studies and who were recruited as part of genetic programs at DeCODE. Individuals with known cardiovascular disease were excluded as controls. Subjects were genotyped with the Illumina HumanHap370 or HumanHap610 SNP chips. I had access to summary level data only and analyses were performed in conjunction with Dr Solveig Grettarsdottir.

Table 12 - AAA studies, data from which has been used in this thesis

Sample Set	Controls			Cases		
	$n_{controls}$	% Female	Age (SD)	n_{cases}	%Female	Age (SD)
AC	5855	50	NA	1596	4	NA
SMART	6352	33	55 (12)	631	22	65 (10)
NZ	718	23	70 (6.6)	1373	20	74 (8.0)

EAS	819	47	64 (5.6)	62	66 (5.6)	74 (8.1)
UTRECHT	1866	56	58 (10.2)	862	10	68.1 (8.3)
Iceland	27,712	62	50.8 (21.4)	425	26	75 (8.3)

2.2 Study Populations Carotid Artery Ultrasound Phenotypes

2.2.1 The IMPROVE study

The IMPROVE study enrolled 3711 individuals (aged from 54 to 79 years) with at least three cardiovascular risk factors, asymptomatic for cardiovascular diseases and free of any conditions that might limit longevity (e.g. cancer) or IMT visualization (e.g. vascular tortuosity). Patients were enrolled from 7 centres in 5 countries in Europe: 1050 from two centres in Kuopio, Finland (general population plus 15% from a diabetic subpopulation), 501 from Paris, France (lipid clinic), 1095 from Italy (lipid/hypertension clinic), 532 from Groningen, the Netherlands (60% from general population and 40% from a diabetic population) and 533 from Stockholm, Sweden (from a general population with an age 65±1 years).

ICCAD is the mean of the inter-adventitial distances of the left and right carotid arteries, measured in the 2nd cm from the carotid bifurcation. For CIMT, the IMPROVE study used identical scanners and protocols in each of the participating centres. The far walls of the left and right common carotid were visualised at three angles (lateral, anterior and posterior) and recorded. For the common carotid artery, a number of measures were recorded. For the analyses presented here, the

maximum IMT value in the 1st cm of common carotid proximal to the bifurcation was used. I had access to individual patient data for all phenotypes and genotypes for the IMPROVE study. There was no abdominal USS for assessment of the aorta in this study.

2.2.2   The Whitehall II Study   (WHII)

The Whitehall II study (WH-II)(149) recruited 10,308 participants   (70% men, age range 35-55) between 1985 and 1988 from 20 London-based Civil service departments. There were relevant genotype and phenotype data from the 2003-2004 screening for 2159 of these participants.

For carotid USS, carotid bulb was identified and longitudinal 2-dimensional ultrasonographic images of the common carotid artery 1 to 2 cm proximal to the carotid bulb were obtained. The optimal longitudinal image was acquired on the R-wave of the ECG, and continuously recorded for 5 seconds. Measurements of the posterior wall of the artery were made from stored images with electronic calipers. CIMT was calculated as the distance between the first bright light (lumen-intima interface) and the leading edge of the second bright light (media-adventitia interface). The 3 maximum measures from the right carotid artery in 3 different frames, and the 3 maximum measures from the left common carotid artery in 3 different frames were averaged and this is the value used in the analyses. I had access to individual level phenotype and genotype information for phase VII of this study.

2.2.3   The SMART Study

The left and right common carotid arteries were examined in the anterolateral, posterolateral, and mediolateral directions. Patients were examined in the supine position, with the head turned 45° from the side being scanned. The reference point for measurement of the IMT was the beginning of the dilatation of the carotid bulb, with loss of the parallel configuration of the near and far walls of the common carotid artery. An R-wave–triggered optimal longitudinal image of the far wall was frozen. On this image, the sonographer traced the leading edges corresponding to the transition zones between lumen-intima and media-adventitia over a length of 1 cm proximal to the reference point. The total intima-media surface of this selected area was calculated online by built-in software of the ultrasound system. The mean IMT of the 6 measurements in each patient was calculated.

I had access to summary level data only. Analyses were carried out in conjunction with Dr Folkert W Asselsbergs.

2.2.4   The Nijmegen Biomedical Study

The Nijmegen Biomedical Study is a population-based survey conducted by the Department of Epidemiology and Biostatistics and the Department of Clinical Chemistry of the Radboud University Nijmegen Medical Centre. 21,756 age and sex stratified randomly selected inhabitants of the municipality of Nijmegen received an invitation to fill out a postal questionnaire and donate an 8.5 ml blood sample. Illumina Human370CNV-Duo BeadChip data are available for 1,980 individuals. The Department of Internal Medicine invited participants aged 50–70

years for a study into noninvasive measures of atherosclerosis (NBS-NIMA); 1,491 individuals participated. In total, 551 participants for which noninvasive measurements of atherosclerosis and Illumina Human370CNV-Duo BeadChip data were included.

Longitudinal images of the most distal cm of both the far wall and the near wall of both common carotid arteries were obtained in the optimal projection (anterolateral, lateral or posterolateral). All measurements were carried out in end-diastole using the R-wave of a simultaneously recorded ECG as a reference frame. The outcome variable was defined as the mean IMT at the optically thickest part of the common carotid. I had access to summary level data only, and analyses were performed in conjunction with Dr Sita Vermeulen.

Table 13 - Demographics of studies included that have data on carotid phenotypes.

	IMPROVE (n=3430)	WHII (n=2110)	SMART (n=3062)	NBS (n=532)
Age (yrs)	64(54-79)	61(50 - 73)	56(49-66)	63(51-72)
Male (%)	48	77	68	51
CIMT (mm)	0.86 (0.16)	0.79 (0.15)	0.88 (0.27)	0.86 (0.11)
CCAD (mm)	7.81 (0.86)	6.17 (0.73)	7.79 (1.1)	6.07 (0.83)
SBP (mmHg)	141 (18)	127 (16)	141 (20)	129 (6)
DBP (mmHg)	82 (9.7)	74 (10)	80 (11)	78 (5)
Current Smokers (%)	15	10	32	15

2.3 Cohorts used for CHD as an outcome

Table 14 shows the baseline characteristics of each of the studies used. Ethics approval was granted for all studies.

2.3.1 Northwick Park Heart Study II (NPSHII)

NPHS-II is a prospective study of healthy middle-aged men (50–64 years) recruited from nine UK general practices (150). In the 2742 Caucasian men with genotype data, by December 2009 there had been 272 CHD events comprising 175 acute CHD events (42 fatal), 74 coronary artery revascularisation procedures and 23 silent myocardial infarctions.

2.3.2 The HIFMECH study

The HIFMECH study (151) compares male survivors of a first MI aged <60 years (excluding patients with FH and insulin-dependent diabetes mellitus) and population-based individuals of the same age and region recruited from four centres in Europe: Stockholm, Sweden and London, England for the North and Marseille, France and San Giovanni Rotondo, Italy representing the south. In all, a total of 598 post-infarction patients and 653 controls were included in the study.

2.3.3 The Simon Broome Study

The Simon Broome Study recruited 409 patients with definite familial hypercholesterolaemia (all with a total cholesterol concentration > 7.5mmol/l) with 127 definite CHD events (152).

2.3.4 The University College London Diabetic Study (UDACS)

The UDACS (153) consists of 1014 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) 2001–02 (629 men; 600 Caucasians with T2DM). All patients had diabetes according to

WHO criteria and analysis was restricted to the Caucasian subjects with T2DM to remove possible heterogeneity within the sample.

2.3.5 The Coronary Artery Bypass Graft Study (CABG Study)

The CABG patients were drawn from the coronary artery surgery inflammation study and are described elsewhere (154). Briefly, all patients undergoing elective first time CABG at the Middlesex Hospital, London, UK, between October 1999 and September 2000 were invited to participate. Subjects undergoing additional surgical procedures (such as valvular surgery or aneurysmectomy), subjects with evidence of a pre-existing inflammatory state or unstable coronary artery disease and subjects who suffered potentially confounding infective postoperative complications or circulatory failure requiring inotropic support were excluded. The CABG group includes 439 people (20% women) having different ethnic origin (83% Caucasians, 8% Asians, 2% Afro-Caribbean, 2% of other ethnicity and 5% of unknown origin). Non-caucasian subjects were excluded from the genetic analysis.

Table 14 - Studies of CHD as an outcome, used in Chapter 3.

	UDACS		NPHSII		Simon Broome		HIFMECH		CABG
	CHD-	**CHD+**	**CHD-**	**CHD+**	**CHD-**	**CHD+**	**CHD-**	**CHD+**	**CHD**
	N=358	**N=135**	**N=2406**	**N=274**	**N=214**	**N=127**	**N=554**	**N=518**	**N= 332**
Age **Years**	66.2 (10.9)	69.5 (9.9)	56.0 (3.4)	56.6 (3.5)	44.6 (13.8)	56.5 (10.4)	51.5 (5.4)	51.9 (5.4)	64.9 (9.2)
% male	56.4 (202)	69.6 (94)	100 (2406)	100 (274)	43.0 (92)	66.9 (85)	100 (554)	100 (518)	81.9 (272)
SBP **mmHg**	142.0 (18.5)	137.9 (20.5)	136.8 (18.6)	141.4 (19.4)	124.1 (16.3)	130.8 (19.9)	128.1 (14.5)	127.7 (16.8)	
DBP **mmHg**	79.5 (10.9)	77.1 (10.5)	84.4 (11.2)	86.8 (11.5)	76.6 (9.9)	78.5 (13.1)	84.1 (8.5)	81.6 (10.2)	
BMI **Kg/m²**	29.2 (5.3)	29.5 (5.3)	26.2 (3.4)	26.6 (3.3)	23.7 (4.0)	25.2 (3.5)	26.1 (3.2)	27.0 (3.3)	28.5 (4.5)
% ever smokers	50.7 (173)	59.9 (79)	67.3 (1618)	77.7 (213)	38.5 (82)	65.4 (83)	61.9 (343)	82.1 (425)	78.0 (259)
Cholesterol **Mmol/l**	5.19 (1.06)	4.68 (1.11)	5.71 (1.01)	6.07 (1.02)	6.85 (1.28)	6.28 (1.32)	5.53 (0.98)	5.39 (1.18)	4.79 (1.10)
Triglyceride **Mmol/l**	1.94 (1.10)	1.96 (1.08)	1.77 (0.93)	2.05 (1.07)	1.24 (0.54)	1.44 (0.67)	1.44 (0.61)	1.87 (0.76)	
CRP **g/l**	1.70 (1.41)	1.86 (1.59)	2.46 (2.43)	3.26 (3.33)	1.19 (1.39)	1.48 (1.72)	1.24 (1.42)	2.23 (2.53)	2.25 (2.75)
Fibrinogen	-	-	2.70 (0.52)	2.80 (0.49)	2.77 (0.82)	3.07 (1.00)	3.41 (0.69)	3.71 (0.92)	3.63 (0.77)

g/l

2.4 Statistical Methods

The statistical methods for each of the studies are described in the respective

chapters.

2.5 Systematic review of the literature

The search strategies used when performing systematic review of the literature are

described in detail in the respective chapters.

3 Replication of the association between a variant in *DAB2IP* and cardiovascular disease

3.1 Background

GWAS should probably be considered a first step in the genetic discovery process, rather than providing definitive and detailed results about the genetic architecture of common diseases. It is common for discoveries made by GWAS to be followed up with replication/validation studies to confirm the observed associations in independent cohorts. This process, combined with meta-analysis with data from the discovery dataset allows a mature estimate of the effect size so the overall impact of such a variant can be assessed. Furthermore, these studies can investigate other mechanisms & biomarkers through which variants exert their effect, and assess their potential role in prediction of common diseases. For example, following the discovery of the 9p21 variants with CHD & AAA a number of follow-up studies have been published. These included simple replication studies (103), studies of subclinical atherosclerosis (155)and vascular remodeling(156), studies of established and emerging cardiovascular biomarkers (157) and studies that investigated the role of the variant in the prediction of CHD(158). The work presented in this chapter is a follow-up study for the *DAB2IP* variant. Most of the results described herein have been published in the *European Heart Journal* (see Appendix 3).

3.2 Introduction

A genome wide association study (GWAS) found a sequence variant in *DAB2IP*
(rs7025486) to be strongly associated with the presence of AAA with a per allele
odds ratio of 1.21 ($P = 4.6 \times 10^{-10}$) (159). The authors also found an association
with early onset myocardial infarction (MI) (OR 1.18, $P = 3.1 \times 10^{-5}$), MI at all
ages (OR $= 1.08$, $P = 0.0012$), peripheral arterial disease (OR $= 1.14$, $P = 3.9 \times$
10^{-5}), venous thromboembolism (VTE, OR 1.12, P=0.0079) and pulmonary
embolism (OR $= 1.20$, P $= 0.0003$), but not with intracranial aneurysm or
ischaemic stroke.

DAB2IP, located on Chromosome 9q33, is a GTPase activating protein thought to
play a role in prostate cancer metastasis (. A SNP in this gene has been associated
with aggressive prostate cancer (160) and *in vitro* functional studies have
demonstrated that loss of this protein leads to enhanced cell proliferation and
reduced apoptosis, via the Pi3-Akt pathway (118). There has not been a great deal
of research regarding the role of *DAB2IP* in cardiovascular disease to date, but it
has recently been shown that deletion of *Dab2ip* in a mouse model of graft
atherosclerosis promotes VSMC proliferation and neo-intima formation (161).
Interestingly the authors demonstrated that the *Dab2ip* KO mice had up-
regulation of interferon-γ (IFN-γ) target genes. IFN-γ signaling promotes vascular
smooth muscle cell proliferation, appears to be highly expressed in both
aneurysmal tissue (73) and atherosclerotic tissue samples (162). One of the most

interesting observations from the discovery study is that the lead SNP was associated with both arterial and venous diseases that have traditionally been considered distinct entities. This suggests that this variant acts on pathways that play a role in both of these disease processes, such as those involved in promoting thrombosis or inflammation.

In the discovery report the authors performed a "phenome scan" in order to generate hypotheses about the potential mechanisms of this variant. They found no convincing association with a range of intermediate phenotypes that included circulating lipid levels, blood pressure and Type 2 Diabetes. There are however, other intermediate phenotypes such as haemostatic or inflammatory biomarkers through which the effect of this variant could be mediated, especially given the association with VTE. An alternative hypothesis postulated by the authors of the discovery GWAS is that the variant effects vascular (arterial & venous) remodeling and this explains the association with a broad range of cardiovascular phenotypes.

The aims of the analyses presented in this study are twofold. First, I sought to replicate the association of this variant with cardiovascular disease (both CHD & AAA) as this is an important follow-up to all novel genetic discoveries. Second, I sought to extend the "phenome scan" performed by Grettarsdottir et al, by including phenotypes not examined in the initial report that could play a role in both arterial and venous diseases. Broadly, these included inflammatory markers,

haemostatic markers, markers of subclinical atherosclerosis and markers of vascular remodeling. This strategy is a useful means of hypothesis generation, with regard to the potential mechanism by which this genetic variant increases risk of vascular diseases. Lastly, because this variant appears to share some mechanistic factors with the 9p21 variants that are associated with cardiovascular disease risk, I examined the role of the potential interaction of these variants in both CHD and AAA.

3.3 Statistical Methods

Allele frequencies between groups were compared using χ^2 test. Mean values for continuous variables were compared between those with and without CHD using unpaired t-tests. Where necessary variables were log-transformed before analysis and results are presented as geometric means with approximate standard deviations for these variables. Categorical variables were compared using chi-squared tests. Genetic associations were tested using regression models using an additive (per-allele) model. Hazard ratios (HRs) were calculated in the prospective study (NPHSII), using Cox regression models with corresponding 95% confidence intervals (CI). Odds ratios (ORs) and their corresponding 95% CI's were calculated from case–control and cross-sectional studies using logistic regression models. Gene-disease associations were adjusted for age as a continuous outcome. In NPSHII, analyses are stratified by general practice. For the conventional model a score was derived based on age, triglycerides, cholesterol, smoking, and systolic blood pressure. A model including both

conventional factors and rs7025486 & rs10757274 genotypes was fitted, and obtained a second score by weighting according to the β-coefficients from the model. The area under the receiver operating curve (ROC) curve was calculated for both scores and the difference between the conventional model and that incorporating genotypes was tested. The effect of adding the genetic variables on the ability of the score to assign participants to the correct risk category using the net reclassification index (163) was also examined.

For meta-analysis, study specific odds ratios and standard errors were obtained and pooled generating a summary estimate and its 95% CI. There was no evidence of between study heterogeneity (I-squared = 0%) therefore a fixed effects model was used. An additive model was used to confirm the association for additive effects found by Gretarsdottir et al (108). All analyses were carried out using Stata version 10 (Statcorp, Texas).

3.4 Results

3.4.1.1 Replication of the association between rs7025486 and Abdominal Aortic Aneurysm

In 622 AAA cases and 6,270 controls from the SMART study, rs7025486 was weakly associated with an increased risk of AAA (OR 1.13, 95% 0.99-1.28, P = 0.07)(Figure 13). In the AC GWAS (109) rs7025486 again showed a modest association with AAA in both discovery and replication phases (OR 1.09, 95% CI 1.02-1.16, P=0.0099). In prospective analyses of clinical endpoints from the SMART study, a similar effect was seen but this did not reach statistical significance (HR AG vs GG 1.19, 95% CI 0.9-0.57, P=0.2: HR AA vs GG 1.26, 95% CI 0.78 – 2, P=0.3) (Figure 14). When the new analysis from the SMART study and AC are combined with data from the discovery GWAS, there was a consistent association between the rare allele and an increased risk of AAA (Figure 13). The association is, however, strongest in the discovery sample with evidence of heterogeneity between the effect size in discovery and follow-up studies (p=0.04), consistent with the "so-called" winners curse.

Study		OR AAA (95% CI)
Discovery		
Iceland		1.25 (1.10, 1.42)
Utrecht		1.17 (1.03, 1.32)
Subtotal (I-squared = 0.0%, p = 0.465)		1.21 (1.11, 1.32)
Follow-up_1		
Belgium		1.15 (0.84, 1.58)
Canada		1.13 (0.81, 1.57)
New Zealand		1.28 (1.10, 1.48)
UK		1.40 (1.15, 1.70)
Subtotal (I-squared = 0.0%, p = 0.614)		1.28 (1.15, 1.42)
Follow-up_2		
Demark		1.17 (0.98, 1.40)
Netherlands		0.82 (0.60, 1.12)
US(Denville)		1.14 (0.93, 1.39)
US(Pittsburgh)		1.20 (0.85, 1.70)
Subtotal (I-squared = 28.5%, p = 0.241)		1.11 (0.99, 1.24)
Follow-up_3		
AC		1.09 (1.02, 1.16)
SMART		1.13 (0.99, 1.28)
Subtotal (I-squared = 0.0%, p = 0.623)		1.10 (1.04, 1.16)
Heterogeneity between groups: p = 0.042		
Overall (I-squared = 26.7%, p = 0.183)		1.15 (1.10, 1.20)

.587   1   1.7

Figure 13 - Association between rs7025486 and AAA, including data from the initial report   (Discovery, Follow-up_1, Follow-up_2) and subsequent studies including the SMART Study.
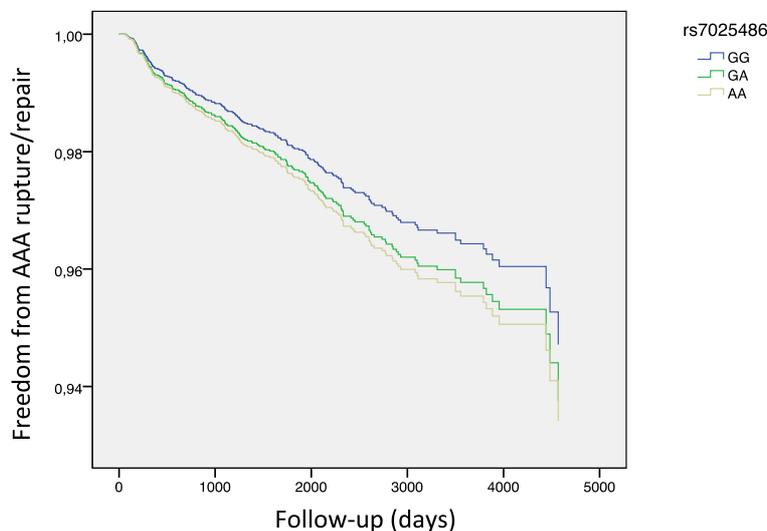
Figure 14 – Freedom from AAA related clinical endpoints by rs7025486 genotype in the SMART study (A rare allele).

3.4.2 Association of rs7025486 with CHD

In each of the newly genotyped study groups the rare allele of rs7025486 was associated with a modest (8-34%) higher risk of CHD, although only one of these (CABG vs healthy controls in NPHSII) was nominally statistically significant using a p-value threshold of 0.05 (OR 1.2, 95% CI 1.03-1.46, p=0.021). Pooled analysis of the results from each of the studies (Figure 15) demonstrated that there was a consistent association between rs7025486 and CHD (OR 1.16, 95% CI 1.05-1.29, p=0.003) in newly genotyped studies. Meta-analysis of data from the original report (108), the newly genotyped studies and *in silico* data from the WTCCC and CVHS GWAS data also demonstrated a consistent association

between rs7025486 and increased risk of CHD (OR 1.1, 95%CI 1.06-1.14, p=3.2

x 10^{-6}, Figure 16).
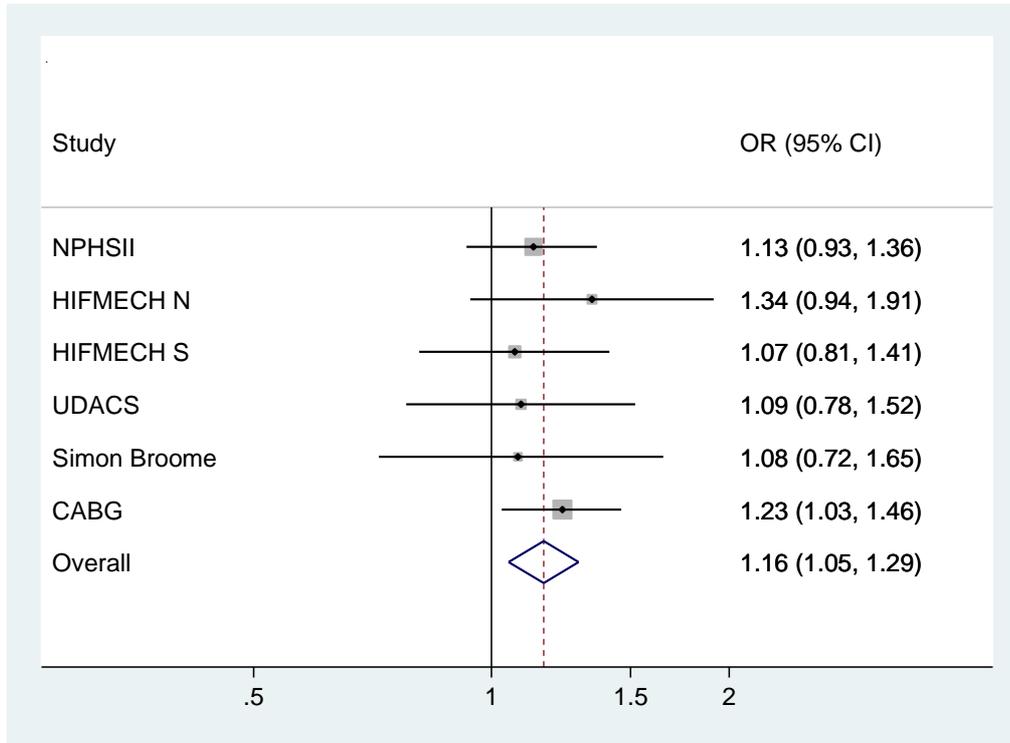
Figure 15 - Meta-analysis of the association between rs7025486 and CHD in newly genotyped studies, pooling data from 1,386 cases and 3,532 controls. Odds ratio per A allele is 1.16, 95% CI 1.05 – 1.29, P = 0.003, $I^2=0$.

Study	OR (95% CI)
Iceland	1.04 (0.97, 1.12)
Italy	1.21 (0.97, 1.51)
NZ	1.13 (0.94, 1.36)
Atlanta	1.15 (0.95, 1.39)
Baltimore	1.12 (0.88, 1.43)
Durham	1.16 (1.00, 1.35)
Philadelphia	1.13 (0.92, 1.39)
NPHSII	1.13 (0.93, 1.36)
HIFMECH N	1.34 (0.94, 1.91)
HIFMECH S	1.07 (0.81, 1.41)
UDACS	1.09 (0.78, 1.52)
Simon Broome	1.08 (0.72, 1.65)
CABG	1.23 (1.03, 1.46)
CVHS	1.14 (1.00, 1.31)
WTCCC	1.06 (0.97, 1.16)
Overall	1.10 (1.06, 1.14)
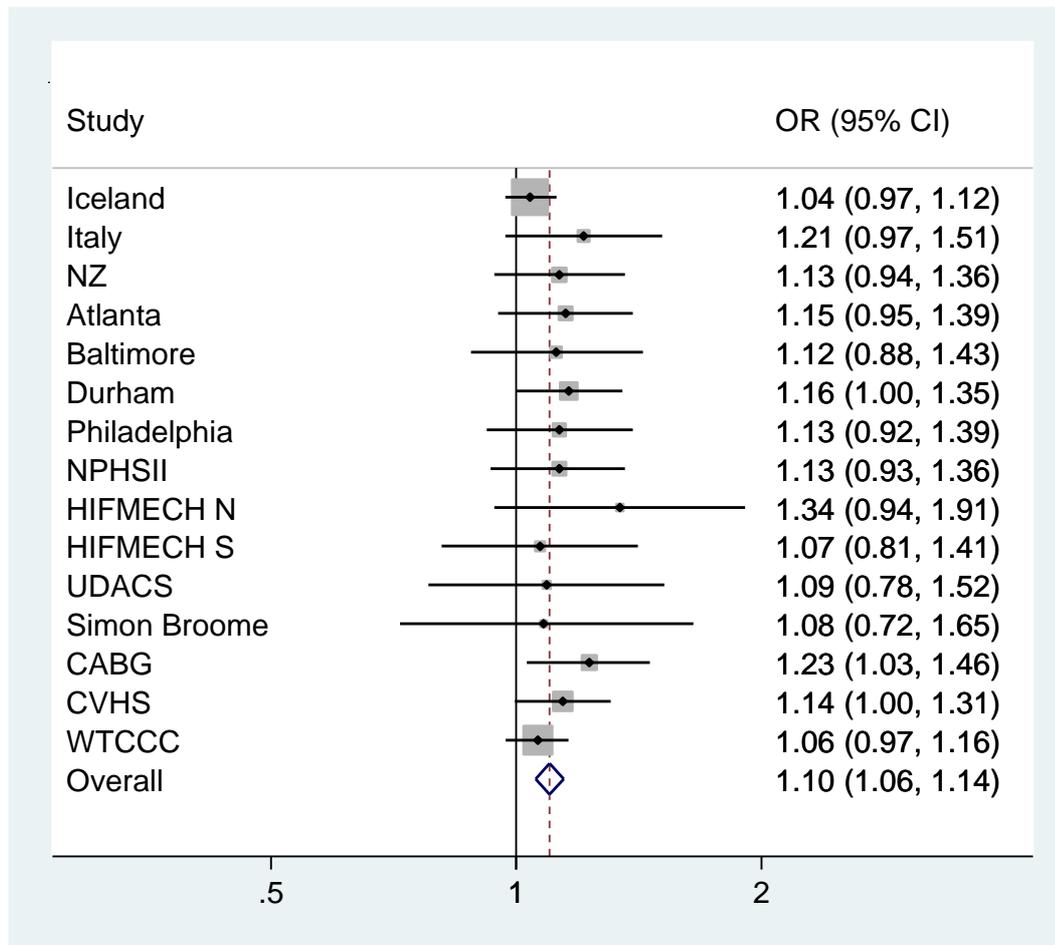
Figure 16 - Meta-analysis of the association between rs7025486 and CHD in newly genotyped studies, those in the published literature and *in silico* studies pooling data from 9968 cases and 20048 controls. Odds ratio per A-allele = 1.10, 95% CI 1.06 – 1.14, P=3.2x10⁻⁶, I²=0.

3.4.3   Association of rs7025486 with inflammatory and haemostatic

   biomarkers

There was no significant association between this SNP and any of the haemostatic

markers (Table 15), Fibrinogen, CRP or IL-6 (Figures 17,18,19).

Table 15 - Association between rs7025486 and haemostatic markers in NPSHII

	GG N=1498	GA N=997	AA N=185	B (se)	P value
Factor VIIc	105.4 (29.2)	106.2 (27.6)	104.6 (26.6)	0.002 (0.008)	0.791
Factor VIIa	1.97 (1.17)	2.05 (1.16)	2.11 (1.28)	0.021 (0.031)	0.493
Factor VIIag	125.2 (34.3)	127.1 (34.9)	121.2 (32.6)	-0.003 (0.008)	0.673
Factor IXp	202.8 (63.0)	198.8 (59.1)	205.2 (62.6)	-0.004 (0.017)	0.791
Factor Xp	77.6 (26.1)	76.2 (30.8)	83.0 27.4)	-0.004 (0.021)	0.832
Factor XIIa	1.79 (0.91)	1.79 (0.93)	1.78 (0.89)	0.015 (0.016)	0.324
F1.2	0.71 (0.29)	0.70 (0.28)	0.71 (0.26)	-0.008 (0.012)	0.535
FPA	1.33 (0.93)	1.29 (0.84)	1.42 (1.21)	-0.001 (0.021)	0.977
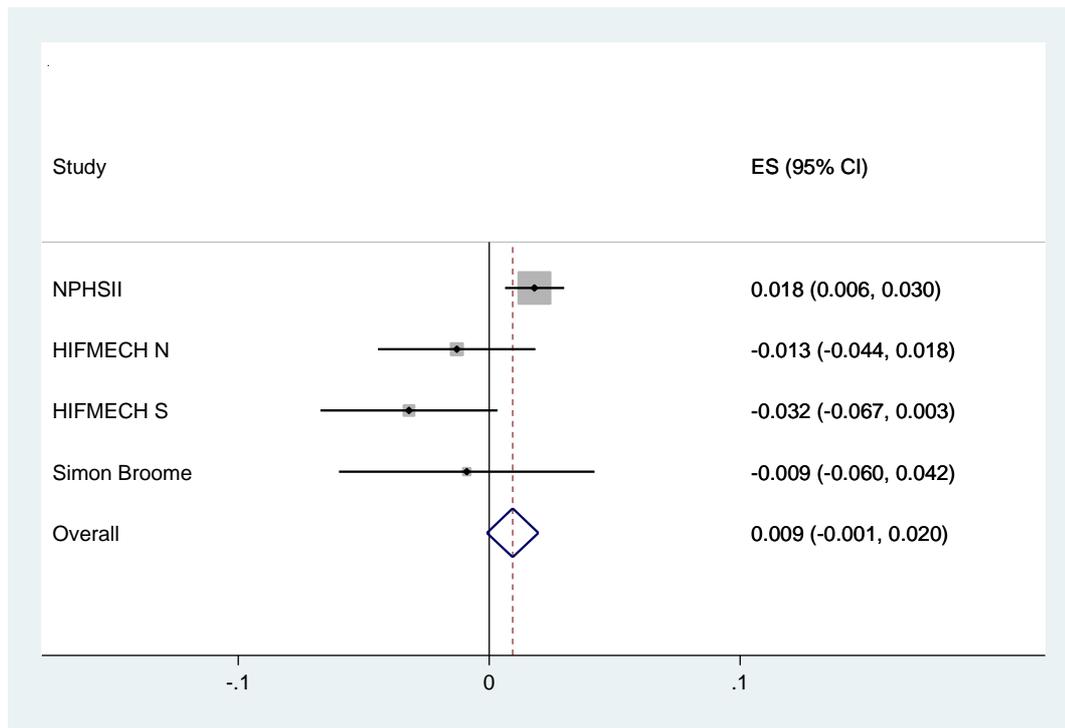
Figure 17 - Meta-analysis of the association between rs7025486 and Fibrinogen concentrations (P=0.08)

Study	ES (95% CI)
NPHSII	0.007 (-0.060, 0.074)
HIFMECH N	-0.038 (-0.242, 0.166)
HIFMECH S	-0.025 (-0.150, 0.100)
UDACS	-0.098 (-0.225, 0.029)
Simon Broome	-0.085 (-0.295, 0.125)
Overall	-0.022 (-0.073, 0.028)
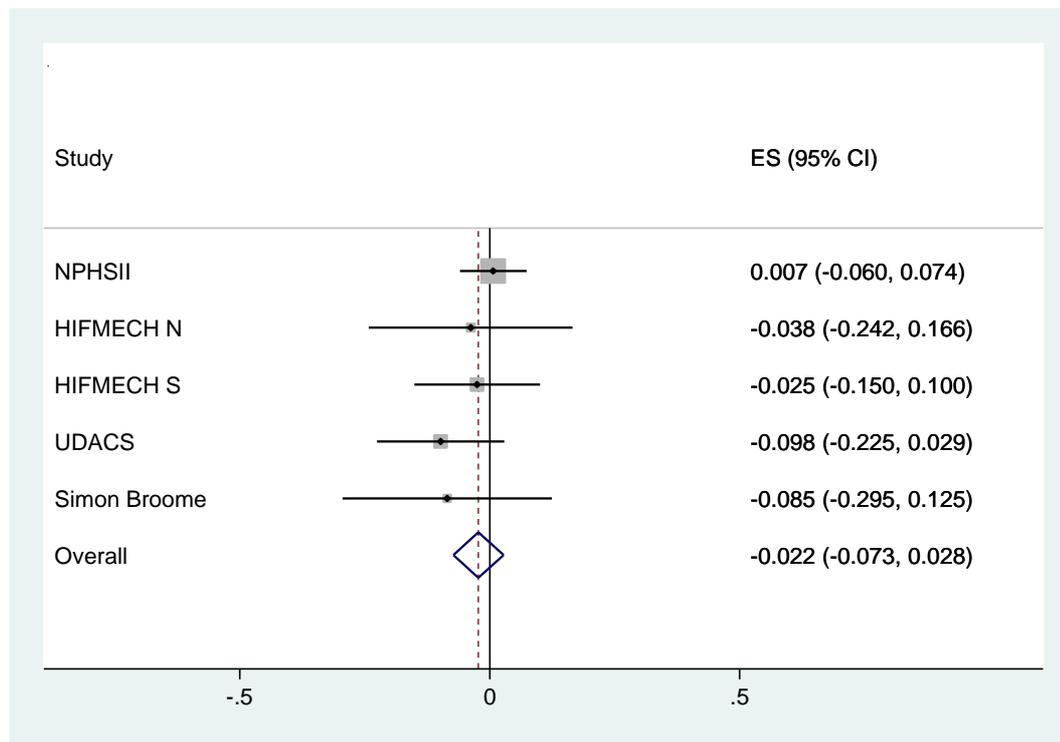
Figure 18 - Meta-analysis of the association between rs7025486 and CRP concentrations (P=0.38)

Study	ES (95% CI)
HIFMECH N	0.060 (-0.036, 0.156)
HIFMECH S	0.049 (-0.067, 0.165)
UDACS	0.018 (-0.092, 0.128)
Simon Broome	-0.057 (-0.173, 0.059)
Overall	0.022 (-0.032, 0.076)
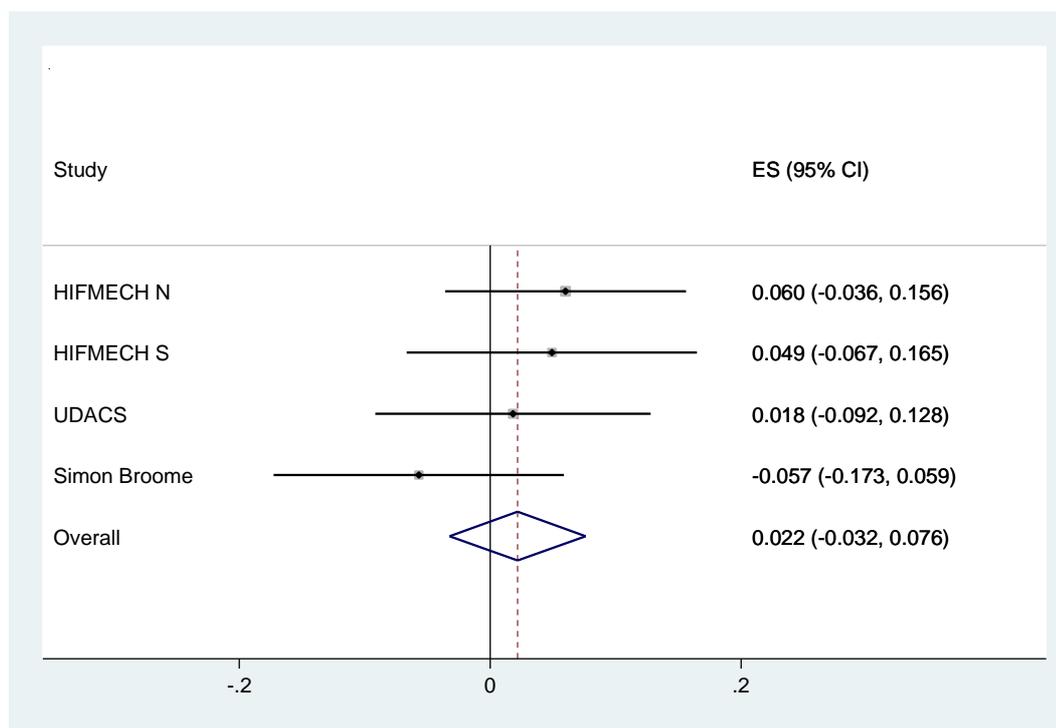
Figure 19 - Meta-analysis of the association between rs7025486 and circulating IL-6 concentrations (P=0.43)

3.4.4 Association of rs7025486 with markers of subclinical atherosclerosis

There was no association between rs7025486 and CIMT in the IMPROVE study

(P = 0.25, n=3,545) or WHII studies (P=0.98, n=2,114). Also, there was no

association with infrarenal aortic diameter (excluding cases of AAA, P = 0.27) or

inter-adventitial common carotid artery diameter (P=0.96 in WHII).

3.4.5 Interaction/additive effects of *DAB2IP* and 9p21.3 variants

As it is biological plausible that the variants at 9p21.3 and *DAB2IP* are having

their effect through common mechanisms, I investigated the potential additive

effects and/or interaction of these variants on cardiovascular disease risk. For the

purposes of theses analyses I will define interaction statistically, as a deviation

from log additive (multiplicative effects) of the variants on disease risk. In the AC dataset, there was no evidence of a statistical interaction between rs7025486 and rs4977574. There was, however, evidence of additive effect and individuals who carry a maximum of four risk alleles at these two loci are at approximately 70% greater risk of AAA than those who carry none (Table 16).

I also examined for an interaction in CHD in the NPHSII study. Again, there was no statistical evidence of interaction between these SNPs (P=0.3) but evidence of an additive effect. This is demonstrated in a Kaplan-Meier plot in Figure 20. As NPHSII is a prospective study, I considered the predictive effect of these two SNPs in combination with the Framingham Risk Score (FRS) for CHD. The A_{ROC} for a model that includes these two SNPs alone was 0.577 (95% CI - 0.540-0.614). The A_{ROC} for the model that included both the FRS and genotypes was 0.64 (95% CI, 0.598-0.675)(Figure 21), which was a small but statistically significant improvement when compared to the value achieved by CRFs alone (p=0.03).

Table 16 - Additive effect of rs7025486 (*DAB2IP*) & rs4977574 (9p21) & risk of AAA in the AC dataset. P for rs7025486 x rs4977574 interaction = 0.49

Number of Risk Alleles	Cases/Controls	OR AAA (95% CI)	P-Value
0	213/780	Reference	NA
1	655/2056	1.17 (0.98-1.39)	0.09
2	717/1856	1.41 (1.19-1.68)	9.6×10^{-5}
3	250/652	1.40 (1.14-1.73)	0.002
4	41/88	1.71 (1.14-2.55)	0.009
Per Allele	1876/5432	1.14 (1.08-1.20)	5.5×10^{-6}
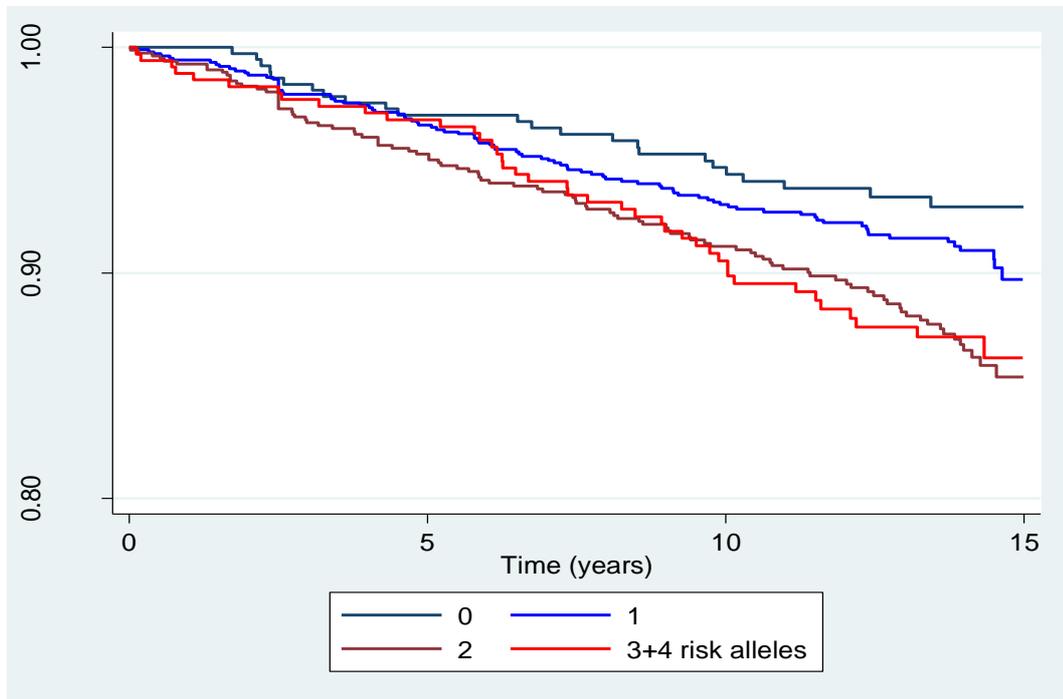
Figure 20 - Kaplan-Meier Plot for CHD and number of risk alleles carried.
Compared to the reference group of those with no risk alleles, the age and practice
adjusted Hazard Ratio (HR)) for those with only one allele = 1.19 (0.78-1.84), p =
0.42, for any 2 alleles = 1.79 (1.16-2.75), p = 0.008, and for 3+4 alleles = 1.75 (1.07-
2.85) p = 0.025. Overall p = 0.005.
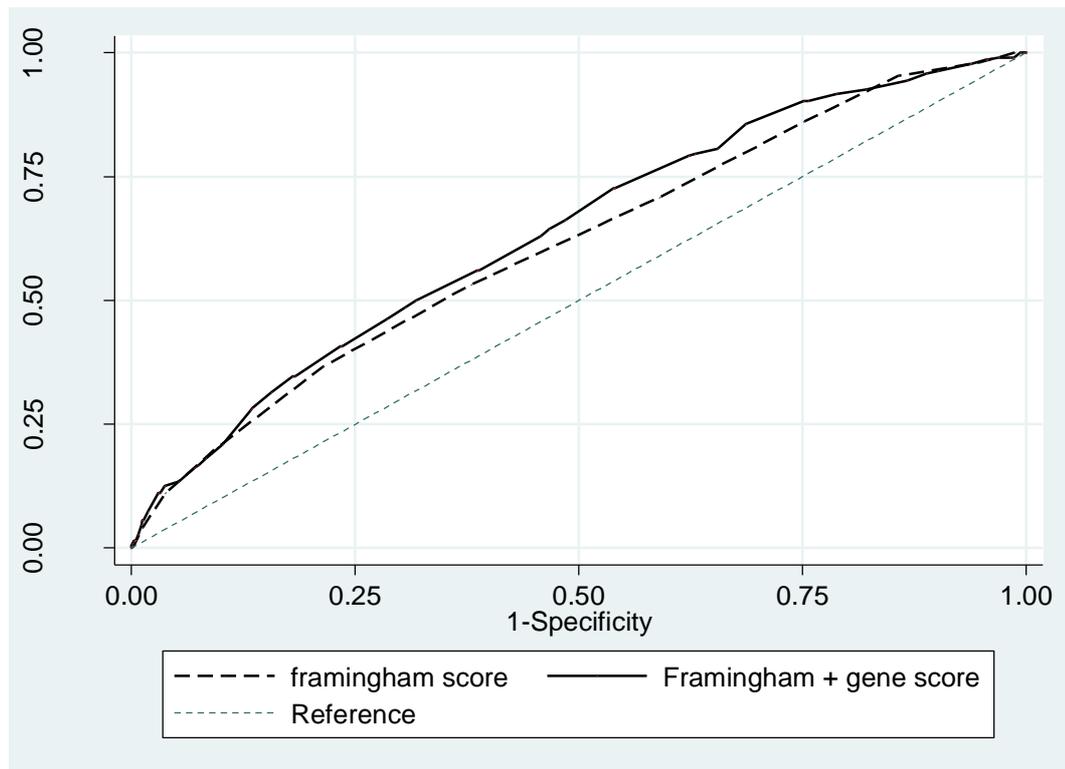
Figure 21 - ROC curve for different prediction models in NPHS-II men. The area under the ROC curve increases from 0.61 to 0.64 when the gene score is added to the Framingham variables (p=0.03).

3.5 Discussion

In this study I have followed up the association between rs7025486 identified by GWAS of AAA (108). The first objective was to replicate the association with AAA and CHD. Although the association with AAA seen in the SMART study was of borderline statistical significance the direction of effect was concordant, but lower magnitude than the discovery study. This is consistent with the "winner's curse" whereby the association seen in the discovery study is often larger than that in subsequent studies (164). Indeed, in meta-analyses of all the available data for the association between rs7025486 and AAA, stratified into

discovery and follow-up studies there is modest evidence of heterogeneity (P =
0.04, Figure 13) between groups, with a smaller effect size in the follow-up
studies.

For CHD, rs7025486 was associated with a modest increase in risk. In the CHD
analysis the effect size of rs7025486 in different groups of subjects is extremely
consistent, even with differing underlying aetiologies such as T2DM or FH, and
in case-control and prospective studies, including previous published GWAS.
Although only one of the fifteen studies included in the meta-analysis passed a
conventional p-value threshold of 0.05 for single SNP-disease association, this is
not unexpected given the minor-allele frequency (MAF) and modest effect size.
The fact that in all fifteen studies the minor allele increased the risk of CHD with
a comparable effect suggests that this association is unlikely to be a false-positive
association, reflected by the combined p-value. Indeed, it is becoming clear that
the initial wave of GWAS have detected only SNPs with the largest effect and
favourable MAFs (the so-called "low-hanging fruit"). Even meta-analysis of
GWAS, involving many thousands of subjects may be underpowered to detect all
SNPs with modest effects sizes. Therefore, single SNP studies guided by the
findings of GWAS are likely to be an important method to decipher which of the
many associations that fail to reach genome wide levels of significance are
actually true positives.

The original report found an association with VTE and pulmonary embolism, so I
hypothesised that this variant may act through pathways that promote thrombosis

but found no significant associations with a panel of haemostatic markers including fibrinogen, PAI-1 and numerous clotting factors. The rest of the phenome scan, which included subclinical imaging markers of atherosclerosis in large cohorts, did not uncover any novel associations that could point to a mechanism by which this variant increases the risk of cardiovascular diseases. This suggests that it does not act through classical pathways involved in atherosclerotic diseases. In many ways this variant is similar to the 9p21 variants associated with both CHD and AAA, which has not shown association with a panel of new and emerging cardiovascular risk factors(155, 157, 165). Functional studies of the 9p21 variants suggest that it promotes excessive smooth muscle cell proliferation (114, 115), which may be the mechanism by which these variants exert their effect on risk of disease. *DAB2IP* is involved in regulating cell survival and senescence, and the association seen here adds to emerging evidence that genetic variants in genes that regulate the cell cycle may be important mediators of cardiovascular disease. Although there is limited functional analysis of *DAB2IP* in the vascular system, the recent paper demonstrating a role for *Dab2ip* in a mouse model of atherosclerosis, and its role in mediation of IFN-γ supports a hypothesis that it has similar mechanism to that of the 9p21 variants. *Moreover, t*he additive effect on risk of CHD seen with these two SNPs implies that accumulation of variants of modest effect in this pathway are important independent mediators of CHD development (as suggested by the common variant hypothesis), and highlight potential targets for development of novel therapeutic options.

It is unlikely that a single SNP of modest effect will improve prediction of cardiovascular disease when compared with a risk score based upon multiple CRFs (158). It is much more likely however, that variants found to be associated with CHD, which do not act through intermediate phenotypes such as lipid traits, which are already included in such risk scores, will be of use when combined with CRFs. It has been reported that addition of the single 9p21 SNP (rs10757278) to the FRS does not improve prediction (as measured by the A_{ROC}) (158). The data presented here suggests that addition of multiple SNPs may improve the overall fit of the predictive model, but this needs to be evaluated in larger studies to determine any potential clinical utility and other studies that have combined multiple genetic variants and clinical risk factors to predict complex diseases have in general provided fairly disappointing results (166, 167).

3.5.1   Conclusion

In this study the effect of a genetic variant in *DAB2IP* on the risk of both AAA and CHD has been replicated which validates the findings of Grettarsdottir et al. An extensive phenome scan of established and novel intermediate phenotypes through which this variant could potentially exert it's effect did not reveal any novel insights suggesting that, like the 9p21 variants, it does not act through traditional pathways of atherosclerotic disease.

4 Genetic evidence that signaling through the interleukin-6 receptor is causal in the development of AAA

4.1 Background

In Chapter 3 I examined the role of a GWAS identified variant on a range of intermediate cardiovascular phenotypes in order to understand its role in the development of cardiovascular diseases. Although there have been two AAA GWAS published to date, it is likely that there are more common variants associated with AAA to be identified and that the discovery phase of each of these studies was underpowered to detect (at the p-value used to select SNPs for replication studies). The most obvious way to identify the true positive associations from large number of false positive, is to combine the datasets in meta-analyses, as has been performed in other cardiovascular diseases (80). Another way to use the large datasets & cohorts developed for GWAS, for a translational purpose is Mendelian Randomisation (MR) analysis, whereby genetic association studies are integrated with classic epidemiological studies to understand causal relationships and identify novel therapeutic targets. In this chapter I use these ideas to investigate the role of inflammatory signaling pathways through the interleukin-6 receptor, in AAA. Most of the results of the work in this chapter are now accepted for publication in the *European Heart Journal* *(appendix 4).*

4.1.1 Inflammation and AAA

One theory of AAA formation is that the dilatation process represents abnormal healing of the aorta following damage by environmental exposures that, in turn

leads to chronic inflammation driven proteolysis in the aortic wall (168). It follows therefore, that the inflammatory processes that underpin this process represent a novel target for pharmacotherapy. A major challenge is to decipher which of the many inflammatory pathways are causal and offer the safest and most effective drug targets.

There is considerable evidence that links inflammation and AAA development. For example, meta-analysis of observational epidemiological studies has demonstrated consistently higher levels of inflammatory biomarkers such as c-reactive protein and fibrinogen in AAA cases than controls (169, 170). An important caveat to these observations is that they come solely from cross-sectional and case-control studies, so the observed differences in biomarker concentrations may simply reflect the underlying disease process (so called reverse causality) and so inferences regarding causality cannot definitively be made from these data. Furthermore, case-control studies are prone to both confounding and bias, which further limits the interpretation of these data with regard to understanding truly causal pathways.

Similarly, microarray studies of AAA tissue compared to non-aneurysmal aorta have demonstrated enrichment of pro-inflammatory pathways such as the natural-killer cell signaling (119). Indeed many tissue studies have found evidence of pro-inflammatory signaling in AAA tissue (171, 172). Tissue removed at the time of surgery represents, however, an end-stage of the disease and it is not possible to

determine if the inflammatory changes are causal, or a result of the underlying disease process, which again limits the translational interpretation of such results.

4.1.2   Interleukin-6 Signaling

Interleukin-6 (IL-6) is a cytokine that plays a role in many inflammatory diseases. IL-6 signaling is initiated by binding of IL-6 to its receptor (IL-6R), which forms a dimer with the ubiquitously expressed signal transducer glycoprotein-130 (gp-130). This, in turn leads to activation of the intracellular receptor-associated kinases and downstream effects via the transcription factor STAT3 (Figure 22). Two forms of IL-6 signaling have been described, namely classical and trans-signaling. In classical signaling, IL-6 binds the membrane bound IL6R (mIL-6R) that is expressed in hepatocytes and cells of the innate immune system. In trans-signaling, IL-6 binds to the circulating soluble IL-6 receptor (sIL-6R), and this complex is capable of binding to the ubiquitously expressed gp130, so can initiate IL-6 signaling in a wide range of cell types.
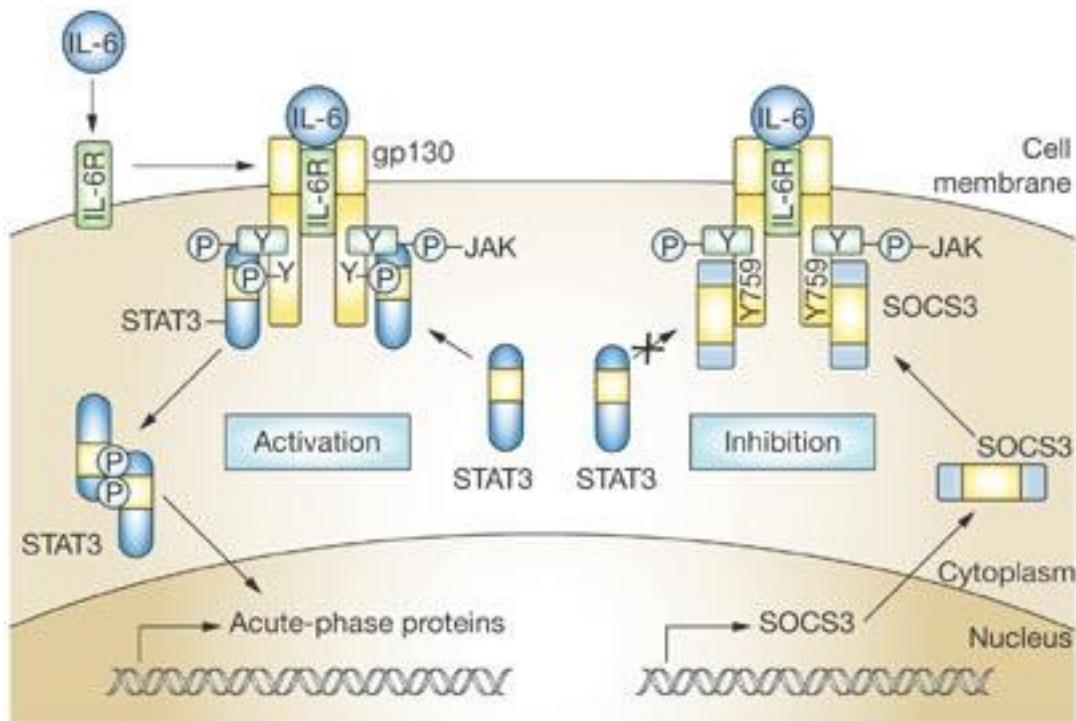
**Figure 22 - IL-6 Signalling – IL-6 has binds to its receptor with binds to the
ubiquitously expressed transmembrane protein gp130 and leads to activation of the
JAK-STAT pathway. SOCS3 – suppressor of cytokine action 3 – from (173).**

4.1.3 IL-6 signaling and abdominal aortic aneurysm

In addition to the biomarker studies that will be reviewed later in the chapter there

are data from tissue studies, genetic association analyses, and animal models that

point to IL-6 and related pathways as playing an important role in AAA. Lindeman

et al showed that expression of IL-6 is considerably higher in AAA tissue than non-

aneurysmal aorta (171), whilst Liao et al showed that expression of downstream

targets of IL-6 signaling (such as STAT1) are higher in AAA tissue compared to

non-aneurysmal aorta (172). In the Ang II infusion model of aortic injury it has

been shown that *Il6* knockout results in reduced aortic inury (174), as does

antagonism of the murine *Il6r* (175), suggesting that it may be a key mediator of

aortic inflammation and injury.

Genetic variants in *IL6* have been previously investigated for an association with

AAA. Smallwood et al investigated the association of three variants in the IL-6

promoter with presence of AAA in 677 cases and 656 controls (176). They found

an increased risk for carriers of a rare variant in the *IL6* under a recessive model

but this finding has not been independently replicated, and did not replicate in the

AC GWAS (p=0.35). One other small candidate gene study found no significant

association between *IL6* variants and presence of AAA (177). Jones et al examined

the role of *IL6* promoter variants and growth rates of small AAA, but again found

no association. They did, however, report an association between a promoter

variant and increased risk of cardiovascular death in patients with small AAA

(178), but this finding has not been replicated in independent cohorts.

4.1.3.1   Mendelian randomization for target validation

Mendelian randomization (MR) has been traditionally used to explore the potential

causal role between circulating biomarkers and disease phenotypes (179). An

extension of the technique has been recently described whereby MR is used to

validate novel drug targets (180). In this approach, functional variants in genes

encoding potential drug targets are used as instruments to explore the utility of

targeting this pathway in specific disease states. For example, a genetic variant that

disrupts the function of a particular receptor could be used as an instrument to

investigate the effects of targeting that receptor pharmacologically. As an example,

variants in *HMGCR,* which encodes the target for statins have been associated with

lower levels of circulating LDL-C and a reduction in the risk of CHD (135). This

approach could potentially help to prioritize targets for novel drug development or guide repositioning of existing pharmacological treatments between disease categories.

A common non-synonymous sequence variant in the *IL6R* (Asp358Ala, rs2228145, also annotated as rs8192284) results in increased proteolytic cleavage of the membrane bound IL-6 receptor (mIL-6R)(181). This variant is strongly associated with higher levels of circulating IL-6 levels, but importantly, lower levels of downstream products of IL-6 signaling, such as C-reactive protein and fibrinogen (182, 183), suggesting that it acts by attenuation of signaling via the IL-6 receptor. This variant or its proxies, therefore represent a useful tool to explore the potential utility of targeting the IL-6R in disease (Figure 23)(184). This is given extra impetus by the existence of a monoclonal antibody to the IL-6 receptor (tocilizumab), currently used for the treatment of rheumatoid arthritis (185). Furthermore, recent large-scale genetic association studies have demonstrated that this variant has a broadly concordant effect as tocilizumab on a broad range of cardiovascular biomarkers as well as a reduced risk of CHD (184), suggesting that targeting the IL-6R may be a useful strategy in cardiovascular disease.
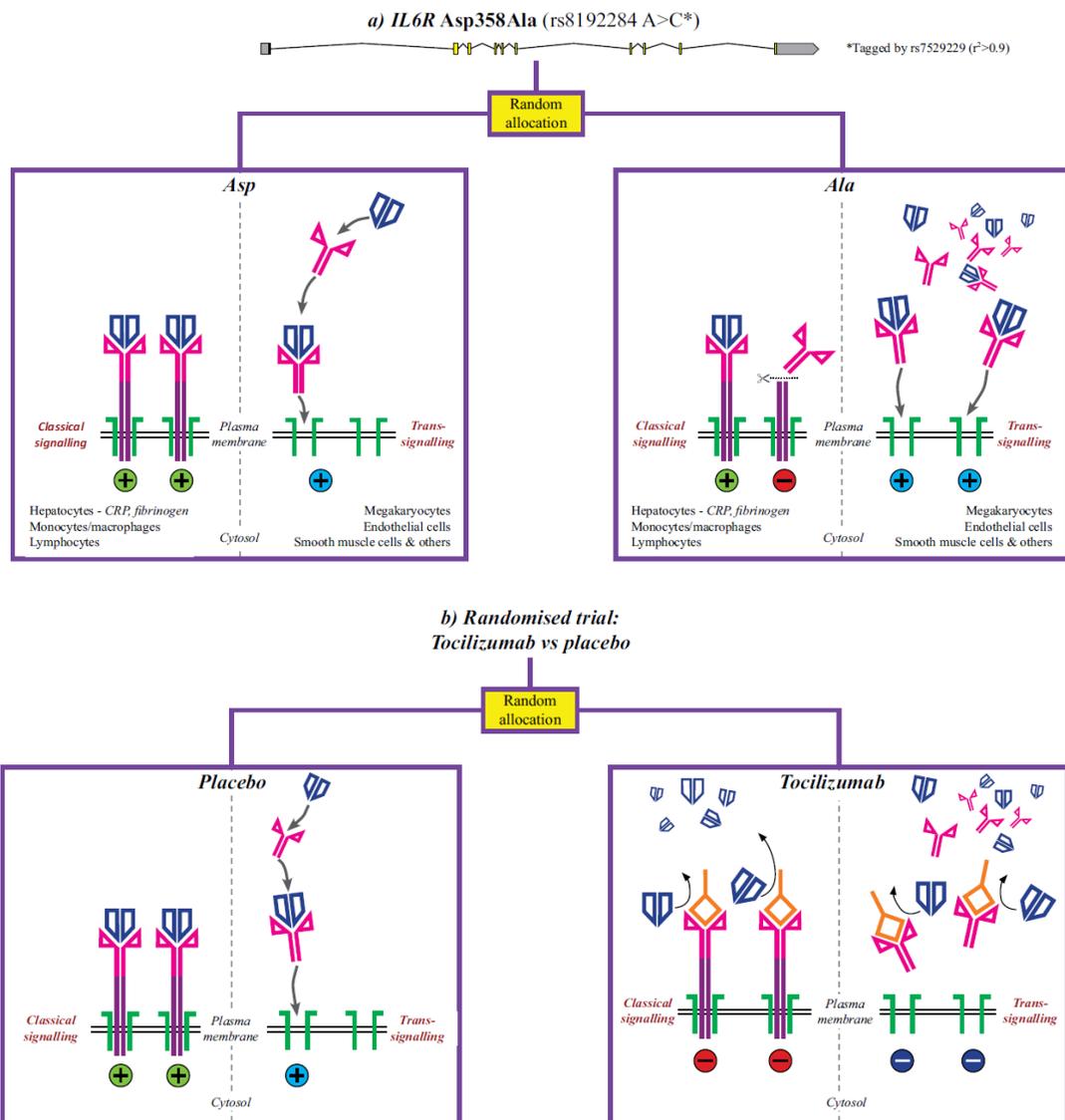
Figure 23 – Mendelian Randomization paradigm for the interleukin-6 paradigm. Random allocation of the Asp358Ala genotype mirrors random allocation of Tocilizumab as in a clinical trial. Figure created by Daniel Swerdlow & re-printed with his permission.

In this study I sought to investigate the potential clinical utility of targeting the IL-6R in AAA. To do this I sought to

1. Establish the association between circulating IL-6 and AAA in the published literature by systematic review and meta-analysis.

2. Use the non-synonymous sequence variant (Asp358Ala) as an instrument to investigate the potential of targeting the IL6R in AAA.

4.2 Methods

4.2.1 Observational Association between Interleukin-6 and AAA

Studies were identified using a two-stage search strategy following PRISMA guidelines (186). In the first stage two electronic databases (MEDLINE & EMBASE) were searched and in the second stage articles were identified by manually searching references of articles identified in the first stage and review articles. The MEDLINE database was searched from January 1966 to January 2012, and the EMBASE from 1980 to January 2012. The search terms used for identification of studies in PubMed is were:-

"abdominal aortic aneurysm[MeSH Terms] AND (interleukin 6[MeSH Terms]"

"abdominal aortic aneurysm[MeSH Terms] AND (cytokine[MeSH Terms]"

In EMBASE the following searches were performed

1) abdominal aorta aneurysm/exp

2) Interleukin 6/exp

3) Cytokine

4) 1 and 2

5) 1 and 3

Selection criteria for studies were the following;-

• Phenotypic definition of AAA(IRAD≥3cm) in cases

• Plasma measure of interleukin 6 in cases and controls

• English language articles

- At least ten cases and controls

4.2.2   Statistical Methods

To estimate the association between circulating IL-6 and AAA a meta-analysis of
summary statistics identified by systematic review was performed. For each study,
we used mean concentrations of IL-6 in cases and controls to determine a
standardized mean difference and standard error. For studies that reported median
concentration levels and interquartile ranges (IQR), the median was taken to be the
mean value and divided the IQR by 1.35 in order to obtain the SDs. Mean
differences in each study were pooled using inverse weighted fixed effect meta-
analysis. Heterogeneity was assessed using the I^2 statistic and Cochrane's Q. SNP-
disease associations in each cohort were determined using logistic regression
adjusted under an additive genetic model. Effects sizes are reported as odds ratios
(OR) and 95% confidence intervals. Analyses were carried out using PLINK
version 1.07 and Stata Version 11. Study quality was assessed using the Newcastle-
Ottawa Score.

4.3   Results

4.3.1   Systematic review and meta-analysis of IL-6 and AAA

Seven studies reporting circulating levels of plasma IL-6 in AAA cases and
controls were identified (Table 17/Figure 24), including one previous unpublished
study (the NZ study)(187-193). Two studies that reported higher IL-6 in cases than
controls, but did not report summary measures of IL-6 (mean & SD or median &
IQR) were not included in the meta-analysis (194, 195). All identified studies
utilised a case-control design and used recognised methods to diagnose and define

the presence of AAA and to measure IL-6 levels. No prospective population

studies were identified. All but one study (196) selected controls that were matched

for age and gender, but only one study matched for smoking status (197) but none

specifically adjusted for these factors in the primary analyses.

In meta-analysis, pooling data from 869 cases and 851 controls, individuals with

AAA had higher circulating IL-6 concentrations than controls; standardised mean

difference (SMD) = 0.46, 95% CI 0.25-0.66, P random effects model = 1. 1 x 10^{-5},

I^2=70% (Figure 25). Concentrations of IL-6 were higher in cases than in controls

in all but one of the studies in which no difference was observed (192). This study

included only small AAA in the case group (3-5.5cm), and reported different

measurement units to all the other studies (ng/ml vs pg/ml). Exclusion of this study

reduced overall heterogeneity   (I^2 = 56%) and increased the statistical significance

of the association (P random effects = 2 x 10^{-8}), however, this study did not overly

influence the overall effect estimate greatly (Figure 26). Possible reasons for

heterogeneity may be related to selection of cases and controls. For example, there

is evidence that IL-6 concentrations are correlated with AAA size (178, 187, 197)

and subgroups analyses demonstrated that when the analysis was restricted to

studies that compared IL-6 in large AAA compared to controls, there was less

heterogeneity (I^2 = 31.5%, Figure 25). Furthermore there was variation in how

controls were selected, e.g. from distinct cohorts such as the general population

(188) or from surgical outpatients clinics (198), which is likely to contribute to

heterogeneity. There was no evidence of publication bias using Begg's adjusted

rank correlation test (P=0.7).
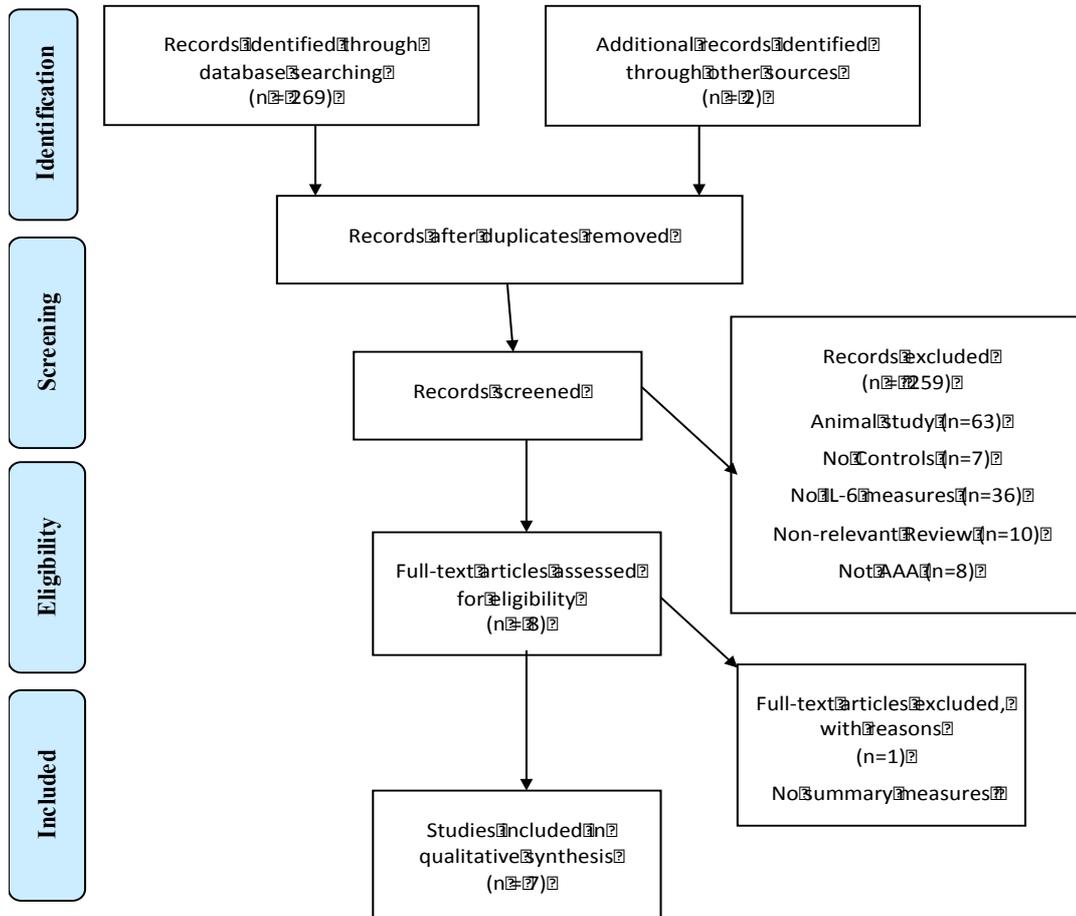
Figure 24 - Flow chart of search strategy for studies reporting IL-6 concentrations in AAA cases and control

Table 17 - Details of the Case control studies identified and included in the meta-analysis of IL-6 levels in AAA

Author	Year	Country	Study Design	Cases/ Controls	Case Definition	Controls	Diagnosis	IL-6 Cases, mean (SD)	IL-6 Controls, mean (SD)
Treska	2003	Czech Republic	Case-Control	74/30	Small (<5cm) = 30 Large (5-8cm) = 38 Very Large (>8cm) = 22 Ruptured/Symptomatic = 16**	Matched for Age & Gender No History of Atherosclerosis	USS	59.3 (134.8)	6.7 (5.1)
Fowkes	2006	UK	Case-Control	89/98	AP Aortic Diameter > 3cm, size distribution NR	Normal USS (<3cm) Matched for Age & gender. Nested case-control within the Edinburgh Artery Study(199)	USS	2.8 (1.62)	1.8 (1.04)
Dawson	2007	UK	Case-Control	27/15	Large AAA undergoing Endovascular Repair	Undergoing other vascular intervention	CT	4.94 (2.49)	2.65 (1.98)
Flondell-Site	2009	Sweden	Case-Control	360/218	Small (<4.5cm) = 122 Medium (4.5-5.5cm) = 108 Large (>5.5cm) = 130	Age & Sex Matched No History of Atherosclerosis/AAA Undergoing routine preventative checks	CT/USS	9.19 (31.91)	2.08 (2.90)
Jones **(Unpublished)**	2012	New Zealand	Case-Control	166/359	All greater than 5cm, awaiting repair	Matched for Age, free from atherosclerosis & AAA. Recruited from screening programme in NZ.	CT/USS	9.1 (6.60)	6.3 (3.93)
Parry	2010	UK	Case-Control	75/90	All AAA < 5.5cm	Matched for Age, Gender & Race Recruited from Surgical Clinics	USS	3.13 (2.87)	3.14 (2.32)
Wallinder	2009	Sweden	Case-Control Case-Control	78/41	Small (<5cm) = 38 Awaiting Elective Repair (>5cm) = 40	Matched for Age, Gender & Smoking Status Volunteers with aortic diameter < 3cm	USS	4.02 (3.79)	2.3 (3.3)

Study	SMD (95% CI)	Cases	Controls
All > 3cm			
Dawson	0.98 (0.32, 1.65)	27	15
Treska	0.46 (0.03, 0.89)	74	30
Parry	-0.00 (-0.31, 0.30)	75	90
Wallinder	0.47 (0.09, 0.86)	78	41
Fowkes	0.74 (0.44, 1.04)	89	98
Jones(Unpublished)	0.57 (0.38, 0.76)	166	359
Flondell-Site	0.28 (0.11, 0.45)	360	218
Subtotal (I-squared = 69.5%, p = 0.003)	0.42 (0.32, 0.52)		
Large AAA (>5cm)			
Dawson	0.98 (0.32, 1.65)	27	15
Wallinder	0.39 (0.01, 0.76)	40	90
Treska	0.47 (0.03, 0.92)	60	30
Jones	0.57 (0.38, 0.76)	166	359
Flondell-Site	0.33 (0.14, 0.51)	238	218
Subtotal (I-squared = 31.5%, p = 0.211)	0.46 (0.34, 0.58)		

-1.65   0   1.65

Figure 25 Association between circulating IL-6 levels and presence of AAA. In pooled analysis of 7 studies reporting IL-6 circulations in cases and controls (869/851) there was consistently higher concentrations in cases (SMD = 0.46, 95% CI 0.25-0.66, P random effects model = 1. 1 x 10^{-5}, *I^2=70%*). Subgroup analyses, comparing IL-6 concentrations in large AAA (>5cm) demonstrated less heterogeneity (SMD = 0.46, 95% 0.34 – 0.58, P = 2.25 x 10^{-14}, I^2 = 32%).
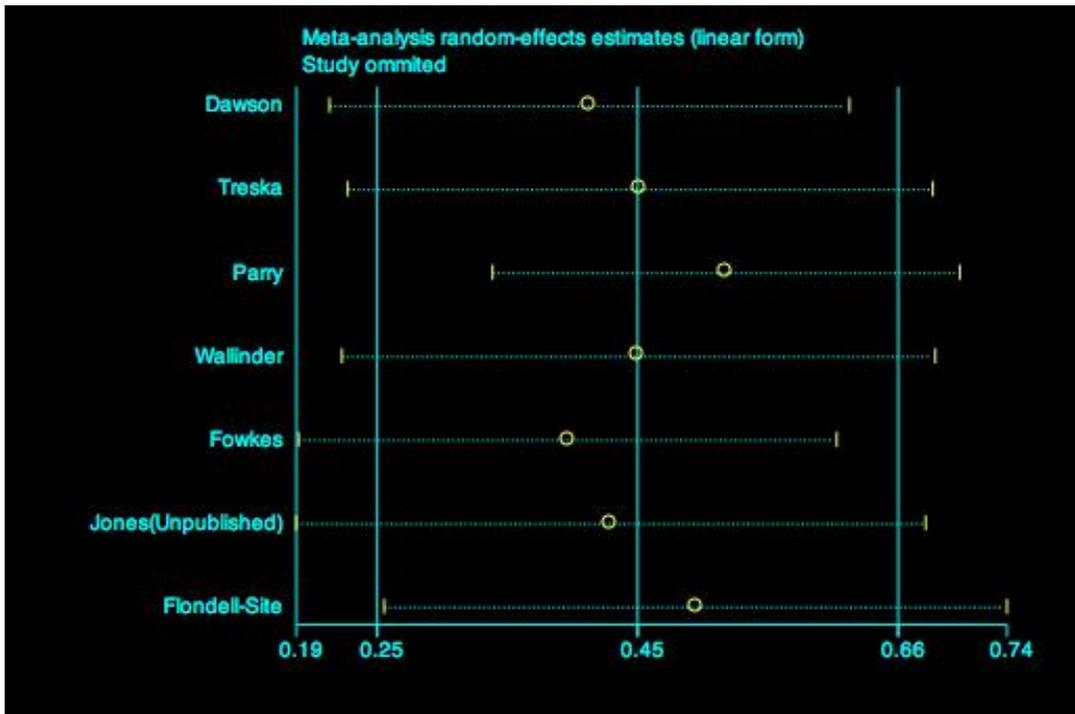
Figure 26 – Influence on removing 1 study at a time on the overall effect estimate for the meta-analysis reporting IL-6 concentrations in AAA cases and controls.

4.3.2 Association of *IL6R* variants with AAA

There was a consistent association between the rare allele of rs7529229 and reduced risk of AAA in all four cohorts. Meta-analysis, pooling data from 4,708 cases and 15,816 controls, demonstrated a per-allele odds ratio of 0.84 (95% confidence interval 0.80-0.89, P = 2.7 x 10^{-11}, I^2=0% fixed-effect meta-analysis) (Figure 27). In time to event analyses of prospective data from the SMART study, using Cox proportional hazard models, adjusted for age and gender, the rare allele was also associated with a reduction in the risk of AAA clinical endpoints (AAA rupture or repair)(Figure 28, per allele HR 0.81, 95% CI 0.67-0.99, p=0.043). There was no evidence of an association between the SNP and AAA diameter (p=0.25) in 7,777 individuals from the SMART study.

Table 18 - Genotyping quality control from each of the studies.

Study	Genotyping Platform	Minor Allele Frequency	Call Rate (%)	HWE p-value
AC	Illumina 670k Beadchip	0.39	>99	0.28
New Zealand	ABI Taqman	0.40	>98	0.89
SMART	Kaspar	0.39	>97	0.77
EAS	ABI Taqman	0.42	>97	0.08
UTRECHT	Illumina 610K	0.38	100	0.97
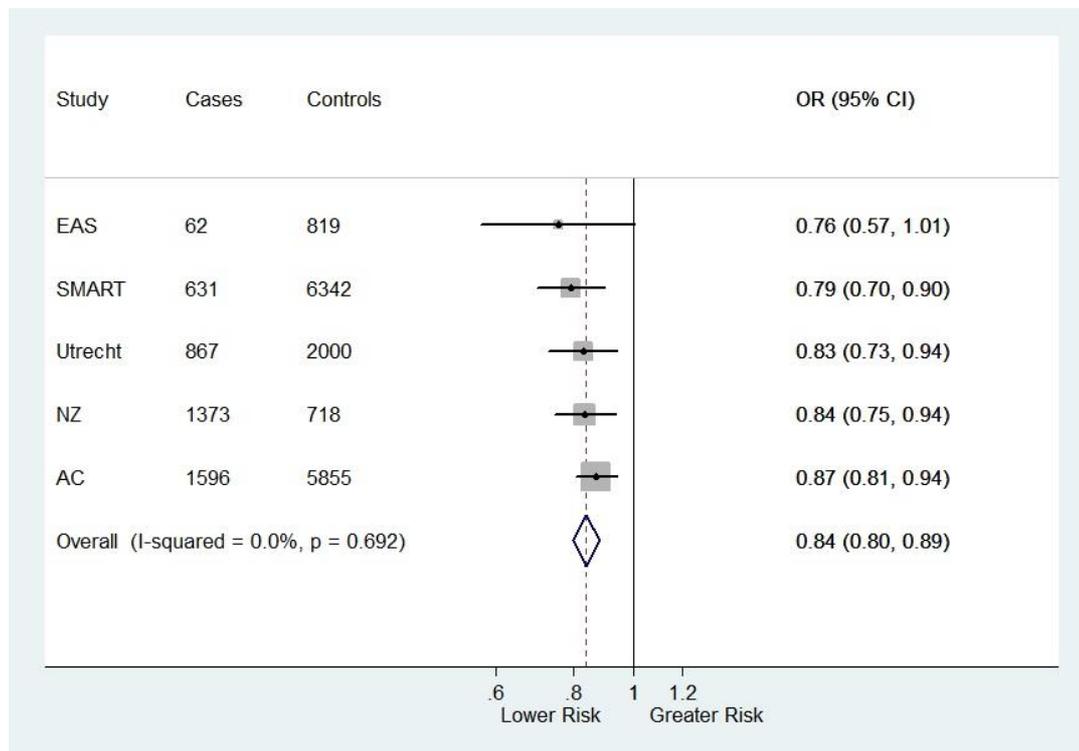
Figure 27 - Association between rs7529229 and AAA following fixed effect meta-analysis of 4 case-control studies pooling data from 4,708 cases and 15,816 controls. Per allele odds ratio = 0.84   (95% CI 0.80-0.89, I^2 = 0, p=2.7 x 10^{-11}).
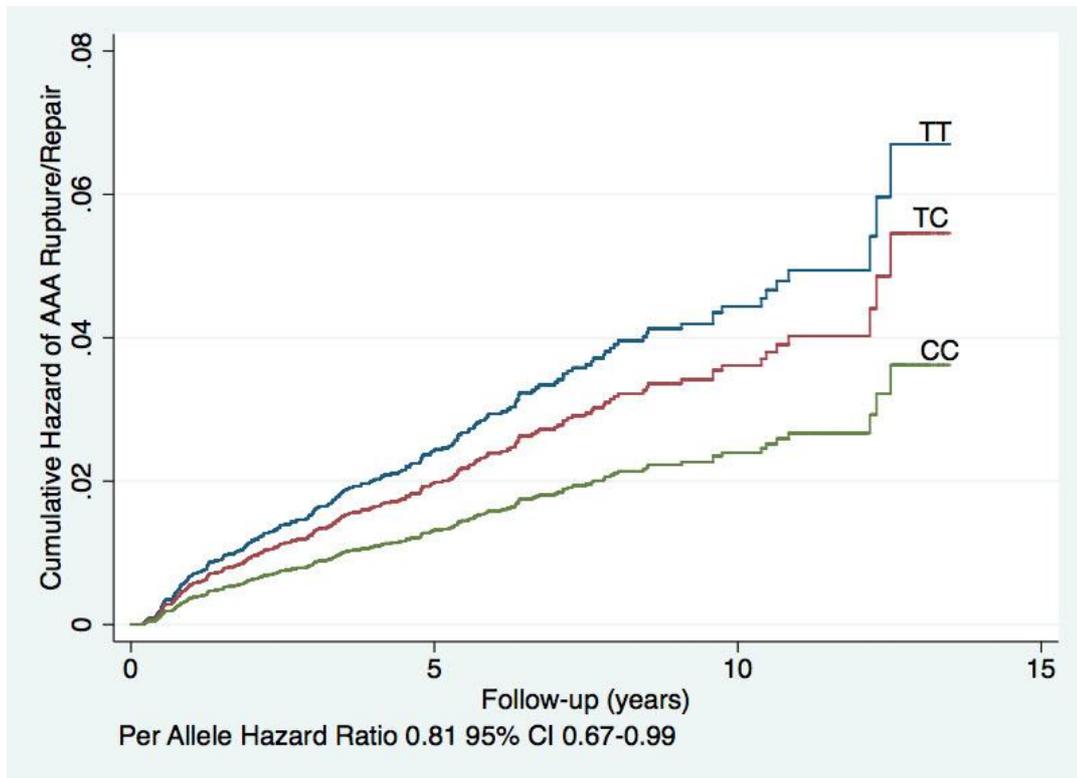
**Figure 28 - Survival curves showing the probability of AAA related endpoint
(Rupture/Endovascular Repair/Open Repair) by genotype at rs7529229 (CC is the
rare homozygote) in 7139 individuals from the SMART study, in who there were 223
events. The hazard ratio per c-allele is 0.81, 95% CI = 0.67-0.99, p = 0.043.) The p-
value was calculated using Cox-regression, adjusting for age and gender**

4.3.3 Functional analyses of the Asp358Ala (rs2228145) *IL6R* variant

(N.B. The functional analyses was performed by Dr Andrew JP Smith (UCL) in

conjunction with the analyses that I performed).

I hypothesized that the mechanism by which this variant reduces risk of AAA is a

genotype-specific reduction in sensitivity to IL-6 in inflammatory cells in the aortic

wall, through reduced levels of functioning mIL-6r. This was tested *in vitro* using a

panel of lymphoblastoid cell lines with known genotype. The aim was to determine

the downstream effects of *IL6R* rs2228145 on expression of IL-6 mediated target genes identified in previous studies (200), namely *STAT3, MYC, BCL3, ICAM1 and ATF3*.

There was no difference in expression of STAT3 targets by genotype, although there was a modest trend towards lower expression for *STAT3* and *ATF3* in the rare homozygotes cells. After IL-6 stimulation, there was a reduction in the expression of *STAT3, MYC, ICAM1* in the rare homozygotes compared to the common homozygotes, and a trend for lower *BCL3* and *ATF3* gene expression (Figure 29). This genotype-dependent difference was abolished by the presence of excess sIL-6R in the media. There was no longer a significant difference in expression of STAT3 target genes by *IL6R* genotype (Figure 29), suggesting that the effect observed with IL-6 stimulation alone is via reducing signaling through the m-IL6R in the presence of rs2228145.

In aortic tissue there was no genotype-specific differences in expression of the IL-6 targets (*STAT3, MYC, ICAM1 and ATF3*) in either aortic adventitia or intima-media. There was however, strong correlation between adventitial *IL6* mRNA levels and expression of *STAT3*, *MYC*, *ICAM1* and *ATF3*, irrespective of genotype. This suggests that tissue production of IL-6 (as opposed to circulating IL-6) is a major determinant of IL-6 signalling pathways in the aortic wall.
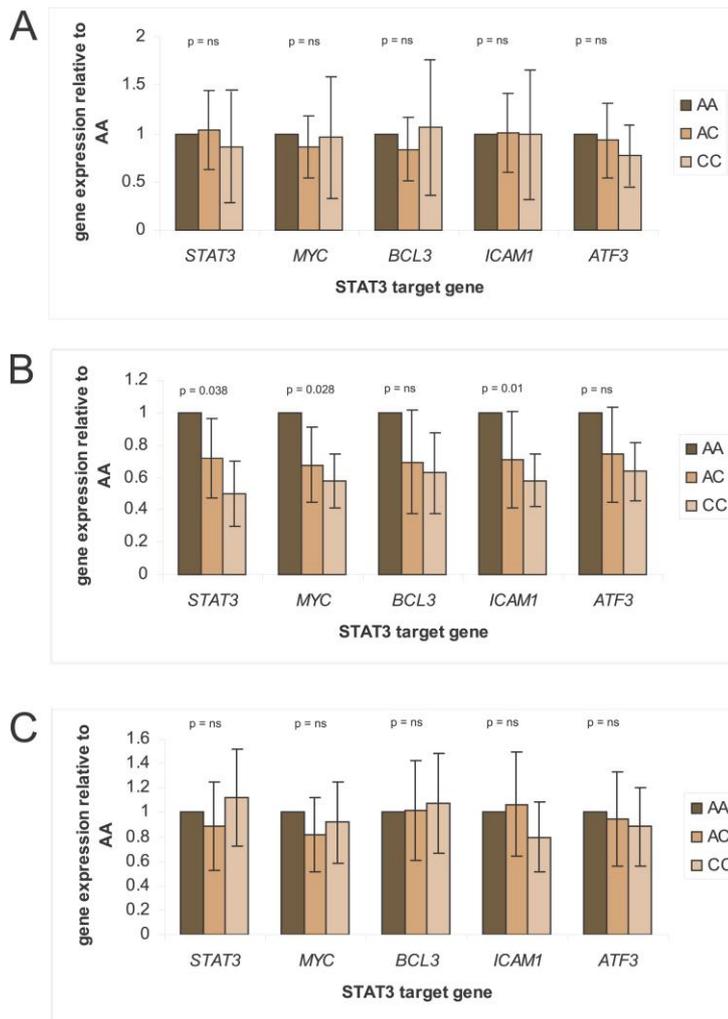
Figure 29 - Gene expression in STAT3 target genes in lymphoblast cell lines of different IL6R rs2228145 (AA = common homozygote, CC rare homozygote). A) Basal gene expression in lymphoblast cell lines shows no statistical difference in gene expression between *IL6R* genotypes. B) Gene expression following IL-6 stimulation. Replacement of IL-6-containing media was carried out periodically to mimic the shedding IL-6R entering the circulation rather than having a local effect. Lower gene expression was observed in all STAT3 target genes in lymphoblasts possessing the C allele. C) Gene expression following IL-6 stimulation and addition of excess sIL-6R. To examine if the effect seen in B was due to receptor shedding, excess soluble IL-6R was added to the stimulated cells. This reversed the genotype effect on gene expression. The results indicate that IL-6 stimulation results in increased shedding of IL-6R from cells possessing the C allele, and reduced gene expression of STAT3 target genes in these cells.

4.3.4   Discussion

In this study the potential role of signaling through the IL-6R in the development of

AAA was evaluated. In the first stage the association between circulating IL-6 and

presence of AAA was consolidated by systematic review of the published

literature. Meta-analysis of case-control studies demonstrated that IL-6 was higher

in subjects with AAA compared to those without. These data support a hypothesis

that IL-6 and associated signaling pathways may play a role in the development of

AAA but it is not possible to directly infer a causal relationship, from observational

evidence such as this alone. For example, many of the studies adjusted for potential

confounders such as smoking status, and previously published reports have found

that AAAs are a source of circulating IL-6(178, 196), suggesting that the observed

association could at least in part be the result of reverse causation. Furthermore,

none of the identified studies were prospective population cohorts, considered the

gold standard in epidemiological studies and there was some heterogeneity in how

cases and controls were selected in the papers identified by the literature search.

Genotype is randomly allocated at conception and not subject to reverse causality

and therefore represents a useful tool to examine causal relationships in complex

diseases phenotypes. The Asp358Ala variant (or close proxy) was selected because

it has previously been shown that it has concordant effects as tocilizumab on a

range of inflammatory and cardiovascular biomarker and therefore may be

considered a useful genetic instrument to investigate the potential for targeting the

IL-6R pharmacologically (184). There was a consistent association between the

Ala358 variant and a reduced risk of AAA, a finding that suggests targeting the IL-6R is a plausible strategy in AAA. This was a statistically robust association, surpassing threshold levels of significance commonly used in genome-wide studies. The *IL6R* variants have not been previously identified in two separate GWAS of AAA (159, 201), however the largest of the discovery cohorts (n_{cases}=1,866) had only 20% power to detect a variant of this effect size at the significance threshold of $p<1x10^{-5}$ used to triage those SNPs to be submitted for analysis in replication studies.

Given the higher levels of circulating IL-6 in patients with AAA, it may seem paradoxical that a variant that is associated with *higher* circulating IL-6 is also associated with a *lower* risk of AAA. However, this variant has previously been associated with reduced concentrations of two downstream markers of IL6R activation CRP and fibrinogen, a pattern consistent with pharmacological blockade of the IL-6 receptor with tocilizumab (184). Furthermore, it is important to note that this study is *not* an MR study of circulating IL-6 and AAA, but uses the MR concept to evaluate the potential utility of targeting the IL-6 receptor in AAA disease.

The functional analysis suggests that the Ala358 variant is associated with a reduction in the sensitivity of immune cells to IL-6 *in vitro*. One possible interpretation of these data is that this variant reduces inflammation in the aortic wall in response to injury, via reduced signalling through the mIL-6R in immune

cells recruited to the injury, resulting in a lower risk of AAA development (Figure 30). Although the lymphoblastoid cell line is useful to understand the functional consequences of this variant *in vitro,* further work on tissue specifically from AAA cases required to understand the *in vivo* mechanism of action, particularly in understating the balance of pro and anti-inflammatory effects on remodelling of the arterial wall in response to damaging environmental exposures that promote vascular injury.
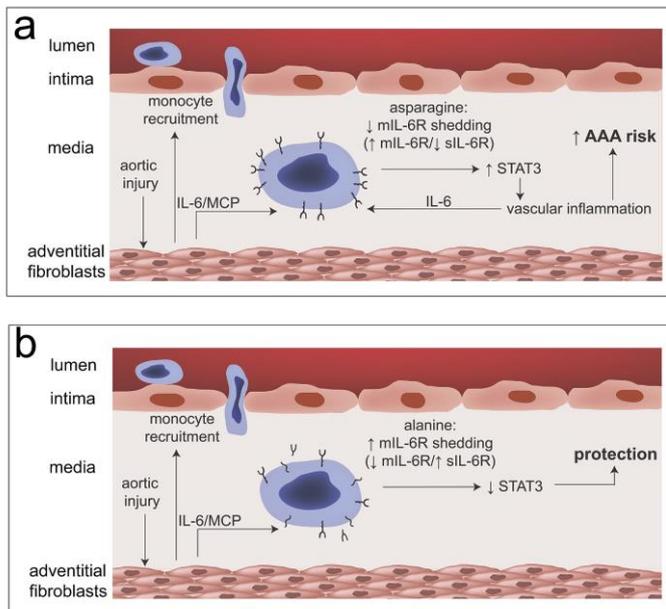
Figure 30 - Proposed mechanism by which this variant influences AAA risk. 1-5a, in the presence of the common allele, vascular injury results in monocyte recruitment and transition to macrophages via an intact mIL6R, with subsequent release of inflammatory proteases. 1-5b, in the presence of the rare allele, reduced levels of mIL6R results in reduced sensitivity to IL-6, and dampened inflammatory responses, conferring protection against aneurysm formation. Figure created in conjunction with Dr Andrew Smith and used with his permission.

These findings have potentially important translational implications as they support a hypothesis that targeting the IL-6R pharmacologically is a strategy that merits

evaluation in AAA. No randomised trials of tocilizumab (a monoclonal antibody to
the IL6R used to treat Rheumatoid Arthritis) have yet examined AAA as an
outcome, although two recent open label studies have reported that in patients with
rheumatoid arthritis, tocilizumab reduces arterial stiffness (202, 203), which
suggests that IL6R blockade has effects on the vasculature. Indeed, a role for
tocilizumab in the treatment of other forms of inflammatory vascular disease such
as Takayasu's arteritis has been postulated, although randomised trials in these
conditions have not yet been reported (204). Furthermore, there is evidence that
targeting inflammatory pathways can both prevent aneurysm formation and regress
already established aneurysms (69) in murine models of the disease and evidence
that tocilizumab acts on this pathway (205). Tocilizumab does, however, have a
number of side effects (such as hypersensitivity and respiratory tract infections)
and the potential for benefit in patients with AAA would of course need to be
balanced against the potential risks.

Although the effect of the *IL6R* polymorphisms on AAA risk is modest, the
potential for clinical benefit from pharmacological intervention at the IL-6R should
not be discounted. Common variants of modest effect have been discovered by
genetic association analysis in therapeutic pathways for other complex disease.
These include SNPs in *HMGCR* (the target for statins) which effect circulating
lipid levels and SNPs in the PPAR pathway (target for thiazolidinediones) affecting
the risk of diabetes. The availability of a specific inhibitor of IL-6R signaling that

has been shown to be safe in large trials in humans adds further weight to the translational potential of the present findings.

In common with two of the three previously identified well-validated loci associated with risk of AAA the *IL6R* locus is also associated with CHD. AAA and CHD have traditionally been considered distinct disease entities that share some common pathobiological pathways. The magnitude of the effect identified in the present analysis (OR 0.86, 95% CI 0.80-0.89) is, however, greater than that reported for the association with CHD risk (OR 0.94; 95% CI, 0.91 to 0.97). Furthermore, in the SMART study where all the control subjects suffered from non-aneurysmal arterial disease, the effect of the *IL6R* variant on AAA risk was consistent with the other studies in which the control group consisted of healthy subjects. The association with CHD does, however, strengthen the case for targeting the IL-6R therapeutically, as the major non-aneurysm related cause of death in patients with dilated aortae is cardiovascular disease.

4.3.4.1   Limitations

It is important to note the potential limitations of this study. The number of cases and controls identified for the case-control analysis of IL-6 levels and AAA is relatively small. I did not have access to tissue from AAA for expression studies. AAA tissue is likely to have a higher content of immune cells than thoracic aorta, which may explain why we did not find genotype -specific difference in the expression studies (as vascular smooth muscle cells and fibroblasts do not express *IL6R*). As there was limited phenotypic/risk factor data for some of the cohorts I

was unable to include the *IL6R* variant in a multivariate regression model including

other covariates. However, this variant has not come up in large well-powered

GWAS of circulating lipids, blood pressure or smoking, suggesting that the effects

are not primarily mediated through, or confounded by these intermediate

phenotypes.

4.3.5   Conclusion

The work in this chapter has demonstrated an association between a functional

variant in *IL6R* and presence of abdominal aortic aneurysm. This provides genetic

evidence that targeting the IL-6 receptor may be a useful and novel strategy in

AAA, and supports future clinical trials in this regard.

5   High Density Lipoprotein Cholesterol and AAA – a Mendelian Randomisation analysis

5.1   Background

In Chapter 4, I was able to use MR analysis to provide robust evidence that genetic variation in parts of IL-6 signalling pathway is strongly associated with risk of AAA. In the next step of the thesis, I apply this methodology to a new biomarker – HDL-cholesterol. Unlike IL-6 where a single non-synonymous variant has a relatively very large effect on levels/signaling, there are a large number of variants that have been robustly associated with modest effects on levels of circulating HDL-C. In this chapter I extend the MR paradigm by considering multiple genetic variants associated with HDL-C and the risk of AAA.

5.1.1.1   HDL-C

Epidemiological studies have consistently demonstrated that higher circulating concentrations of HDL-C are associated with a lower risk of clinical events related to atherosclerosis, including AAA(206, 207). There has therefore been considerable interest in HDL-C raising therapies to prevent events in patients at high risk for cardiovascular disease. The assumption underlying this strategy is that low HDL-C is causal for atherosclerotic disease, which may not be valid because causal inferences cannot be definitively made from observational epidemiological studies alone.

Previous meta-analysis of the observational literature has reported that AAA patients consistently have lower concentrations of HDL-C than disease free controls (207). Furthermore, it has recently been shown that augmentation of HDL-C prevents initiation and progression of AAA in two separate animal models of AAA (208, 209), suggesting it may be a valid target in the treatment/prevention of AAA. To my knowledge there have been no randomized trials of HDL-C raising therapies that have defined AAA specifically as an endpoint and it is possible that HDL-C raising therapies are a novel paradigm for the prevention of AAA and its related complications. The research presented in this chapter of the thesis will describe an MR analysis of HDL-C and AAA.

5.1.2   Protective Mechanism of HDL-C

HDL-C, like other lipoproteins consists of an outer layer of phospholipids and apolipoproteins, and an inner core that carries lipids. The major apolipoproteins are apo A-I and apo A-II, but not apo B (the major apolipoprotein on LDL-C). Apo A-I can leave the HDL-C particle and interact with other tissues (mainly via the ATP-binding cassette (ABC) transporter ABCA1, which leads to transfer of lipids from the tissue to HDL-C.

Modified LDL-C in atherosclerotic lesions is taken up by macrophages leading to the formation of foam cells that may play a role in plaque instability and progression to clinical events. HDL-C can mediate the removal/clearance of cholesterol from these macrophages. This process is known as reverse cholesterol

transport, and is thought to be a key mechanism through which higher HDL-C levels could protect from vascular diseases.

In addition to the reverse cholesterol hypothesis, there are data to suggest that HDL-C has independent vasoprotective mechanism including an anti-inflammatory effect (210). Other molecules that have anti-oxidant/anti-inflammatory effects such as paraoxanase (PON1) are also carried on HDL, and PON1 may be able to prevent accumulation of lipid oxidation products that lead to recruitment of inflammatory cytokines such as interleukin-8 (211). Furthermore, *in vitro* work suggests that HDL-C may be able to attenuate the pro-inflammatory effect of tumour necrosis factor in cultured endothelial cells(212), though the mechanism here is not entirely clear. Specifically for AAA pathology, Tornsey et al demonstrated that reconstitution of HDL-C in animal models of AAA led to alteration in expression of a number of inflammatory and vascular remodeling genes in the aortic wall (209) while it has also been shown that HDL-C has anti-elastase properties *in vitro* (213).

5.1.3   HDL-C Raising Therapies

5.1.3.1   CETP Inhibition

Cholesterylester transport protein (CETP) transfers cholesteryl esters from HDL to LDL or VLDL in exchange for Triglycerides   (Figure 31). Inhibition of CETP leads to an increase in HDL-C, reductions in LDL-C and Triglycerides, and preclinical studies indicated that this correlated with a reduction on atherosclerotic

disease burden (214). A number of CETP inhibitors have been developed for use in humans, including Torcetrapib, Dalcetrapib and Anacetrapib. Torcetrapib has been subject to a randomized controlled trial for prevention of coronary heart disease but this trial was stopped early due to an excess number of events in the group receiving Torcetrapib (137). Although these data suggest that CETP inhibition might not provide the expected clinical benefit, a number of observations have suggested that the deleterious effect of Torcetrapib may have been the result of "off-target" effects of the preparation such as an increase in systolic blood pressure (180). Newer formulations such as Anacetrapib and Dalcetrapib do not appear to have similar off-target effects(215, 216), but during this research project a large phase III study of Dalcetrapib (DAL-plaque) was stopped early because of a lack of efficacy in patients with ACS and the manufacturers (Roche) decided to abandon further development of this preparation (press release, http://www.theheart.org/article/1395141.do). The reasons for this are not yet clear because the interim analyses have not yet been published. To my knowledge there are no plans to shelve development of Anacetrapib, which is being evaluated in Phase III trials and is a stronger CETP inhibitor. It is possible therefore that the failure of Dalcetrapib, like Torcetrapid before it was molecule rather than mechanism specific.
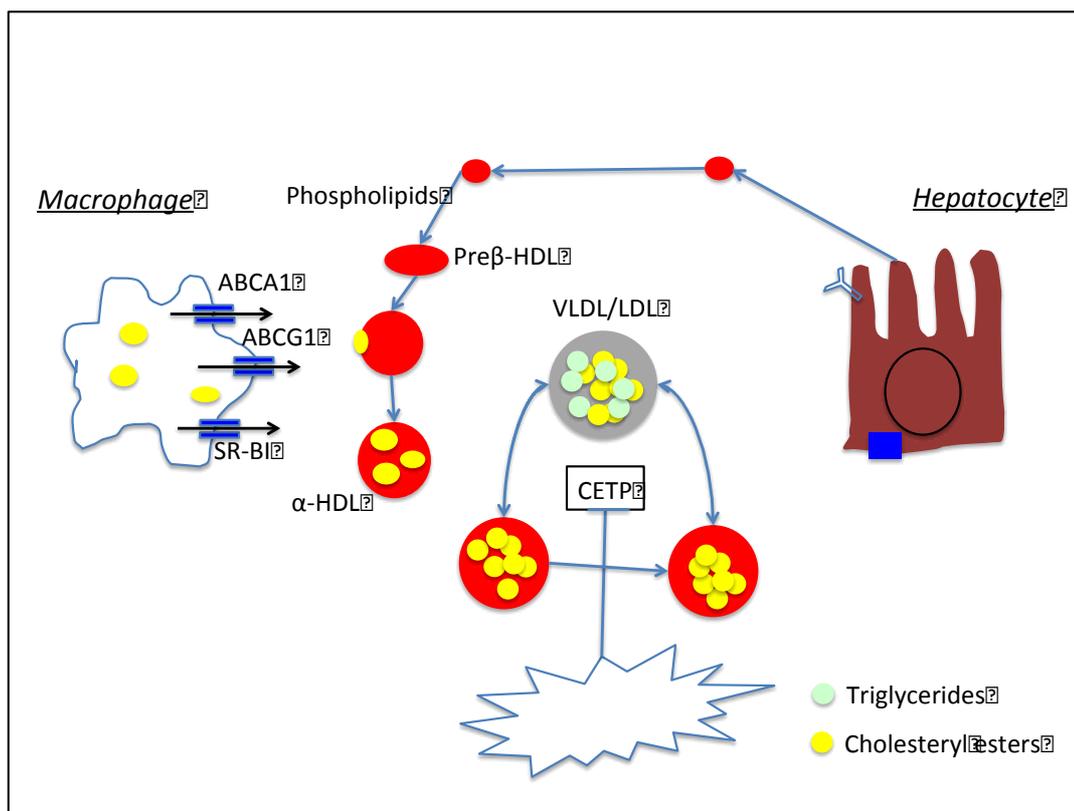
Figure 31 - Mechanism of CHD in HDL-C metabolism. ABCA1, ABCG1, SR-BI are cholesterol efflux proteins. VLDL is very low density lipoprotein cholesterol.

5.1.3.2 Other HDL-C raising therapies

Direct augmentation of Apo AI using a variety of methods, has been described in the literature. These include infusions of recombinant apo A-I, purified native apo A-I and autologous de-lipidated apo A-I. These have shown promising pre-clinical results but none has yet demonstrated efficacy in large phase III randomized controlled trials (reviewed in (217).

Niacin is an antidyslipidaemic medication that leads to not only increased HDL-C but reduced concentrations of both Lp(a) and LDL-C and so clinical trials reporting a beneficial effects (217, 218) cannot be sole ascribed to the HDL-C raising properties.

5.1.4   Genetic Determinants of HDL-C

A recently published meta-analysis of GWAS of circulating lipid concentrations in over 100,000 individuals reported 35 loci that showed association with HDL-C levels at genome wide levels of $(P<5x10^{-8})$(135). This is the largest genome wide study of genetic variants associated with circulating lipid levels in the literature to date. In combination these SNPs explain approximately 12% of the total variation in HDL-C levels.

5.1.5   MR of HDL-C and CHD

A number of MR studies, utilizing a variety of different approaches, have been published in the literature in recent years (Table 19). In general the findings of these studies refute a casual relationship between genetically raised HDL-C and coronary disease. The notable exception to this is HDL-C raising variants in *CETP* that have been found to be associated with CHD (219). These variants, however, have pleiotropic effects and are associated with lower LDL-C and Triglyceride levels so attribution of the association with CHD purely to HDL-C raising is not possible. Variants in *CETP* may however be useful instruments to investigate the effect of CETP inhibition (180).

Table 19 - Mendelian Randomisation analyses of HDL-C and CHD.

Author	Gene	OR for HDL-raising Variant	Support Causal Role for HDL-C
Thompson(219)	CETP - TaqIB	0.95 (0.92-0.99)	Yes
	CETP - I405V	0.94 (0.89-1)	Yes
	CETP - -629A>C	0.95 (0.91-1)	Yes
Haase(220)	APOA1	NR, but 95% CI Crossed 1	No
Haase(221)	LCAT	NR, but 95% CI Crossed 1	No
Voight(143)	LIPG	0.99 (0.88-1.11)	No
	Genetic Risk Score	0.93 (0.68-1.26)	No

5.1.6   MR using Multiple Genetic Variants

To date the genetic variants associated with HDL-C levels have either been
common in the population with modest effects on HDL-C exclusively, common
with larger effects on HDL-C but also effects on other lipid parameters such as
LDL-C and Triglycerides, or rare in the population with large effects exclusively
on HDL-C levels. Individually therefore, these variants may not represents strong
instruments. It has been proposed that the power of MR analyses can be improved
by using multiple genetic variants; either combined into a single instrument or used
as separate instruments (147). Both strategies have been used in recent publications
(Table 20), and methodological papers have reported that they are likely to give
similar results but when there are a large number of variants associated with the
intermediate phenotype (as with HDL-C) there is a risk of finite sample bias that
can affect the results (146). I have therefore decided upon combining variants into

a single instrument in this chapter. Genetic risk scores (GRS) can be "weighted",
where the individual contribution of each genetic variant is taken into account or
"unweighted", where the score is simply the sum of the risk alleles carried.
Although both scores are likely to provide similar results, it has been shown that
when the number of variants contributing to he score is large, a weighted score is
less likely to be effected by bias and so I have chosen to use a weighted score in the
analysis presented in this chapter.

**Table 20 - Studies using genetic risk scores and multiple genetic instruments in
cardiovascular diseases**

Studies Using Genetic Risk Scores as a single instrument

Reference	Finding
(143)	GRS for HDL-C raising variants does not associate with risk of CHD
(222)	GRS of BP raising alleles associates with risk of intracerebral haemorrhage
(42)	GRS of 2 Lp (a) variants associated with risk of cardiovascular diseases

Studies using multiple instruments for MR Analysis

(223)	5 variants associated with raised fasting glucose levels also associated with risk of CHD supporting causality
(224)	3 variants associated with BMI also associate with risk of CHD supporting causality

5.2 Methods

5.2.1 Literature Search

Studies were identified using a two-stage search strategy following PRISMA guidelines. In the first stage MEDLINE & EMBASE were searched using a web-based search engines, and in the second stage articles were identified by manually searching references of articles identified in the first stage and review articles. The MEDLINE database was searched from January 1966 to December 2011. Studies were included if they reported summary measures (mean, median & standard deviation) of plasma HDL-C in cases and controls.

5.2.2 Construction of an HDL-C Raising Genetic Risk Score

SNPs for inclusion in the GRS were identified using the largest published meta-analysis(135). If the reported SNPs were not directly genotyped, a proxy was used ($R^2 \geq 0.8$) when available. If more than one SNP was reported at a single locus then only the SNP with the strongest association with HDL-C was included in the score. A priori, SNPs/loci previous associated with AAA by GWAS ($p<1 \times 10^{-7}$) were excluded from the analysis but there were none.

Genotypes were coded (0,1,2) with 1 & 2 as the HDL-C raising alleles. Each individual was given a score according to how many HDL-C raising alleles they carried. A weighted risk score was also calculated, whereby the SNPs were weighted by their respective beta-coefficients on HDL-C in the original report

(135). This score was then standardized (mean 0, SD 1) prior to statistical analyses, and results reported are odds ratios for AAA, per one sd increase in weighted GRS.

5.2.3   Statistical Analysis

To estimate the association between circulating HDL-C and AAA data identified by literature review was pooled. For each study, mean concentrations of HDL-C in cases and controls to determine a standardised mean difference and standard error. For studies that reported median concentration levels and interquartile ranges (IQR), the median was taken to be the mean value. The IQR was divided by 1.35 in order to obtain the SDs. Furthermore, studies that reported an odds ratio   (OR) for AAA per unit increase in HDL-C, adjusted for clinical risk factors were analysed separately. Study-specific ORs and standard errors (SE) were pooled generating a summary estimate and its 95% confidence interval. All data were pooled using inverse-variance weighted meta-analysis in Stata version 11. Heterogeneity was assessed using Cochranes Q and the I^2 statistic. For analysis of the GRS data, mean allele counts were compared between cases and controls using a t-test. The standardized weighted GRS was then used as a continuous independent variable in a logistic regression for AAA. Other covariates included in the regression were MDS co-ordinates and gender. SNPs that did not meet pre-defined QC thresholds were excluded from the analysis, as were individuals with missing genotypes at any of the SNPs. Effect sizes are expressed as odds ratios +/- 95% confidence intervals. A p-value <0.05 was considered statistically significant.

5.3 Results

5.3.1 Systematic Review and meta-analysis of the association between HDL and AAA

The literature search followed PRISMA guidelines. The following terms used to identify suitable papers for inclusion in the meta-analysis –

Abdominal Aortic Aneurysm[MeSH term] *and*Lipoprotein[MeSH Term]

Or

Abdominal Aortic Aneurysm[MeSH term] *and* High density lipoprotein cholesterol[MeSH Term]

Inclusion criteria were;-

Selection criteria for studies were the following;-

- Phenotypic definition of AAA   (IRAD≥3cm) in cases

- Plasma Measure of HDL-C in cases and controls

- English language articles

- At least ten cases and controls

In addition to the results obtained from the search, data were obtained from recent reviews on risk factors for AAA and from established studies in the

epidemiological literature of AAA. Contact was also initiated with studies that had

not published the association between HDL-C and AAA previously   (i.e. the

SMART study). A flow diagram of the literature search is included in Figure 32.

The search identified 13 studies that reported circulating concentrations of HDL-C

in AAA cases and controls (Table 21)(33, 37, 225-234). Two studies stratified

results in males and females and three studies included only males. Five studies

reported the results from logistic regression analysis with AAA as the outcome

variable and HDL-C as a predictor variable, with other covariates such as age and

gender included (Table 22)(33, 231, 234, 235).

Meta-analysis of studies reporting HDL-C in cases and controls, pooling data from

2,573 cases and 23,412 controls, demonstrated a weighted mean difference of -

0.16mmol/l (95% CI -0.20 - -0.11, P_{random} = 3.59 x 10^{-13})(Figure 33). There was,

however, evidence of considerable heterogeneity (I^2 = 82%). Meta-analysis of five

studies, with a total 1,056 cases and 21,450 controls that reported an odds ratio for

AAA per unit increase in HDL-C, adjusted for covariates demonstrated a consistent

association between higher HDL-C levels and a lower risk of AAA (OR per SD

increase in HDL-C = 0.69, 95% CI 0.63 – 0.76, I^2=0, P_{random} = 4.11 x 10^{-16})

(Figure 33) with little evidence of heterogeneity. In subgroup analysis the effect

sizes were similar in case-control studies, cross-sectional screening studies and

prospective cohort studies (Figure 34). This suggests that at least some of

heterogeneity in meta-analysis including all studies results from gender and age

mix.
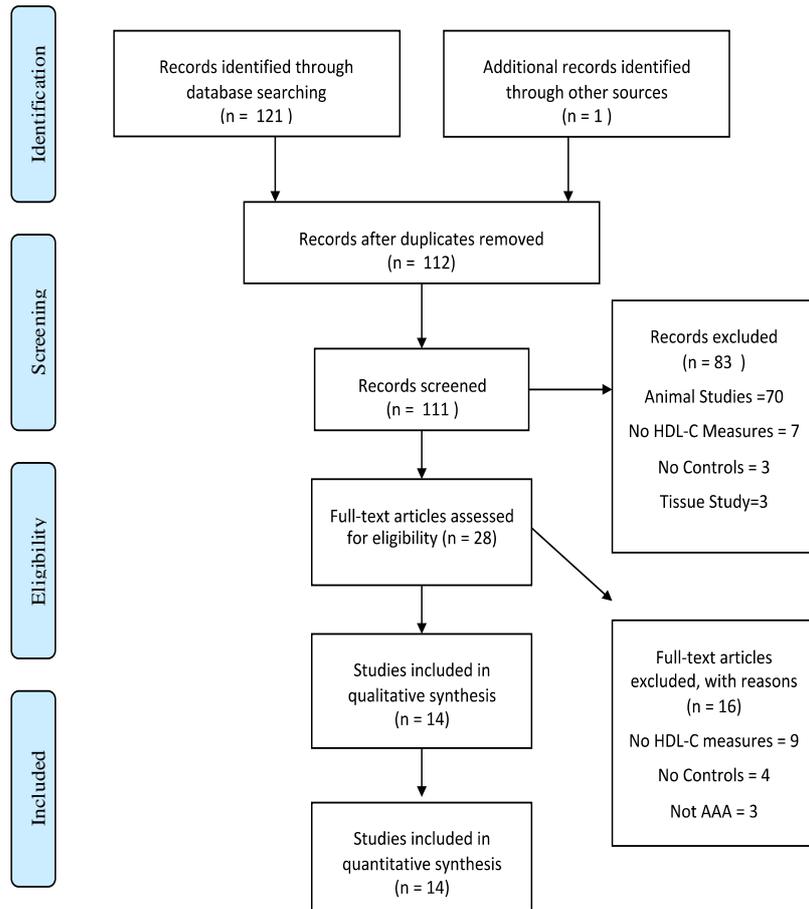
Figure 32 – Search Strategy used to identify HDL-C AAA papers

Table 21 - Studies reporting HDL-C and AAA

Author	Year	Cases	Control	Design	%Male Cases	%Male Control
Ahnstrom	2009	343	214	Case-Control	79	46.3
Hobbs	2003	206	252	Case-Control	100	100
Lee	1999	40	200	Case-Control	82.5	82.5
Rizzo	2007	30	26	Case-Control	100	100
Simoni	1996	69	1460	Population	90	50
Asselbergs	2012	230	6916	Population	NA	
Freiberg Men	2008	252	1701	Population	100	100
Freiberg Women	2008	164	2617	Population	0	0
Singh Men	2001	263	2699	Population	100	100
Singh Women	2001	74	3350	Population	0	0
Jones	2008	576	472	Population	78	45
Naydeck	1999	25	241	Population	NA	NA
Wanhainen	2005	35	140	Case-Control	NA	NA
Golledge	2010	245	3082	Population	100	100
Blann	1998	21	42	Case-Control	86	61
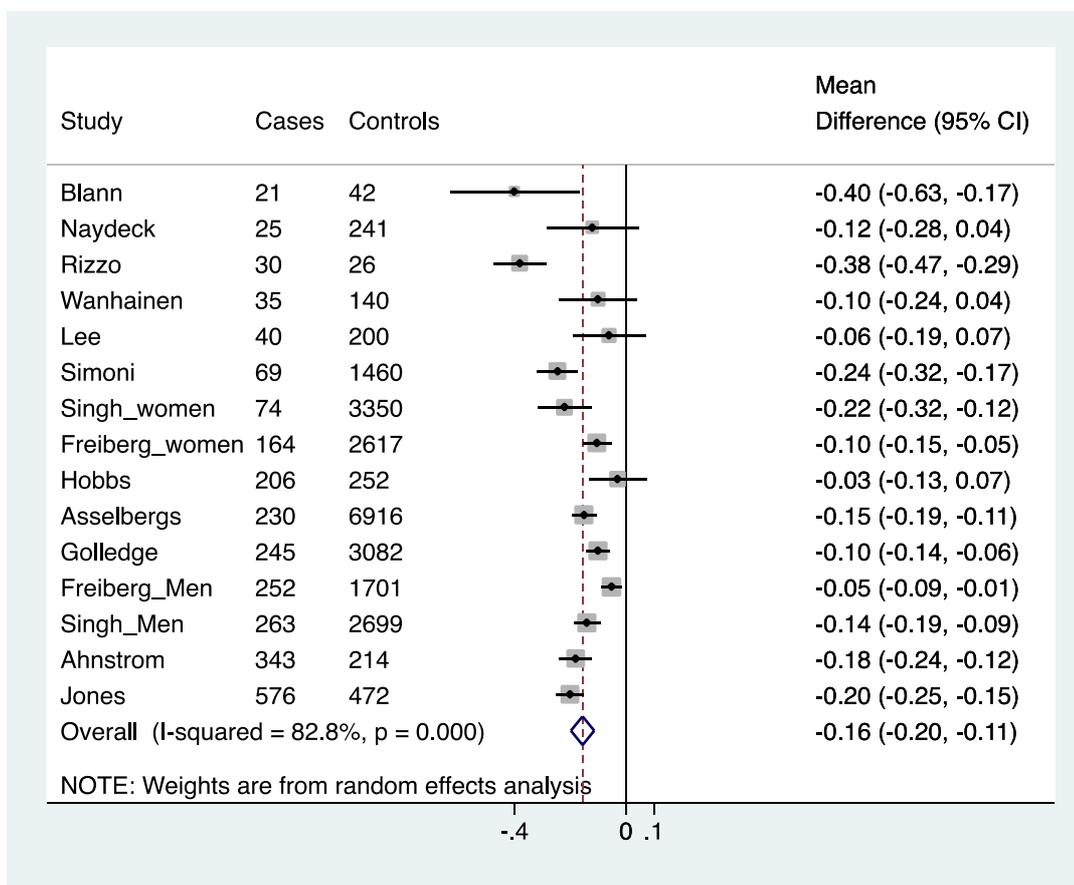
Figure 33 - Meta-analysis of the association between HDL-C concentrations and AAA in the published literature pooling data from 2,573 cases and 23,412 controls. The overall estimate demonstrates that, on average cases of AAA have an HDL-C concentration 0.16mmol/l lower than controls (95% CI -0.20- -0.11, P for random effects meta-analysis = 3.59 x 10^{-13}).

Table 22 - Covariates included in the logistic regression models of studies that reported an odds ratio for AAA per unit increase in HDL-C.

Author	Setting	Covariates included in Model
Blanchard	Recently diagnosis of "focal widening of the aorta"; only 90% IRAD>3cm	Age, gender, pack-years, family history & diabetes
Wanhanien	Nested Case-Control from population screening	Age, gender, atherosclerosis
Golledge	Population screening men aged	Age, smoking, diabetes, CHD, lipid-lowering therapy, waist-hip ratio
Singh	Population screening men and women, aged greater than 25	Age, gender stratified, diastolic bp, Fibrinogen, smoking
Asselbergs	Screening individuals with previous history of cardiovascular disease	Age, gender
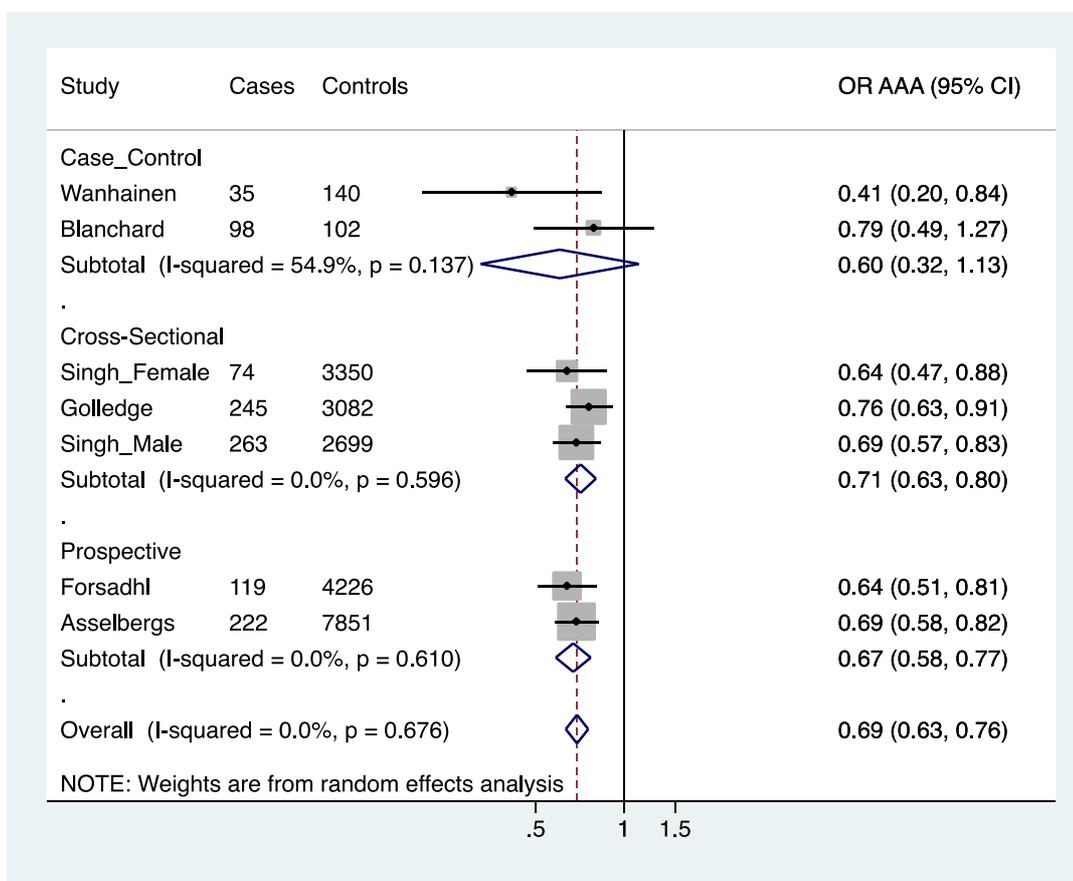
Figure 34 - Meta-analysis of studies that reported an Odds Ratio of AAA per SD increase in HDL-C, pooling data from 1,056 cases and 21,450 controls). Overall, per SD increase the OR for AAA was 0.69   (95% CI 0.63 – 0.76, I^2=0, P for random effects = 4.11 x 10^{-16}). The effect seen in prospective, cross-sectional and case control studies was similar.

5.3.2   Construction of an HDL-C genetic risk score   (GRS)

Genotype data were available for 35 HDL-C raising SNPs in the AC dataset and

Icelandic datasets (Table 24). Lipid measures were available for the Iceland dataset

but not the AC. As expected the score associated strongly with HDL-C levels, but

also with Triglyceride and LDL-C levels, though the effect was comparatively

much smaller (Table 23).

Table 23 - Effect of HDL-C GRS on lipid subfractions in the Iceland dataset in mmol/l.

Trait	n	Effect	SD	P	R2	F
HDL	12459	0.0850	0.0037	3.7×10^{-111}	0.04	517.1
Triglyceride	12104	-0.0459	0.0078	4.0×10^{-09}	0.0028	34.66
LDL	3889	-0.0500	0.0150	0.00083	0.0026	11.19

5.3.3 Association of HDL-C raising alleles with AAA

Evaluation of the SNPs individually demonstrated that 5 of the 35 SNPs were nominally associated with AAA (P<0.05) (Table 25), but only the *CETP* was a significant association when multiple testing is considered. In all cases the HDL-C *raising* allele was associated with a lower risk of AAA.

Table 24 - HDL-C associated SNPs at 35 loci identified by GWAS. The R-squared column is the LD between the lead and proxy SNPs used in the analysis. The weight is the effect of the SNP on HDL-C (per allele in mg/dl), from the discovery GWAS

Gene	Chr	SNP	Other	MAF	Weight	Proxy_SNP	R-squared
PDE3A	12	rs7134375		0.42	0.4		
ZNF664	12	rs4765127	TG	0.34	0.44	rs7307277	1
IRS1	2	rs2972146	TG	0.37	0.46	rs2943645	1
KLF14	7	rs4731702		0.48	0.59		
SCARB1	12	rs838880		0.31	0.61	rs838878	0.96
LRP4	11	rs3136441		0.15	0.78	rs5896	1
LILRA3	19	rs386000		0.2	0.83	rs103294	0.89
SBNO1	12	rs4759375		0.06	0.86		
LCAT	16	rs16942887		G/A/0.16	0.12	rs10773003	1
LIPC	15	rs1532085	TC, TG	0.39	1.45		
CETP	16	rs3764261	TC, LDL, TG	0.32	3.39		
CITED2	6	rs605066		0.42	0.39	rs668459	1
LACTB	15	rs2652834		0.2	0.39	rs2652840	1
PGS1	17	rs4129767		0.49	0.39		
AMPD3	11	rs2923084		0.17	0.41		
ABCA8	17	rs4148008		0.32	0.42		
MC4R	18	rs12967135		0.23	0.42		
TRPS1	8	rs2293889		0.41	0.44	rs2737217	0.97
MVK	12	rs7134594		0.47	0.44		

Gene		SNP						
CMIP	16	rs2925979		0.3	0.45		
ANGPTL 4	19	rs7255436		0.47	0.45	rs2278236	0.97
UBE2L3	22	rs181362		0.2	0.46	rs4821112	0.96
ZNF648	1	rs1689800		0.35	0.47	rs1689803	1
PABPC4	1	rs4660293		0.23	0.48		
STARD3	17	rs11869286		0.34	0.48	rs1877031	0.96
ARL15	5	rs6450176		0.26	0.49	rs7736354	1
C6orf106	6	rs2814944		0.16	0.49		
GALNT2	1	rs4846914	TG	0.4	0.61	rs10779835	0.97
LOC55590 8	19	rs737337		0.08	0.64		
SLC39A8	4	rs13107325		0.07	0.84		
PLTP	20	rs6065906	TG	0.18	0.93	rs7679	1
ABCA1	9	rs1883025	TC	0.25	0.94		
PPP1R3 B	8	rs9987289	TC, LDL	0.09	1.21	rs2126259	0.8
LIPG	18	rs7241918	TC	0.17	1.31	rs7239867	1
HNF4A	20	rs1800961	TC	0.03	1.18		

Table 25 - Of the 35 HDL raising SNPs 5 showed association with AAA at P<0.05 in combined analysis from the AC and Icelandic dataset.

Gene	SNP	OR	95%CI	P
CETP	rs3764261	0.86	0.8-0.93	9.0×10^{-4}
LIPG	rs7239867	0.89	0.82-0.98	0.013
SCARB1	rs838878	0.9	0.83-0.98	0.012
LCAT	rs1689803	0.93	0.86-1	0.049

PLTP	rs7679	0.91	0.84-0.97	0.039

5.3.3.1   Association of HDL-C genetic risk score with AAA

In the AC cohort there was small but statistically significant difference in the un-weighted GRS between cases and controls (40.57 Vs 40.28, mean difference 0.29, 95% CI 0.10 – 0.48, p = 0.003). When five SNPs with pleiotropic effects on lipids were removed from the score, the association remained statistically significant but the effect size was smaller   (OR 0.93, 95% CI 0.86-0.99, P=0.015). A one SD increment in the weighted GRS was associated with a lower risk of AAA (OR 0.88, 95%CI 0.84-0.94, P=1.8 x 10^{-5}, Table 26). Individuals in the fourth quartile of GRS had an OR for AAA of 0.72 compared to those in the lowest quartile (95% CI 0.61 – 0.84, P = 1.3 x 10^{-5}). In the Icelandic cohort a one SD increase in weighted GRS was associated reduction in risk of AAA but the 95% confidence interval for the effect spanned the null effect (OR 0.93, 95% CI 0.85 – 1.012, P=0.121). Combined analysis of the two studies demonstrated a strong association between the weighted risk score and presence of AAA (OR 0.89, 95% CI 0.86 – 0.93, P=4.5x10^{-7})(Table 26).

Table 26 - Association between 1 SD increase in weighted GRS and AAA in the AC and Icelandic Cohorts.

	Cases/Controls	OR	(95% CI)	P-Value
AC	1887/5437	0.88	(0.84 - 0.92)	1.4x10^{-5}
Iceland	480/39000	0.93	(0.85-1.02)	0.12

Combined	2367/44437	0.89 (0.86-0.93)	4.5×10^{-7}

5.3.4 Instrumental variable regression

The association of the weighted GRS and HDL-C in Whitehall II was used for the instrumental variable regression analysis. Here, a 1 SD increase in wGRS was associated with a 0.11 mmol/l increase in HDL-C and the estimate of the weighted GRS with AAA (a 1SD increase in wGRS associated with an AAA OR of 0.89, 95% CI 0.86 – 0.93, P = 4.5 x 10^{-7}) were used to triangulate the "causal" association between HDL-C and AAA. Using a Mendelian triangulation technique that takes into account the imprecision in the gene-HDL-C and gene-AAA associations (236), it is estimated that for every 1 mmol/L increase in HDL, the causal OR for AAA was 0.35 (95%CI: 95%CI: 0.24, 0.50). This was consistent with the meta-analysis of observational (non-genetic) studies, scaled to the same difference in HDL-C (1 mmol/L), which yielded an OR per increase in 1 mmol/L HDL-C of 0.40 (95%CI: 0.32, 0.50).

5.3.4.1 Association of *CETP* variant with AAA

A common variant in the promoter of *CETP* (rs3764261) was found to have the strongest association with HDL-C levels in the Teslovich analyses. The rare allele of this variant was also found to associate with higher total cholesterol, lower LDL-C and lower TG concentrations, a repertoire of effects seen with pharmacological CETP inhibition. This variant is therefore a useful instrument to investigate the

potential for CETP inhibition in AAA. This variant was genotyped in 5 cohorts of

AAA. The HDL-C raising allele was associated with a lower risk of AAA in meta-

analysis (OR 0.91, 95% CI 0.86 – 0.95, $I^2 = 15\%$, P=1.3x10^{-4})(Figure 35).

Considered individually, only the AC cohort showed a strong association between

rs3764261 and AAA, however the direction of effect was consistent in all studies.

Removing the AC from the overall meta-analysis diminished the overall effect size

(OR = 0.94) but the association was still statistically significant (P=0.05)(Figure

36).

Study	Cases	Controls	OR AAA (95% CI)
AC	1596	5855	0.86 (0.79, 0.93)
New Zealand	593	594	0.94 (0.79, 1.12)
Utrecht	862	1866	0.92 (0.81, 1.04)
ICELAND	479	36910	0.88 (0.77, 1.01)
New Zealand_2	729	805	1.05 (0.90, 1.23)
SMART	630	6452	0.94 (0.82, 1.06)
Overall (I-squared = 15.4%, p = 0.315)			0.91 (0.86, 0.95)

.7   .8   1   1.2
OR
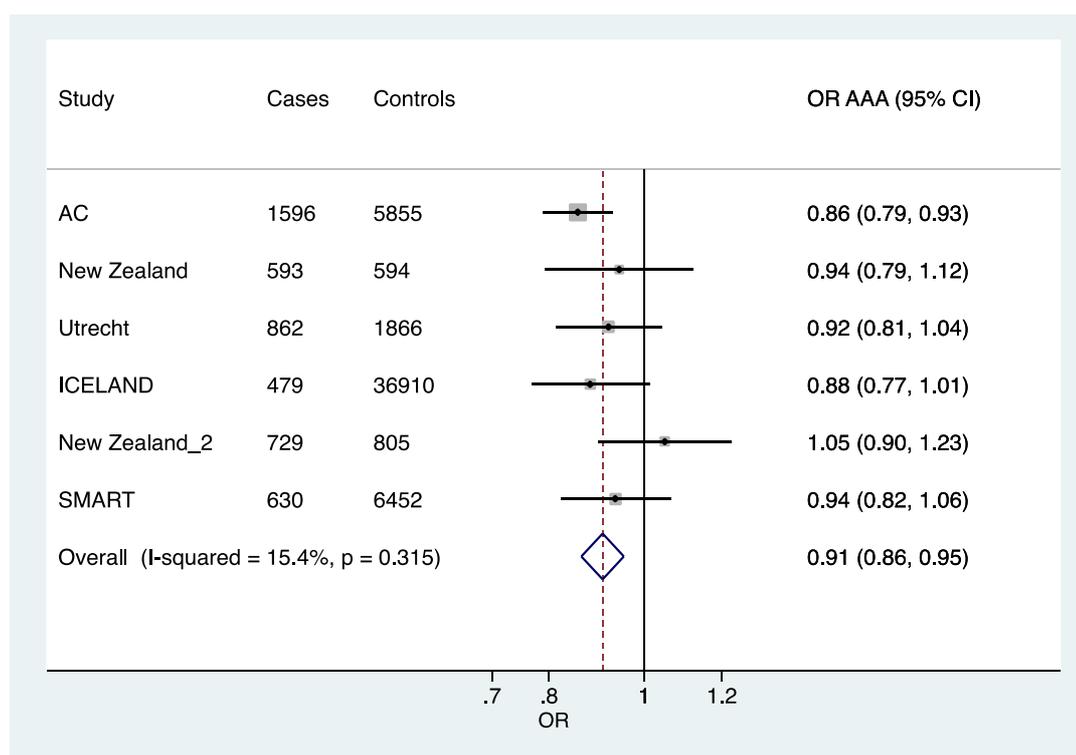
Figure 35 – Association of rs3764261 with AAA in meta-analysis of studies, pooling data from 4,889 cases and 52,482 controls. Per allele Odds Ratio is 0.91, 95%CI 0.86 – 0.96, P=0.00013, I^2=15%.
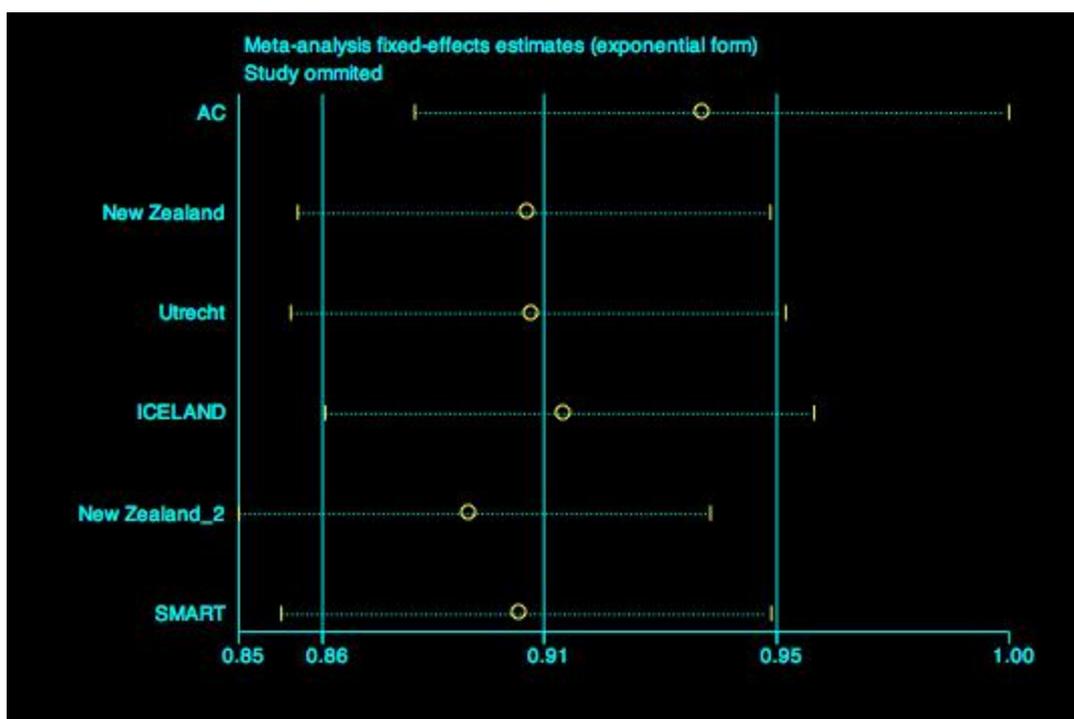
Figure 36 – Effect of removing one study on the overall meta-analysis effect size. The AC had the largest individual effect, but removing this there was still an association between the HDL-C raising allele and a lower risk of AAA (OR 0.94, 0.88-1, P=0.05).

5.4   Discussion

The role of HDL-mediated pathways in cardiovascular disease is controversial. The epidemiological literature reporting the inverse association between HDL-C levels and CHD risk is extensive and the association statistically robust, yet previous MR analyses refute a causal role and late stage clinical trials have up to now have failed to demonstrate an incremental benefit of HDL-C raising over established cardiovascular risk prevention. Taken together this would suggest that lower HDL-C levels are not themselves causal for CHD. It is recognized that AAA has an overlapping yet distinct risk factor profile to CHD and therefore it does not

necessarily follow that they will fail in AAA, therefore the role of HDL-C raising

therapies in AAA prevention/treatment warrants consideration.

In this study I have performed an updated meta-analysis of the association between

HDL-C levels and AAA. Although a meta-analysis was published relatively

recently, the present analysis adds considerably to this. Specifically, the number of

cases and controls has more than doubled   (12,406 to 25,985). Furthermore, two

large prospective studies   (the gold standard in epidemiological literature) were

included and the risk of AAA, adjusted for age and gender has been quantified

precisely for the first time.

The association between HDL-C levels and AAA appears to be statistically robust.

However, given the controversy that accompanies the HDL-C – CHD relationship

it is possible that the observed association does not reflect causality. Therefore to

examine this I employed an MR framework of investigation. In two cohorts there

was a strong association between a score composed of a panel of common variants

of modest effects that raise HDL-C and a lower risk of AAA. Furthermore the

effect size was concordant with that seen in the observational literature, consistent

with a hypothesis that HDL-C mediated pathways are causal in the development of

AAA. Although the score has pleiotropic effects on other lipid sub-fractions, it is a

much stronger instrument to study the effects of HDL-C and for that reason the

result is likely to be valid. The HDL-C score based on the GLGC consortium data

of over 100,000 participants, and is therefore an unbiased and robust genetic instrument for HDL-C raising alleles, a fact that adds to the validity of the current analysis.

Given the observations made with the HDL-C GRS, I sought to evaluate the potential role of CETP inhibition for AAA. To do this a variant in the promoter region of *CETP* that has been shown to be the largest effect on HDL-C by the GLGC consortium was used as a genetic instrument. This SNP has pleiotropic effects, and is associated with lower LDL-C levels and lower Triglyceride levels at genome wide levels of statistical significance. In meta-analysis of five case control studies we found a consistent association between the rare allele of rs3764261 and a lower risk of AAA. Although individually, this association was only nominally significant in the AC, this is the largest cohort with the greatest power to detect an association. These results suggest that a pattern of effects mediated by CETP inhibition may be of benefit in the treatment of AAA and clinical trials may be warranted even if they turn out to be negative for CHD as the primary outcome.

These data support recently published manuscripts that report regression of AAA in a murine model via reconstitution of HDL-C (209). The authors of this article also showed HDL-C augmentation resulted in alteration in the expression of inflammatory & remodeling genes in the aorta. Interestingly, these changes were noted only in the area of the aorta that develops AAA in theses models. This could

potentially be one reason that there appears to be a positive result from the MR

analyses in AAA but not CHD. These results also indicate that a trial of CETP

inhibition specifically in AAA patients may be warranted. The DAL-plaque phase

two studies demonstrated that Dalcetrapib is safe for use in patients with

cardiovascular disease(215). Furthermore, a number of surrogate outcomes were

studied including arterial inflammation measure by CT-pet scanning. Interestingly

the authors reported an improvement in this parameter following treatment with

Dalcetrapib. In Chapter 4, it was demonstrated the effect of the anti-inflammatory

polymorphism in the IL-6R was considerably stronger in AAA than in CHD

suggesting that inflammatory pathways could play a larger role in AAA than CHD.

This is supported by observational studies reporting higher levels of inflammatory

markers such as IL-6 and CRP in AA patients that AAA free CHD patients. It is

possible, therefore that the anti-inflammatory effect of HDL-C is greater in AAA

than CHD, which would explain the differential results from the MR analysis of

both phenotypes. In the present analysis each copy of the rare allele was associated

with approximately 10% lower risk of AAA, compared to 4-5% lower in risk

observed in a recent analysis, suggesting that the effect of CETP-inhibition could

be greater in certain forms of cardiovascular disease such as AAA.

5.4.1.1 Limitations

Due to lack of availability of HDL-C measures in AC, I relied upon an external

association of GRS with HDL-C for the Mendelian triangulation. Although this

limited the statistical approach to summary data (rather than individual participant

data), use of an external source may in fact prevent over-fitting bias. Ideally there would be a large dataset with validated measures of aortic expansion in the context of the HDL-C raising genotypes and assess whether or not this could provide clinically useful information to guide surveillance programs, but this is not available at present.

5.4.2 Conclusion

This study provides evidence that HDL-C plays a causal role in the development of AAA. Trials of HDL-C raising therapies in the prevention of AAA development and/or progression may be warranted.

6   Genetics of vascular remodeling and risk of AAA

6.1   Background

In chapters 4 & 5 I used established variants associated with circulating biomarkers to understand novel pathways in AAA. In this chapter I extend the methodology by first using large scale genomic analyses to identify variants associated with an intermediate imaging biomarker, and then investigate if these are associated with with AAA risk.

6.1.1   Vascular Remodeling and AAA

The study of continuously distributed intermediate phenotypes strongly associated with complex diseases in population-based studies has been used to augment and add to discoveries made by traditional case-control genetic studies(130). The intermediate phenotypes used may be circulating biomarkers, anthropometric traits or imaging studies of subclinical disease. The use of imaging studies is of particular interest because it may be possible to study the subtle initiating factors for complex diseases on a population level. For example, in coronary disease it has been shown that that genetic variants associated with carotid intima media thickness (CIMT), a marker of atherosclerotic disease burden, are also associated with CHD (133). Another example is the study of coronary artery calcification, which has uncovered novel associations in CHD (134). Furthermore, it is not just in cardiovascular disease that this approach has reaped rewards;- the study of mammographic breast density has uncovered novel genetic associations in the study of breast cancer (136, 237). These examples all serve to exemplify the

potential power of this approach in the search for the genetic variants that associate

with risk of common complex diseases.

Arteries are dynamic vascular conduits that can remodel in response to

atherosclerosis (238). Cardiovascular disease is characterized by thickening of the

intima media portion of the vessel and plaque formation, reduced vessel elasticity

and increased vessel size. The process by which the vessel enlarges to maintain

flow through is diseased lumen is known as expansive vascular remodeling (238).

This is generally considered to be a beneficial physiological response but may

actually have deleterious effects such as plaque instability and aneurysm formation

(239). For example, *excessive* expansive arterial remodelling in the coronary

circulation has been associated with an increased risk of coronary heart disease

events (240) and may be associated with development of aneurysms(241) (Figure

37) and plaque instability.
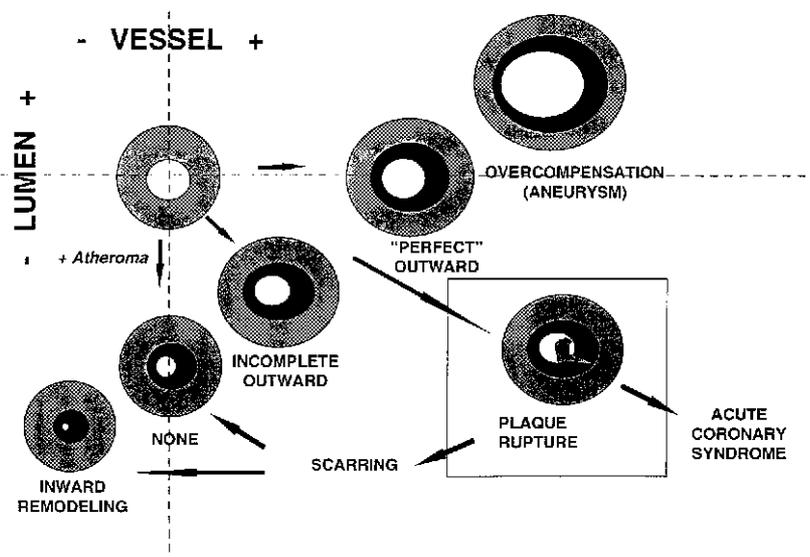
Figure 37 – Putative mechanism of vascular remodeling in response to atherosclerotic disease of the intima-media. In order to maintain luminal flow, proteolysis occurs but this can result in plaque instability or even aneurysm formation in some cases.Hypothesis and diagram from(239)

In AAA, genetic association studies have been limited to either case control

designs or studying growth rates of small AAA and to date there have been no

studies of continuous intermediate phenotypes associated with the disease

endpoint. The question is, however, if one is to take the intermediate phenotype

approach, what imaging phenotype is the most appropriate to study with regards to

AAA? Perhaps the most obvious phenotype is infra-renal aortic diameter (IRAD)

given that the criterion for definition of AAA comes from this measurement.

Furthermore, it has been shown in prospective population studies that IRAD is a

strong predictor of future aneurysmal disease (37, 242) as well as other

cardiovascular endpoints(27). Unfortunately, I did not have access to adequately

powered studies that had both genome-wide genotype and phenotype data

available, so was unable to study this. Carotid intima media thickness (CIMT) does

not show a consistent association with AAA (48) and is therefore not an

appropriate candidate to study for this purpose. The common carotid artery may,

however, have properties that could be exploited in the search for genetic

determinants of AAA. The common carotid artery enlarges in response to

progressive atherosclerotic disease (243) and inter-adventitial carotid artery

diameter (ICCAD) correlates strongly with the presence of cardiovascular risk

factors (244, 245). This suggests therefore, that ICCAD may be considered a proxy

marker of large vessel vascular remodeling in response the atherogenic

environmental exposures. Furthermore, a larger ICCAD has been associated with

an increased risk of AAA; in the prospective Tromso study an incremental increase

in CCAD was an independent risk factor for presence of AAA (246) (Figure 38).

Furthermore, it has also been shown that patients with AAA have larger carotid

arteries than age-matched controls and cases of thoracic aortic disease (247). These

data support the hypothesis that pathways involved in systemic vascular

remodeling may also play a role in the development of AAA as previously

postulated (see Figure 37)(241). It therefore, may be a suitable imaging phenotype

to study in relation to AAA development, which is the aim of this study.

Specifically the hypotheses that I examined in this chapter are;-
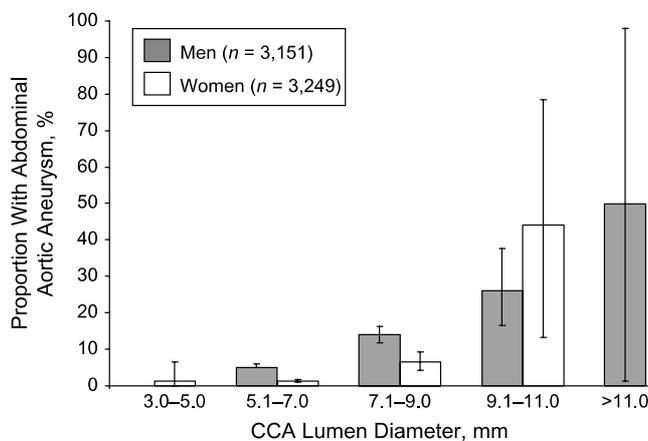
Figure 38 – Proportion of subjects in the Tromso study with AAA by incremental increase in carotid artery diameter(246)

1. What are the genetic determinants of common carotid artery diameter?

2. Do these variants associate with risk of abdominal aortic aneurysm?

6.2 Methods

A flow diagram of the overall study design is demonstrated in Figure 39. ICCAD

was normally distributed and therefore not transformed. Association analysis was

performed using linear regression, adjusting for age and gender with the

assumption of additive genetic effects using PLINK v1.07

(http://pngu.mgh.harvard.edu/~purcell/plink/). The PLINK scripts used for the primary

analysis are included in the appendices. For IMPROVE additional adjustment for

the first three multidimensional scaling (MDS) components was applied to control

for population structure. Meta-analysis was performed using a fixed-effect model

with inverse variance weighting and a calculation of two homogeneity statistics:

Cochran's Q and I^2. The *a priori* threshold for array-wide statistical significance

was established as $P<8.39\text{x}10^{-7}$ through approximation of the total number of uncorrelated SNPs on the Metabochip with the number of principal components explaining >99.5% of the total SNP variation, using a block size of 8192 SNPs (248, 249). This number was then used for a standard Bonferroni correction to set the threshold for array-wide significance for a two-sided test at the 5% level. The calculations for definition of array wide significance and QC of the data was undertaken by statisticians and bioinformaticians at UCL and the Karolinska Institute, funded by the IMPORVE study group. For case-control analysis of AAA, summary effects from each of the studies were combined using fixed effects meta-analysis. Aortic diameter was log transformed prior to analysis. To determine if the identified loci were associated with expansive remodeling in response to atherosclerosis, interaction analyses with CIMT was carried out in all SNPs showing a suggestive association on the discovery analyses $(P<1\text{x}10^{-5})$. The main effects of the SNP and CIMT were included in the regression equation   (1). The interaction term   (SNP*CIMT) is the product of the number of alleles at the locus (1,2,3) and standardized CIMT.

1. $\text{ICCAD} = \beta_0 + \beta_{age} + \beta_{sex} + \beta_{SNP} + \beta_{CIMT} + \beta_{SNP*CIMT}$

For SNP-AAA association, logistic regression adjusted for age and gender was performed. In the AC dataset, additional covariates were multidimensional scaling co-ordinates to adjust for population substructure as previously described (109). Study specific odds ratios and corresponding standard errors were pooled using inverse weighted meta-analysis using the "metan" command in Stata V10.
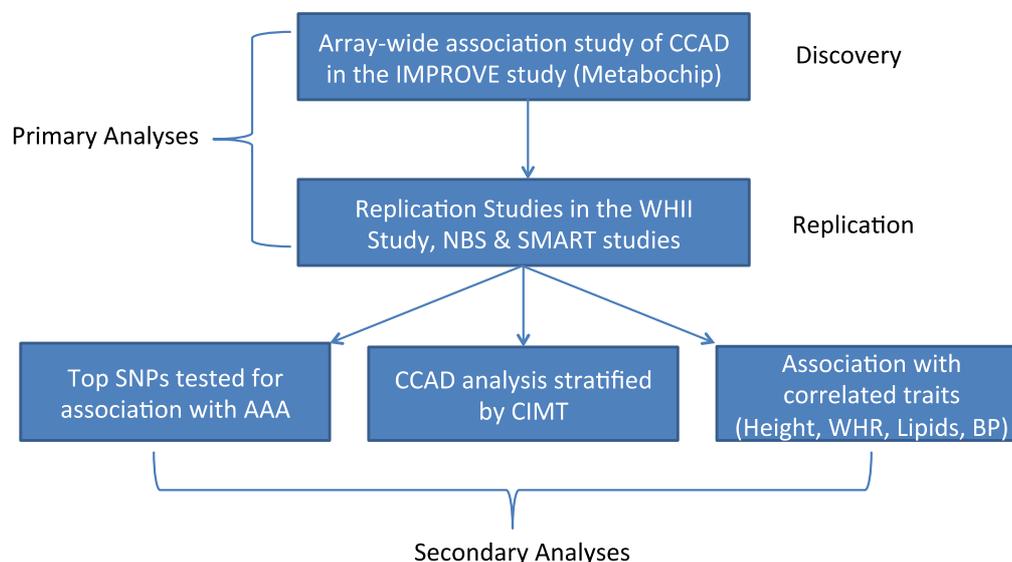
Figure 39 - Study flow used in this chapter

6.3 Results

6.3.1 Genetic variants associated with CCAD

Genotyping quality control results, and some basic demographic details for the

studies are provided in Tables 27 & 28. The results for the 127,730 SNPs tested in

the IMPROVE discovery cohort are shown in Figure 40, organised by chromosome

and genomic position in a Manhattan plot. There was a strong association between

variants at 1q24.3 and greater ICCAD (Figure 41). Details of all SNPs that showed

a suggestive association ($p < 1 \times 10^{-5}$) with ICCAD are provided in the Table 29. The

lead SNP in the discovery analysis was rs3768445 (MAF 18%) and for each copy

of the rare allele ICCAD increased by 0.13mm (95% CI 0.08-0.18mm, P=8.2 x 10^{-8}). Rs4916251 was selected for further study because of genotype availability in follow-cohorts. Minor allele frequencies in each cohort are shown in Table 5. This SNP is in high LD with rs3768445 (R^2=0.99) with a similar effect size in the discovery study (0.12 mm per allele, 95% CI 0.08 – 0.17, P = 1.89 x 10^{-7}). In follow-up studies there was no association between rs4916251 and ICCAD in analysis from 5,755 individuals from three cohorts (per allele change in ICCAD = 0.02 mm, 95% CI -0.02 – 0.06, P=0.28). There was no association between this SNP and other anthropometric traits, or strong LD between the lead SNP and others at this locus that have come up in previous GWAS of other traits/diseases (Table 31) .

Table 27 – Quality control filters for the metabochip data in the IMPROVE and WHII studies

	IMPROVE	WHII
Individuals with Relevant Phenotype	3516	2158
Individuals removed following IBD analysis	86	64
SNPs removed HWE filter	293	823
Genotyping Rate	0.99	0.99
SNPs removed after missingness filter	177	3
SNPs removed after MAF filter	57,324	61,179

SNPs in final analysis	127,998	130,216

Table 28 – Demographics of the carotid phenotype cohorts

	IMPROVE (n=3430)	WHII (n=2110)	SMART (n=3062)	NBS (n=532)
Age (yrs)	64 (54-79)	61 (50 - 73)	56 (49-66)	63 (51-72)
Male (%)	48	77	68	51
CIMT (mm)	0.86 (0.16)Mean 1.17 (0.33) Max	0.79 (0.15) Max	0.88 (0.27) Mean	0.86 (0.11) Max
CCAD (mm)	7.81 (0.86)	6.17 (0.73)	7.79 (1.1)	6.07 (0.83)
SBP (mmHg)	141 (18)	127 (16)	141 (20)	129 (6)
DBP (mmHg)	82 (9.7)	74 (10)	80 (11)	78 (5)
Current Smokers (%)	15	10	32	15
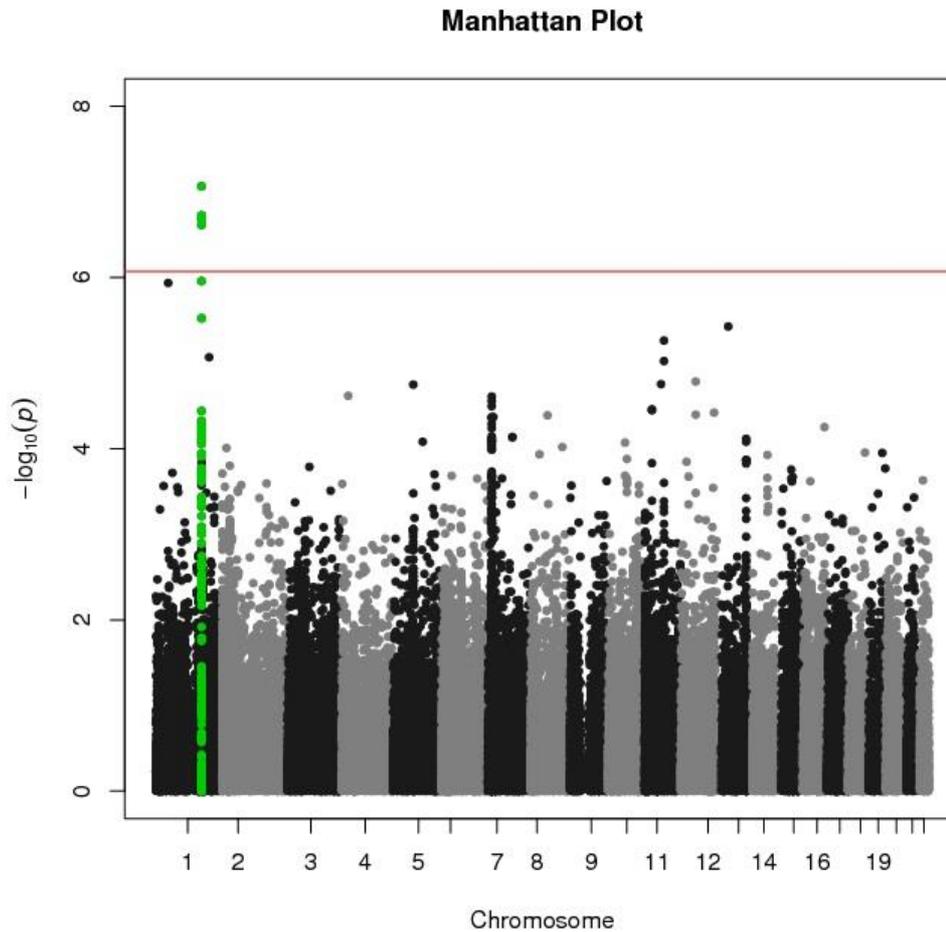
Manhattan Plot

Figure 40 - Manhattan plot for association results by chromosome between 127,998 SNPs and CCAD in the IMPROVE study   (red-line = array wide significance, 8.39 x 10^{-7}). Each point on the plot represents the –log10 p-value for the association between individual SNPs and CCAD. Manhattan plots created in R statistical package (http://www.r-project.org/).
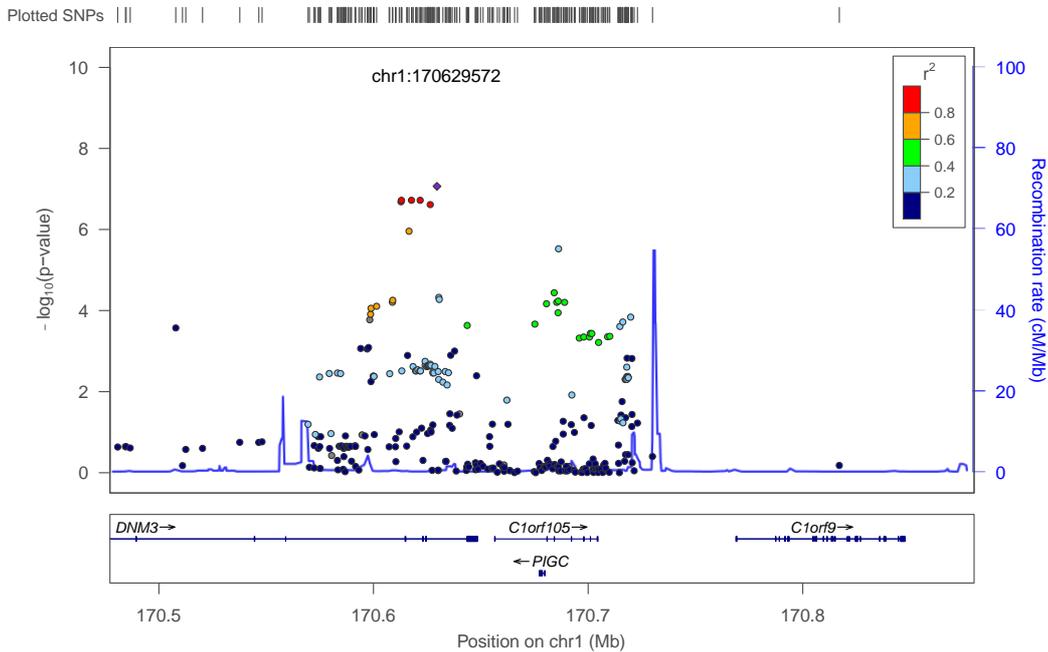
Figure 41 - The lead SNP value (rs3768445, β=0.11, *P*=1.67E-07) is represented by the purple diamond, while the other points are colour coded by their LD with the lead SNP (as measured by R^2). Figure created using Locuszoom (http://csg.sph.umich.edu/locuszoom/).

Table 29 – SNPs showing association with CCAD at array wide significance.

CHR	SNP	Gene	β (95% CI)	P	MAF
1	rs3768445	*DNM3*	*0.11 (0.02)*	1.67×10^{-07}	0.18
1	rs9425586	*DNM3*	*0.11 (0.02)*	4.67×10^{-07}	0.18
1	rs4916251	*DNM3*	*0.11 (0.02)*	4.67×10^{-07}	0.18
1	rs1023479	*DNM3*	*0.11 (0.02)*	4.67×10^{-07}	0.18
1	rs6676665	*DNM3*	*0.11 (0.02)*	5.0×10^{-07}	0.18
1	rs2213731	*DNM3*	*0.11 (0.02)*	5.93×10^{-07}	0.18

6.3.2 Interaction with CIMT & Association with expansive arterial remodelling

To investigate the whether or not the variants associated with ICCAD in the IMPROVE discovery were associated with expansive vascular remodelling further analysis, accounting for CIMT as a marker of atherosclerotic disease burden was performed. In all studies there was a strong association between CIMT and ICCAD as expected. There was evidence of interaction between the lead SNPs on Chromosome 1q24.3 and CIMT in the IMPROVE study ($P_{interaction} = 4 \times 10^{-4}$). There was also evidence of interaction in the WHII ($P_{interaction} = 0.004$), but not the SMART Study ($P_{interaction} = 0.34$) or the NBS ($P_{interaction} = 0.79$). Graphical representations of the interactions in IMPROVE and WHII are included in Figure 42 & 43. For each copy of the rare allele the change in ICCAD accompanied by a one standard deviation increase in CIMT was approximately 0.08mm greater (95% CI = 0.04 – 0.13). In subgroup analysis, by tertile of CIMT, there was evidence of stronger effect for the SNP in the top tertiles of CIMT with a stepwise increase in the meta-analysis effect with each increment in CIMT tertile (Figure 44), again suggesting that this variant is associated with a remodelling response to atherosclerosis. When the two highest tertiles of CIMT are considered together, carriage of the rare allele was associated with 0.09mm increase in ICCAD (95% CI 0.06 – 0.13, $P = 1.87 \times 10^{-7}$), although there was evidence of heterogenetiy ($I^2 = 66\%$) (Table 30), which is probably the result of heterogeneity with regard to CIMT between the cohorts. I also investigated subgroups based upon a threshold CIMT value of 0.75mm (previously shown to be a value above which risk of

events increases (250)). In individuals with a CIMT greater than this (n=5,468)

there was evidence for an association with larger ICCAD (beta = 0.10, 95% CI

0.06 – 0.14, P = 1.15 x 10^{-6}) but evidence of considerable heterogeneity between

studies (I^2 = 78%, Q = 0.004). There was no evidence of interaction with systolic

blood pressure, height, smoking status or gender in the IMPROVE and WHII

studies.
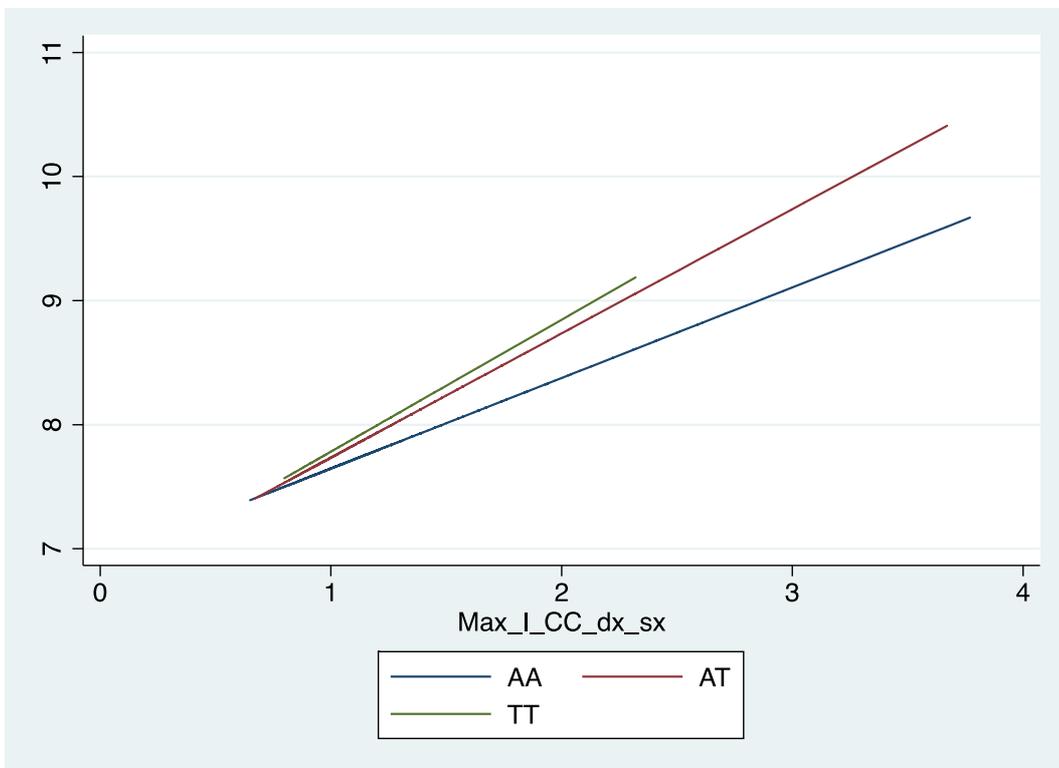
Figure 42 – Interaction in the IMPROVE study
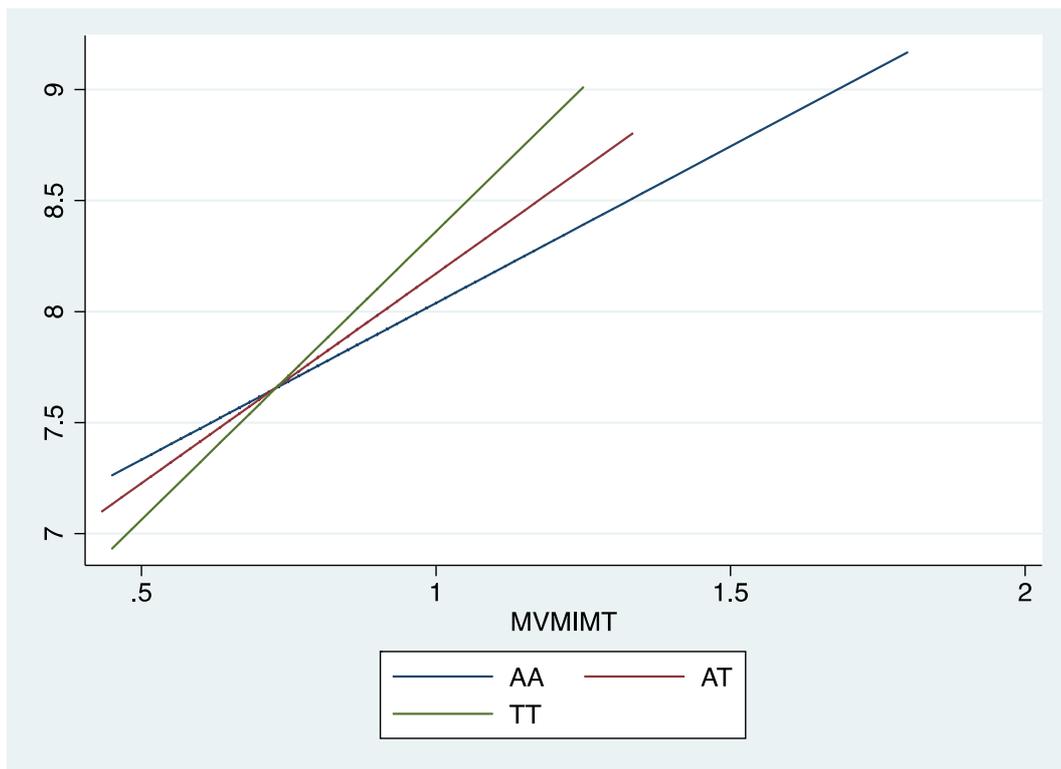
Figure 43 - Interaction in the WHII study

Table 30 – Subgroup analyses of the association between rs4916251 and ICCAD, by tertile of CIMT measure.

STUDY	Subgroup of CIMT	Beta	SE	N	P
IMPROVE	1st Tertile of IMT	0.05	0.03	1126	0.153
WHII	1st Tertile of IMT	0.09	0.04	739	0.043
SMART	1st Tertile of IMT	-0.01	0.04	1014	0.82
NBS	1st Tertile of IMT	-0.05	0.08	176	0.67
Overall	**1st Tertile of IMT**	**0.03**			**0.12**
IMPROVE	2nd Tertile of IMT	0.10	0.04	1126	0.011
WHII	2nd Tertile of IMT	0.08	0.05	677	0.085
SMART	2nd Tertile of IMT	0.02	0.05	978	0.77
NBS	2nd Tertile of IMT	0.05	0.09	177	0.65
Overall	**2nd Tertile of IMT**	**0.07**			**0.005**
IMPROVE	3rd Tertile of IMT	0.18	0.04	1141	0.000039
WHII	3rd Tertile of IMT	0.07	0.05	698	0.165
SMART	3rd Tertile of IMT	0.09	0.06	1011	0.13
NBS	3rd Tertile of IMT	-0.11	0.13	181	0.13
Overall	**3rd Tertile of IMT**	**0.11**			**2.4×10^{-6}**
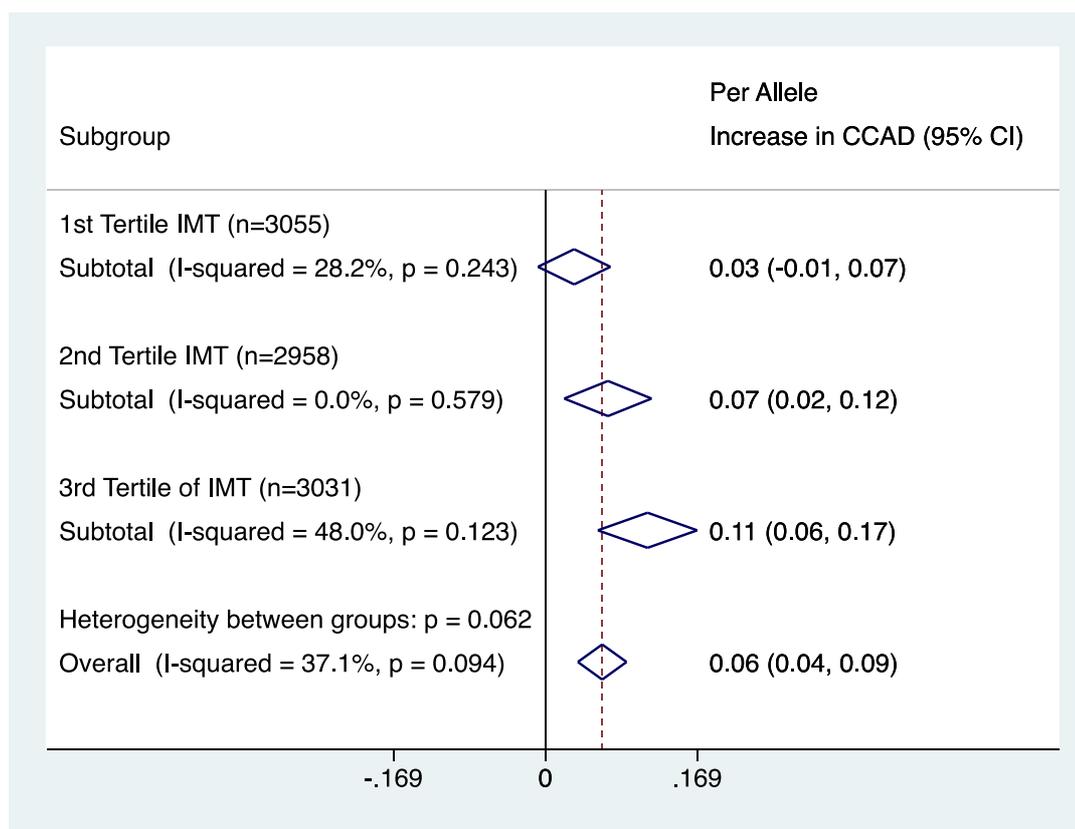
Figure 44 - Meta-analysis of the association between rs4916251 and CCAD, by tertile of CIMT. ES is the effect size, in mm per, using an additive (per allele) genetic model. The overall effect is 0.05 (95%CI 0.03 – 0.08, P=1 x 10^{-4}). In the 1st tertile of CIMT, there is no evidence of association between rs4916251 and CCAD with evidence of heterogeneity (beta = 0.002 95% CI -0.04 – 0.04, P=0.9). In the 2nd tertile of CIMT, rs4916251 is associated with greater CCAD (beta = 0.08 95%CI 0.03 – 0.12, P=0.001) with evidence of heterogeneity. In the third tertile of CIMT, there is a n association between rs4916251 and greater CCAD (beta=0.11, 95% CI 0.06-0.17, P=7.4x10^{-5}).

6.3.3 Association of rs4916251 with AAA

In keeping with previous studies (246), in the SMART study a larger ICCAD was

strongly associated with presence of AAA (OR for AAA per SD increase in ICCAD =

1.67, 95% CI 1.46 – 1.93, P = 6 x 10^{-13}). As AAA may represent a consequence of

excessive expansive arterial remodelling in response to atherosclerotic stimuli, I examined

the possibility that this SNP may also be associated with risk of developing AAA. In meta-

analysis of 5 case-control studies pooling data from 5,007 cases and 43,630 controls,

rs4916251 was associated with presence of AAA 1.10, 95% CI 1.03-1.17, p=2.8 x 10^{-3}, I^2=18.8, Q=0.30)(Figure 45). There was no association between this SNP and aneurysm diameter (β=0.016, *P*=0.075) in analysis of aneurysm size from 2,906 individuals with AAA.
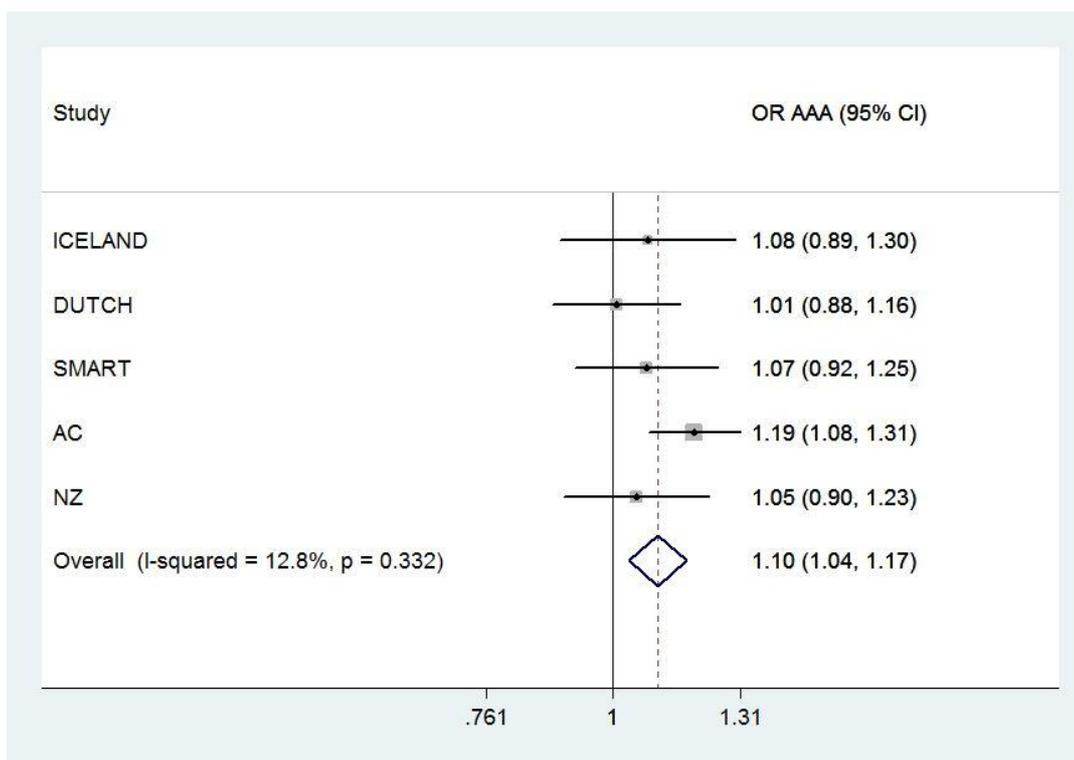
Figure 45 – Association between rs4916251 and AAA.

6.3.4   eQTL analysis in Aortic Tissue

To determine whether or not SNPs at this locus were having an effect on gene eQTL studies of nearby genes (*DNM3, PIGC, C1ORF105* and *C1ORF9)* in aortic tissues were performed. The results shown are for rs1023479, which is in high LD with rs4916251(R^2=0.99), as this SNP was not available for analysis. There was an association between the risk allele and increased expression of *PIGC* in aortic media (*P*=4.4 x 10^{-3}, Figure 46) and aortic adventitia (*P*=0.04). Further *in vitro*

analysis will be required to delineate the functional variant(s) at this locus and the

mechanism by which it could effect expression of *PIGC* in the vasculature.

**Table 31 - Correlation between rs4916251 and other SNPs identified by GWAS for
other traits. Source - http://www.broadinstitute.org/mpg/snap/ldsearch.php**

	R^2	Trait
rs10914144	0.016	Platelet-size
rs678962	0.035	Height
rs1011731	0.27	Waist-hip ratio
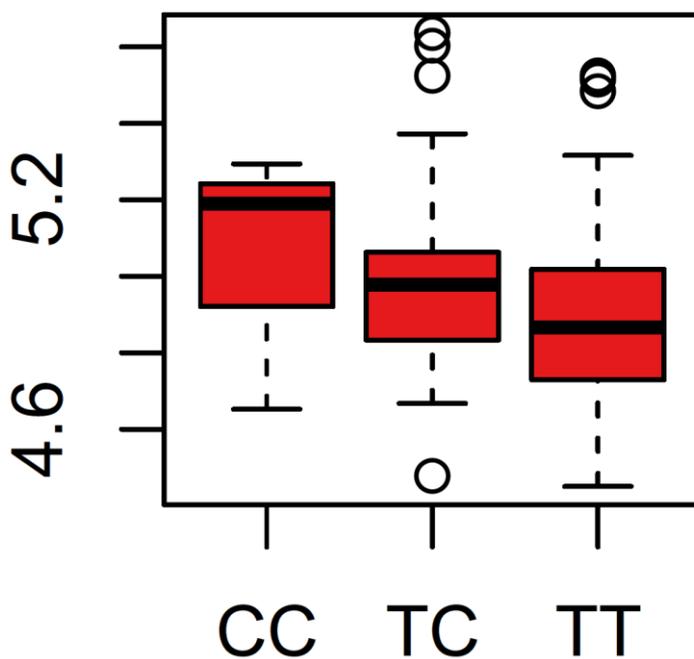
**Figure 46 - Association between rs1023479 (proxy for rs4916251) and expression of
PIGC in aortic media (*P*=4.4x10^{-3}). CC - n=3, TC - n=47, TT - n=83. This analysis
was performed primarily by Lasse Folkersen, upon my request.**

6.4   Discussion

AAA is a complex disease with a considerable heritable component that remains
largely unexplained. Although case-control GWAS are a useful means of
discovering genetic variants associated with complex diseases, an alternative
strategy to augment these is to study intermediate phenotypes associated with the
disease end-point. In this study I chose to investigate carotid artery
diameter/remodeling as a trait associated with AAA. This approach is justified by
literature reporting a correlation between carotid artery diameter and AAA risk.
Furthermore, the previously described association between cardiovascular risk
factors, atherosclerosis and CCAD suggests that it may be considered as a proxy
measure of vascular remodeling, which has been hypothesized to play a role in the
development of AAA. In the discovery analysis a significant association between
variants at 1q24.3 and larger CCAD in the IMPROVE study was found. Although
the association with CCAD did not replicate in the follow-up studies, a potential
reason to explain this is heterogeneity in the study populations. Specifically, the
participants in the IMPROVE study were selected highly recruited on the basis of
high cardiovascular risk (all the participants have at least 3 cardiovascular risk
factors (251), but free from prevalent disease. This is in contrast to both the WHII
study and NBS that recruited healthy population cohorts, or the SMART study that
enrolled subjects who had already experienced a cardiovascular event.
Compensatory arterial enlargement occurs early in the evolution of atherosclerotic
disease before visible plaque is apparent, and the stimulus is thought to be

increasing intima media thickness (243). The analyses stratified by CIMT as a

marker of atherosclerosis, demonstrate that the effect of this SNP on CCAD

increases in an incremental fashion through increasing tertiles of intima-media

thickness in both the discovery and follow-up cohorts. One potential interpretation

of these data is that this SNP is associated with expansive carotid artery remodeling

rather than the diameter *per se* (i.e. an anthropometric trait).

The suggestive association with AAA provides putative genetic evidence to

support the hypothesis that pathways involved in systemic vascular remodeling

may also play a role on the development of AAA. These data also demonstrate that

in genetic studies of AAA, approaches focusing on traits such as carotid diameter

may compliment more traditional case-control study designs. Although the

association between rs4916251 and AAA was only nominally significant (P <0.05)

in one of the cohorts tested (the AC), it is important to note that the AC was the

largest study with the greatest statistical power, and that the meta-analysis estimate

showed little evidence of heterogeneity (I^2 = 13%).

Variants at 1q24.3 have previously been found to be associated with adiposity,

height and platelet size (131, 252, 253), but no association between the lead SNPs

for CCAD and either height or waist-hip ratio was found, and the LD between

these variants and rs4916251 was low (Table 31). Chromosomal deletions at 1q24-

25 result in a phenotype characterised by short stature and skeletal abnormalities

(254) which suggests that this locus plays a role in extra-cellular matrix

remodeling, but the precise underlying mechanism remains unclear. The eQTL

results demonstrate that the variants identified in this study are associated with

altered expression levels of *PIGC* in both aortic tissue. *PIGC* encodes

phosphatidylinositol N-acetylglucosaminyltransferase subunit C, which forms part

of the glycosylphosphatidylinositol (GPI) lipid anchor, a post translational

modification that allows anchorage of proteins to the plasma membrane.

Understanding how *PIGC* is involved in vascular diameter and/or remodeling will

require further experimental analysis, and presently there are few data to provide

biological insights into the observed association.

6.4.1   Limitations

The limitations of our study should be considered. The 200,000 SNP chip has in-

depth coverage of a large number of genes but it does not provide genome-wide

coverage and it is likely that there are other variants associated with this trait that

are not examined. It would also be relevant to have expression data for genes at the

1q24.3 locus in tissue from early and late stage aneurysms, but this is not currently

available. Perhaps the largest limitation is between-study heterogeneity with regard

to phenotype measurement. Although the measurement of carotid variables was

well standardised within studies, this may not be the case between studies. In

particular the inter-tertile analysis of CIMT may be hampered by heterogeneity in

the values of CIMT in each group. This highlights the difficulties in population-

based genetic studies of vascular remodelling traits in which the phenotypic

differences are often subtle. Ideally, there would have been full phenotypic

information from all cohorts, including measures of both CCAD and aorta in the same individuals, but again this was not available in all studies. There was evidence the association with was primarily driven by the AC cohort   (sensitivity analysis in supplementary data) but this was the largest study with the greatest power to detect a modest effect. Although the association between rs4916251 and AAA was only nominally significant (P <0.05) in one of the cohorts tested (the AC), it is important to note that the AC was the largest study with the greatest statistical power, and that the meta-analysis estimate showed little evidence of heterogeneity   ($I^2 = 13\%$). For the AAA association, there was limited phenotype data available, so multivariate regression models including other covariates such as blood pressure and lipid parameters was not possible. Finally, for the expression studies were from thoracic aortic tissue and not the abdominal aorta, which may have a different pattern of expression.

6.4.2   Conclusion

This study has identified variants at 1q24.3 that show association with carotid artery remodeling and the risk of developing an AAA. This may be due to an allele-specific effect of expression of *PIGC* in the vasculature. These results provide suggestive genetic evidence that pathways involved in the systemic vascular remodeling in response to atherosclerosis may play a role in AAA risk.

7 Discussion & Future Work

In this thesis, I have attempted to use genetic epidemiology studies to further our understanding of the pathogenesis of AAA. Broadly this has involved Mendelian randomization analyses and quantitative trait genomics, two strategies that have not been previously utilized in AAA. There are opportunities for some of these studies to act as a template for development of translational studies, particularly that in Chapters 4 & 5. In this chapter I will highlight these opportunities and discuss potential further work in the genetics of AAA.

7.1 Inflammation and AAA; a therapeutic opportunity?

In Chapter 4 of this thesis I examined the role of a common, functional polymorphism in *IL6R* in the pathogenesis of AAA. These data, combined with meta-analysis of biomarker data from the published literature, provided robust evidence that the IL-6 mediated pathways play a causal role on the development of AAA. More broadly, this study also provides evidence that inflammation plays a causal role on the development of AAA and is therefore a bona-fide therapeutic target in humans. Assuming that a potential treatment for AAA results in attenuation of growth, rather than full aneurysm regression, the features of an ideal treatment are listed in Figure 47.

7.2 Tocilizumab – from bench to bedside

Following the observation that IL-6 levels were associated with various forms of inflammatory arthritis, a monoclonal antibody to the mouse IL-6R was developed that appeared to reduce the severity of arthritis in small animal models of the disease (255). This led to engineering of tocilizumab, which underwent phase I & II trials in patients with RA, demonstrating encouraging results(256). There have now been a number of randomized comparative studies that have demonstrated the efficacy and safety of tocilizumab in RA over that of placebo +/- other disease modifying agents (257). A role for tocilizumab in other inflammatory diseases such as systemic lupus erythematosus, polyarteritis nodosa and Crohn's disease has been postulated (258), but there have been no large-scale randomized trials in these conditions to date.

The fact that Tocilizumab, a monoclonal antibody to the IL-6R used in the treatment of rheumatoid arthritis (259), has a broadly concordant effect on a range of biomarkers as the Asp-358 genetic variant (184)(Figure 47), suggests that it could have a therapeutic role to play in both CHD and AAA.
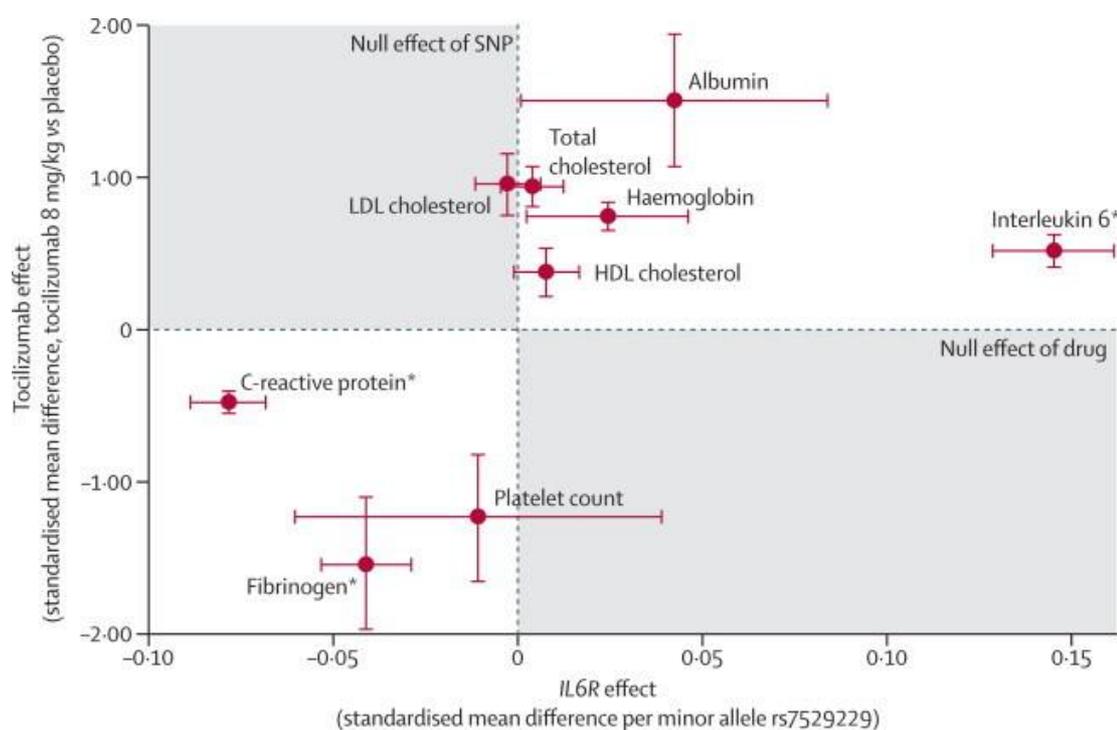
Figure 47 - Concordant effect of the Asp-358 variant and tocilizumab on a range of biomarkers (184)

The definitive way to evaluate this would be a randomized controlled trial of tocilizumab in patients with small AAA, but there are a number of challenges to this approach. Assuming growth rates similar to that seen in the UK small aneurysm trial (8), such a trial would require recruitment of approximately 400 AAA to have 80% power to detect a 25% difference in AAA growth rates over approximately 5 years follow-up. Indeed, this is a common barrier to trialing of new therapies in cardiovascular disease, and it has been suggested that imaging markers of disease may be useful adjunct in early phase studies (260).

It may be that before such a trial became a reality, a Phase II study, examining the safety & practicality of tocilizumab *exclusively* in AAA patients is necessary. Such a trial would not provide definitive evidence of efficacy, over and above current

best management protocols, but could examine specific endpoints such as compliance, safety, acceptability and proxy AAA end-points such as inflammation in the aneurysm wall plasma markers of inflammation (Figure 48). For example, the dal-VESSEL and dal-PLAQUE studies employed this study design, whereby the safety and efficacy of CETP-inhibitors was investigated in cohorts of patients at high risk of cardiovascular disease. Rather than focus on the incidence of events (as would be required in a Phase III study), the authors investigated safety endpoints as well as a range of surrogate markers of cardiovascular disease (Table 32).

Table 32 – Surrogate markers studies in Phase II studies of Dalcetrapib in coronary heart disease.

Classification	Outcome
Biochemical	HDL-C
	LDL-C
	Triglycerides
	Total Colesterol
Inflammation	hs-CRP
	IL-6
	PAI-1
	ICAM-1, Vcam-1
Other	Ambulatory Blood Pressure
Imaging Surrogates	Carotid MRI
	CT-Pet Scanning
	Flow Mediated Dilatation

Of particular interest in a study of AAA would be imaging marker of inflammation in the aneurysm, as this is the specific pathway that is targeted with Tocilizumab. It has been shown that progression and rupture of AAA is associated with histological evidence of inflammation in the wall. Higher 18F-fluorodeoxyglucose (18F-FDG) uptake, measure by computed tomography positron emission tomography (CT-PET), has been associated with higher macrophage infiltration and histological evidence of inflammation in clinical studies of AAA, using both

human and small animal models (261-263)(Figure 48). Importantly, it has also been shown that interobserver variability in measurement of 18F-FDG uptake is low (264), a fact that supports its use in clinical trials. It may, therefore, be a useful surrogate to study in a Phase II trial Tocilizumab in AAA.
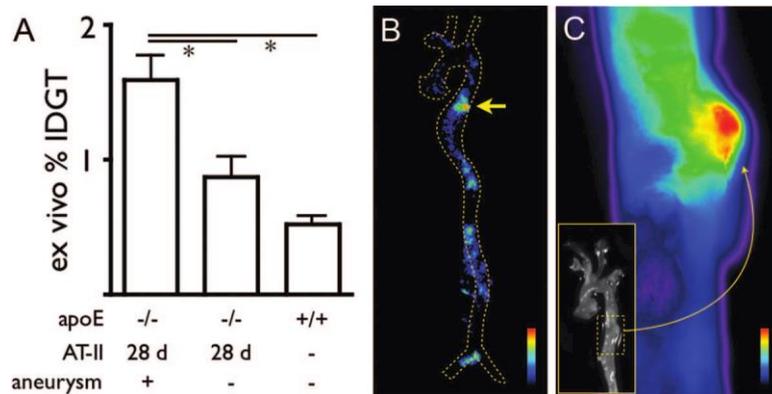
Figure 48 – 18f-FDG uptake in a developing AAA, in the AngII ApoE -/- model of AAA, from(262)

An alternative/adjunct proxy measure would be to measure ultrasmall superparamagnetic particles of iron oxide (USPIO) by magnetic resonance imaging (MRI), which has also been found to correlate with growth rates and inflammation in AAA (265, 266)

- Effectively reduces the growth rate of small AAA, therefore reducing the need for invasive surgery
- Minimal adverse effects & excellent safety profile
- Acceptable to the patient
- Oral formulation, once daily
- Cost effective
- Minimal requirement for monitoring
- Few interactions with other medications (particularly cardiovascular drugs)
- Beneficial to the cardiovascular system, over and above the AAA

Figure 49 – Features of the ideal treatment for AAA
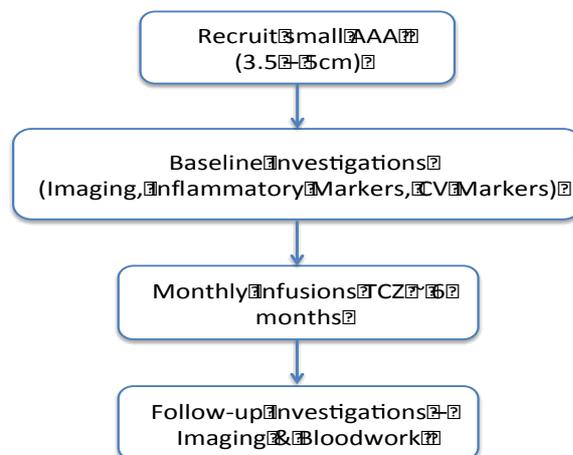
Figure 50 – Basic design of a non-randomised, phase II clinical trial of tocilizumab in AAA. Baseline investigations would include USS +/- other imaging of the AAA, inflammatory markers such as CRP & IL-6, & cardiovascular risk factors such as lipid parameters.

If tocilizumab is evaluated against the criteria in Figure 49, then it is clear that there are a number of challenges over and above establishing efficacy for attenuation of AAA growth. It is given as an intravenous infusion monthly that requires patients to attend a hospital/clinic, and expertise to provide the infusion. This is likely to impact negatively upon cost effectiveness and patient compliance. Furthermore, there are a number of important side effects that warrant consideration. For example, respiratory tract infections occur relatively commonly, which is a concern amongst AAA patients, many of whom have a history of smoking and lung disease. Lastly, there are data that tocilizumab leads to increased

circulating LDL-cholesterol (267), though this is specifically in patients with RA, and whether this is an on or off target effect remains to be clarified.

In summary, although there are considerable challenges to be overcome, there is strong evidence that attenuation of IL-6 signaling in AAA will be of benefit and that trials evaluating this are perhaps the only way that we can answer this question.

7.3   HDL and AAA; implications for therapeutics

The data described in Chapter 5 of this thesis provide evidence that HDL-C mediated pathways play a causal role in the development of AAA. Furthermore, a genetic variant in the promoter of *CETP* that has a range of effects similar to pharmacological CETP inhibition was associated with a lower risk of AAA, suggesting that this may be a beneficial strategy in AAA. It remains to be seen whether or not HDL and dyslipidaemia play a role in AAA growth rates, and a recent meta-analysis did not identify an association between any lipid fraction and AAA growth rates (7). Therefore, it cannot be assumed that CETP inhibition will necessarily be of benefit in attenuating the growth of larger AAA. The data do, however, suggest that CETP inhibition could play a role in prevention of AAA development at an earlier stage. This is potentially important information, from a translational viewpoint, as the need for a future AAA repair can be very accurately predicted with a single baseline USS (37) therefore it may be possible to identify a sub-group of men in the screened population that could benefit from HDL-C raising/CETP inhibition. Although the results of comparative studies have so far

failed to identify a role for CETP inhibition in coronary heart disease, this has not been evaluated in AAA yet. Furthermore, there is now strong evidence that CETP inhibition, with either dalcetrapib or anacetrapib (215, 216, 268), in patients at risk of cardiovascular disease is safe, which opens the door to a potential comparative trial in AAA. It is also important to note that CETP inhibition also satisfies many of the qualities of an "ideal" AAA treatment listed in Figure 50.

7.4 Future Directions in genetics of AAA

7.5 The genetics of AAA expansion/rupture

One of the major questions yet to be answered in the field of AAA is whether or not there are separate pathways involved in aneurysm initiation, progression and rupture. If there are, then it is possible/likely that there is a genetic component to the pathways involved in AAA growth and rupture, and understanding these is of paramount importance. Before this can become a reality, there is an urgent need for researchers in the area to collaborate and develop a large cohort of AAA patients, with standardized methods and time intervals to measure AAA growth (perhaps through the quality controlled national aneurysm screening program in the UK) and dense phenotyping of other important covariates such as age, gender and smoking status. Furthermore, it is important to understand which is the most important metric to study, as it is clear that AAA growth is not linear in many cases, so time to event analysis may be more appropriate than studying individual growth rates, which show a great deal of inter-individual heterogeneity (Figure 51).
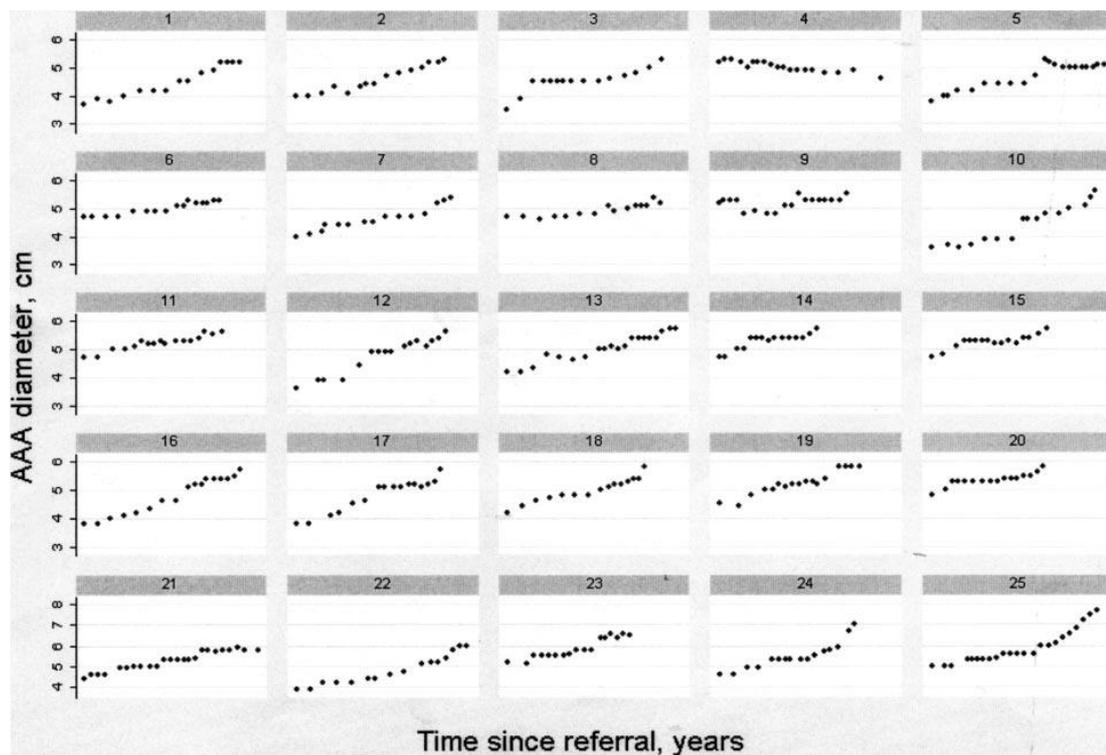
Figure 51 – Individual growth rates of 25 patients with small AAA in the UK Small Aneurysm Trial(8)

7.6 Next Generation Genomic Studies

The field of complex disease genetics has moved rapidly from genome-wide association studies that focus on common genetic variants (MAF>5%) with modest effects on disease risk, to the identification of variants that are less prevalent in the population that may have a greater effect on disease risk (Figure 52). To discover these variants, a number of strategies are being employed by researchers such as "exome chips" that contain both common and rare exonic variants throughout the human genome, or next generation sequencing (either whole exome or whole genome) that is becoming less prohibitively expensive. Although the theory of this approach is attractive, as comprehensive coverage of the genome can be achieved,

there are a number of challenges over and above the cost of such approaches, which need to be overcome before these strategies are to provide definitive answers. Firstly, for comparative purposes, sample sizes may need to be even bigger than those currently employed for GWAS, because by definition the variants are rare in the population. Second, consideration must be given to which statistical tests are to be used and whether variants are compared by gene, by function or by locus(269). Finally, being able to accurately predict the likely pathogenicity of the rare variants, and classify variants based upon this prediction, is likely to be very important as early studies have suggested that there are an unexpectedly high number of non-synonymous and loss of function mutations being identified(270).
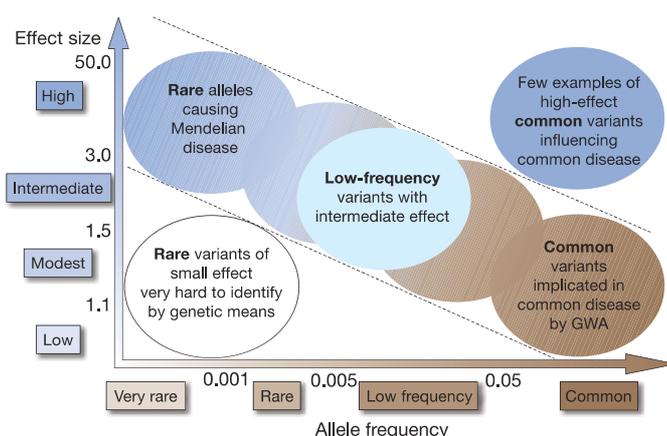
Figure 52 – Theory of differing effect sizes by minor allele frequency (MAF), from(81)

7.7 Genetic Risk Prediction & AAA

Large prospective randomised controlled trials of population-based screening for AAA, in men aged 65-74, have demonstrated that screening with an abdominal

ultrasound scan (USS) reduces aneurysm related mortality (11). As a result, USS

screening programs are currently being introduced in the UK (153). One of the

major hopes for genome wide research is the development of tests that can

accurately predict the onset of common diseases, allowing individualised

preventative measures. Direct to consumer genetic testing for the 9p21 SNP for

AAA is already available though private companies, despite the fact that any single

SNP of modest effect will have very poor predictive indices (158). For example,

this SNP will have a positive predictive value of just 6% for AAA (i.e in a

population of men aged 65-74, only 6% of those who test positive for the SNP will

go on to develop AAA). Combining common SNPs of modest effects into a "gene

score" (271) is one potential method to improve the predictive accuracy of genetic

tests but the results to date have been disappointing in terms of predictive ability

measured by the c-statistic (reviewed(167)). This is a reflection of the fact that

most events tend to occur in people with intermediate risk, and there is little reason

to believe that the performance of SNPs for AAA will perform any better than

those for other common diseases (272, 273). USS for AAA is an excellent

screening tool in terms of diagnostic accuracy, acceptability and cost-effectiveness

and it is highly unlikely that a panel of low-penetrance SNPs will ever match the

performance of this gold standard. However, it might be possible to "enrich" the

pool of subjects being screened using genetic tests, in an attempt to improve cost-

effectiveness, but experience with "high-risk" screening strategies (such as

selective USS screening in current smokers) does not appear to improve upon

population- based methods with regard to aneurysm related mortality (274). An

alternative strategy, suggested by Pharoah et al for breast cancer (275), would be to tailor the age of screening dependent on genetic risk, which may be applicable to AAA given that a single USS (rather than repeated screening as in other diseases) is the preferred strategy. For example, since almost a fifth of AAA rupture surgery occurs in men under the age of 65(276)(the age for a single screening test in the UK), it is conceivable that genetic tests could identify a sub-group of the population for early and/or intensive USS screening.

If, however, a paradigm shift were to occur whereby *prevention* of AAA, rather than diagnosis and prevention of rupture became the focus, it may be possible to use genetic tests to stratify the population into risk groups and offer targeted preventative measures to these groups. Presently, this would involve generic cardiovascular risk factor modification   (statins, smoking cessation, BP control) and it seems unlikely that a "high risk" strategy for AAA would be any more useful than population-based programs for targeting cardiovascular risk factors, such as the cardiovascular health check being introduced in the UK(277). If, however, a specific preventative treatment for AAA (CETP inhibition for example) were to be developed then using a genetic test may prove useful in determining who would receive this treatment, with the caveat that a genetic risk profile based upon common low-penetrance is likely to less predictive than a family history of AAA.

7.8 Pharmacogenetics

Pharmacogenetics refers to the use of genotype information to predict individual response to pharmacotherapy, from which personalised therapeutic plans could be

developed. Early research in this area has, however, had limited success and a recent systematic review revealed methodological issues in the field with a preponderance of small studies, lack of focus on any gene/drug combination and potential publication/reporting bias (278). Novel therapeutic strategies are under investigation for the stabilisation of small AAA to prevent expansion. One example is angiotensin converting enzyme inhibitors (ACE-inhibitors), however studies show conflicting associations with both increased and decreased rates of AAA expansion and rupture rates (279, 280). However, it is possible that an adequately-powered RCT of ACE-inhibitors in AAA could detect subgroups of patients in whom the drug has stronger effects based upon genotype profiles. Since the *intended* effect is still expected to be directionally consistent in individuals irrespective of their genotype, it remains likely that treating all individuals (irrespective of genotype) will have the greatest impact(281).

Another emerging field that could have relevance to patients with AAA is that of "peri-operative genomics" whereby genotype based information is used to predict surgical outcomes. For example, the 9p21 SNP is associated with more severe myocardial injury following coronary artery bypass surgery (CABG), independent of disease severity, while inclusion of the 9p21 genotype in the EuroSCORE model improved overall prediction of mortality within 5 years of CABG(282, 283). In AAA there has been considerable research into predicting outcome following surgical repair and it is possible that genotype may provide another tool in this

regard, whereby pre-operative interventions and decisions as to which operative intervention is most appropriate may be guided in part by genotype.

7.9 Further Genomic Research in the determinants AAA – study design to refine and augment signals

Meta-analysis of available datasets is likely to identify more variants that have not currently met the stringent levels of statistical significance in the initial GWAS, albeit with small effect sizes. In addition to larger GWAS, refinement of study design may also uncover novel genetic insights. For example, most genetic studies of AAA have used case-control designs with a phenotypic definition of an infra-renal aortic diameter of 3cm for AAA. Within the population, infra-renal aortic diameter is a continuously distributed phenotype (skewed to the right)(13), with AAA rupture (the clinical end-point of interest) in aortas less than 4cm almost unheard of. As illustrated in Figure 53, using a 3cm cut-off may be considered arbitrary, with many individuals in the "near-case" group. Alternative analysis strategies could include using extreme phenotypic selection, (Fig 53) i.e. selecting out "supercases" (AAA>4.5cm) and "supercontrols" (<2.5cm). This may be particularly useful in sequencing studies looking for rare variants, as has been used for triglyceride levels (284).

i) Conventional case-control analysis

3cm

Controls   Cases

ii) Extreme Selection

2cm   4.5cm

"Super" controls   "Super" cases

iii) Population based study of trait across the range of variation

Quantitative trait
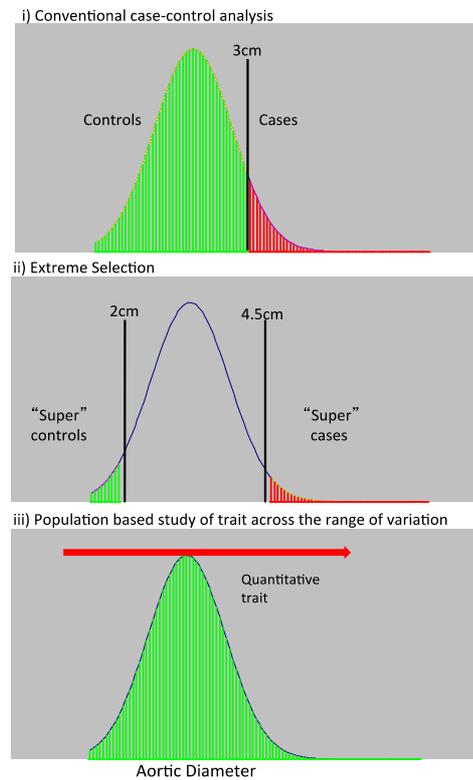
Aortic Diameter

Figure 53 – Study design, focusing on either extreme phenotypes, or aortic diameter as a quantitative trait, to augment classical case-control studies of AAA (122). Adapted from (130)

7.10 Conclusion

Although the field is still at a relatively early stage in its development, it is clear that studying the genetics of AAA has the potential to considerably improve our understanding of the disease, which could ultimately translate into improvements in patient care. Ongoing collaborative research efforts and integration of multiple disciplines, including epidemiology, genetics, statistics, basic science and clinicians is likely to continue to be the key factor that drives new discoveries. This

thesis has provided important data that can act as a template for future translational

studies.

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