An investigation into genetic polymorphisms and abdominal aortic aneurysms

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Abstract

Introduction

A wide variety of genetic polymorphisms have demonstrated a significant association to the disease abdominal aortic aneurysm (AAA). Polymorphisms in rs217120 (Cathepsin C gene *CTSC*) and rs1466535 (low density lipoprotein receptor related protein 1 gene, *LRP1*) have both been associated with AAA, rs1466535 from a genome wide association study (GWAS).

Materials & Methods

Associations were first confirmed then exons in close proximity to the polymorphisms were re-sequenced in order to detect new mutations. Protein levels were assayed and tissue was stained in order to determine the arterial layer that the LRP1 protein resides within. TaqMan genotyping was undertaken in order to determine allele frequency of the SNPs of interest in cohorts from Belfast (211 AAA, 262 controls), Viborg (473 AAA, 195 controls), Leeds (214 AAA and 249 controls) and Leicester (266 AAA, 143 controls). Subsequently a list of linked SNPs was generated and the same samples were analysed in order to fine map the gene(s) of interest.

Samples from Leicester (96 AAA, 96 controls) were sequenced. Serum protein concentrations were measured with an ELISA (43 AAA, 26 controls). Immunohistochemical analysis of aortic biopsies (n = 6) was also performed.

Results

rs217120 failed to associate with AAA in 3 of 4 the replication cohorts and meta-analysis of all data was also not significant, odds ratio 1.17 (95% Confidence Interval 0.98-1.39), P value = 0.08. rs1466535 did not significantly associate with AAA in the replication cohorts however when the data was meta-analysed with the discovery phase of the GWAS (1866 AAA, 5435 controls) the association was significant, odds ratio 1.23 (95% Confidence Interval 1.14-1.33), P value $1.25x^{10-7}$. A second SNP within LRP1, rs11172114, was found to have a more significant association, odds ratio 1.23 (95% Confidence Interval 1.14 to 1.31), P value $3.87x^{10-8}$. Re-sequencing of the 3 LRP1 exons did not detect any novel mutations. Serum LRP1 levels between cases and controls were not different (mean concentration 583.4 v 631.3ng/ml, P value = 0.69). There was also no significant difference when samples were analysed by SNP genotype, rs1466535 P value = 0.07. Analysis of aortic tissue biopsies did not determine an association between LRP1 genotype and cellular location.

Conclusion

SNPs within LRP1 associate with AAA, with rs11172114 being most significant. However the polymorphism is not associated with protein levels in serum and is not clearly localizing to any given layer of the arterial wall. More investigation is required to determine how LRP1 affects aneurysm development and growth.

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning"

Albert Einstein.

Statement of Originality

The work on which this thesis is based is my own independent work except where acknowledged.

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Contents

Abbreviations

A	Adenine
AAA	Abdominal aortic aneurysm
ACE	Angiotensin Converting Enzyme
ADAM	Aneurysm Diagnosis and Management
agLDL	Aggregated Low Density Lipoprotein
AGT	Angiotensinogen
AGTR1	Angiotensinogen II Receptor Type 1
AHCY	Adenosylhomocysteinase
ALI	Acute Limb Ischaemia
ARB	Angiotensin Receptor Blocker
BDKRB2	Bradykinin Receptor 2
C	Cytosine
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CASS	Collaborative Aneurysm Screening Study
CCR5	Chemokine {C-C motif} receptor 5
CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2
CHD	Coronary Heart Disease
CNTN3	Contactin 3
COL3A1	Type 3 Collagen
COPD	Chronic obstructive pulmonary disease
CRP	C reactive protein
CST3	Cystatin 3
CT	Computerised tomography
CTSC	Cathepsin C
CTSH	Cathepsin H
DAB2IP	Disabled homolog 2-interacting protein
ddNTP	Dideoxynucleosidetriphosphates
DM	Diabetes Mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleictriphosphates
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme linked immunsorbant assay
ELN	Elastin
ENCODE	Encyclopedia of DNA elements
EUROSTAR	European Collaborators on Stent/graft techniques for aortic aneurysm repair)
EVAR	Endovascular aneurysm repair
FAM	Fluorescein Amidite
G	Guanine
GWAS	Genome Wide Association Study

H&E	Haematoxylin and Eosin
HCY	Homocystein
HLA	Human Leukocyte Antigen
HMG co-A	Hydroxymethylglutaryl-coenzyme A
HRP	Horseradish Peroxidase
HWE	Hardy-Weinberg Equilibrium
ICD-10	International Classification of Diseases 10^{th} edition
ICH GCP	International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use Good Clinical Practice
IHC	Immunohistochemistry
Il-1	Interleukin 1
Il-1A	Interleukin 1A
Il-1B	Interleukin 1B
Il-2	Interleukin 2
Il-6	Interleukin 6
Il-10	Interleukin 10
IL-1RA	Interleukin 1 receptor antagonist
kDa	Kilo Dalton
LDL	Low Density Lipoprotein
LDLR	Low-density lipoprotein receptor
LRP1	Lipoprotein Receptor Related Protein 1
LRP1B	Lipoprotein Receptor Related Protein 1B
LRP5	Lipoprotein Receptor Related Protein 5
LRP6	Lipoprotein Receptor Related Protein 6
MASS	Multicentre Aneurysm Screening Study
MMP1	Matrix Metalloproteinase 1
MMP2	Matrix Metalloproteinase 2
MMP3	Matrix Metalloproteinase 3
MMP9	Matrix Metalloproteinase 9
MMP10	Matrix Metalloproteinase 10
MMP11	Matrix Metalloproteinase 11
MMP12	Matrix Metalloproteinase 12
MMP13	Matrix Metalloproteinase 13
mRNA	Messenger RNA
MTHFD1	Methylene-tetra hydrofolate dehydrogenase 1
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methyltetrahydrofolatehomocysteine methyltransferase
MTRR	Methionine Synthase Reductase
NAAASP	National AAA screening programme
NCBI	National Centre for Biotechnology Information
NICE	National Institute for Clinical Excellence
NVD	National Vascular Database
PAI	Plasminogen Activator Inhibitor
PAP	Plasmin-Antiplasmin complexes
PCR	Polymerase Chain Reaction
PDGFβ	Platelet Derived Growth Factor beta
PSRC1	Proline/serine-rich coiled-coil protein 1

RAAA	Ruptured AAA
RCT	Randomised Control Trial
REC	Regional Ethics Committee
RNA	Ribonucleic acid
RT	Room temperature
SNPs	Single Nucleotide Polymorphisms
SORT1	Sortilin 1
SREBP	Sterol regulatory binding protein
T	Thymine
TAA	Thoracic Aortic Aneurysm
TGF	Tissue Growth Factor
TGFBR2	TGF beta receptor 2
TIMP1	Tissue Inhibitors of Matrix Metalloproteinase 1
TIMP2	Tissue Inhibitors of Matrix Metalloproteinase 2
TIMP3	Tissue Inhibitors of Matrix Metalloproteinase 3
TIMP4	Tissue Inhibitors of Matrix Metalloproteinase 4
TKI	Tyrosine kinase inhibitors
TNF	Tumour Necrosis Factor
tPA	tissue Plasminogen Activator
TSP1	Thrombospondin 1
TSP1	Thrombospondin 1
TYMS	Thymidylate synthetase
UCSC	University of California, Santa Cruz
UKSAT	UK Small aneurysm Trial
uPA	urokinase Plasminogen Activator
USS	Ultrasound scan
VLDLR	Very low density lipoprotein receptor
VSMC	Vascular Smooth Muscle Cell

Chapter 1: The Aorta and the Aneurysm

1.1 Aorta

The aorta is the largest artery of the body; it originates directly from the aortic valve of the left ventricle of the heart. Extending cranially the aorta then arches over to the left and descends caudally, becoming the descending thoracic aorta. This then ultimately becomes the abdominal aorta upon transition through the aortic hiatus of the diaphragm, at the level of the twelfth thoracic vertebral body (Figure 1).

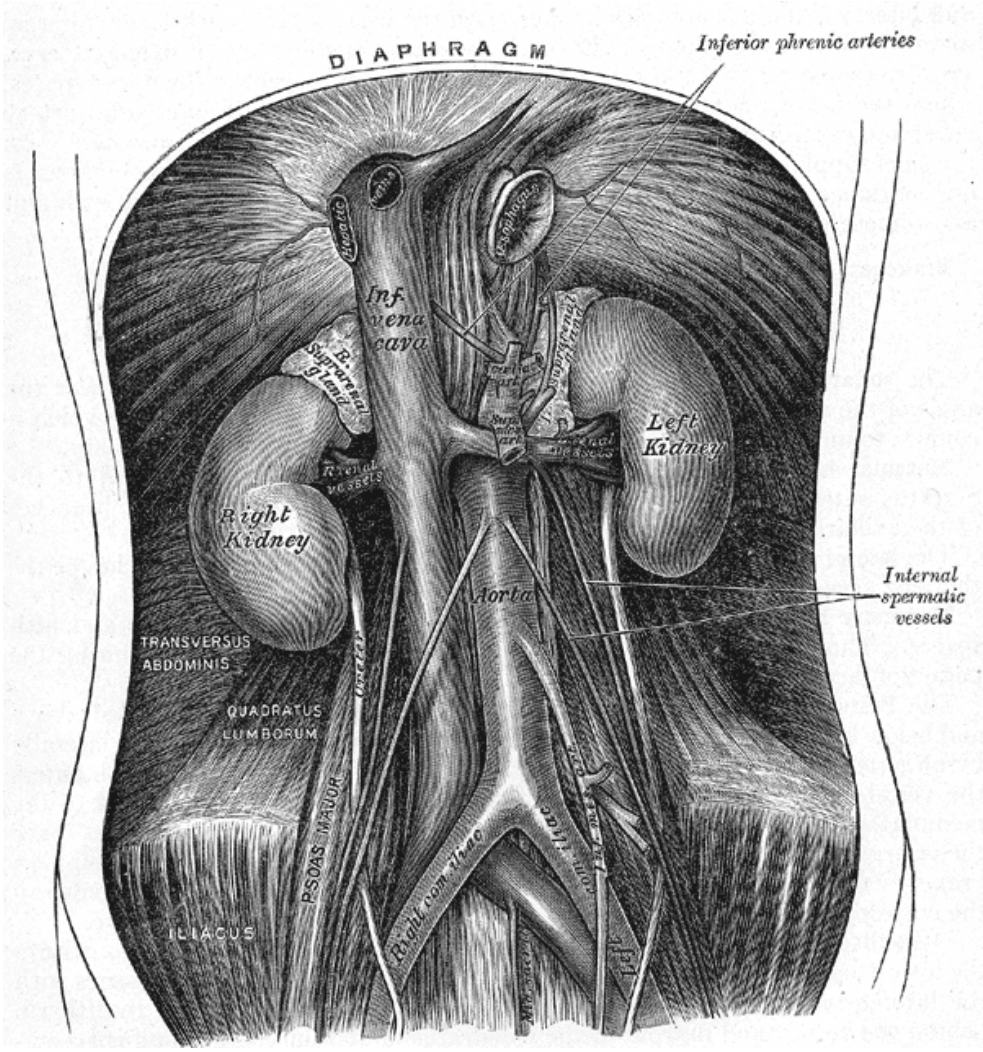

Figure 1.1: Abdominal Anatomy, taken from Gray H. Anatomy of the Human Body. Philadelphia: Lea & Febiger, 1918; Bartleby. Com, 2000. 20th ed.

1.2.1 Aneurysm

An aneurysm is defined as an abnormal dilatation of an artery. The word aneurysm is from the Greek ἀνεύρυσμα (*aneurysma*,"a widening, an opening"), from ἀνά (*ana*,"up") & εὐρύς (*eurys*,"wide").

1.2.2 Historical Perspective

It is clear that mankind has been suffering from aneurysmal disease for several centuries. The first recorded description of an aneurysm is attributed to the second century AD Roman physician Galen in the works of the 7th century Byzantine physician Paulus Aegineta. A translation by Dr Francis Adams of 1846 reads; "an artery having become anastomosed (i.e. dilated) the affection is called an aneurism; it arises also from a wound of the same, where the skin that lies over is cicatrized, but the wound in the artery remains, and neither unites nor is blocked up by flesh. Such affections are recognized by the pulsation of the arteries, but if compressed the tumour disappears in so far, the substance which forms it returning back to the arteries." [Stehbens 1958].

There is a belief that the ancient Egyptians had experience with aneurysms, with Ebbell listing aneurysm and arteriovenous aneurysm amongst the diseases described in the Ebers Papyrus, however other Egyptian scholars do not agree [Stehbens 1958]. Despite this long experience, there has only be reliable treatment for the condition in the last half century.

1.2.3 Classification of aneurysms

There are a variety of methods utilised to classify aneurysms using features such as location and aetiology to group the condition.

1.2.3.1 Location

Aneurysms can be classified by their site. Anatomical locations where aneurysms have been located include the cerebral arteries, coronary arteries, the thoracic and abdominal aorta, the iliac vessels, the femoral artery and the popliteal artery, though an aneurysm could be located in any blood vessel.

1.2.3.2 True and False aneurysms

A true aneurysm involves all three layers of the arterial wall, intima, media and adventitia. A false or pseudo aneurysm does not involve all the layers of the artery (Figure 1.2).

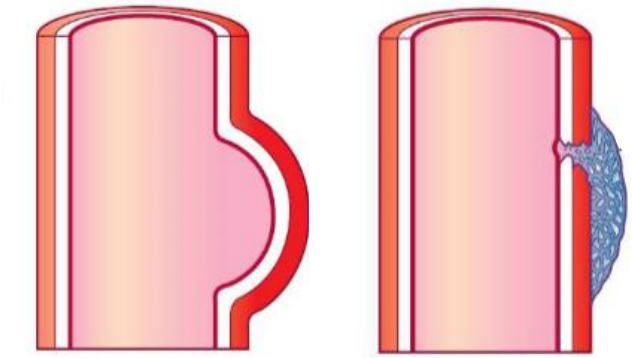

Figure 1.2; Aneurysm Morphology. Schematic of a true aneurysm (left) and a false aneurysm (right).

1.2.3.3 Aneurysm morphology

The macroscopic appearance of a true aneurysm is also used for classification. Fusiform aneurysms are variable in length, symmetrical and are spindle shaped, tapering from the normal vessel diameter to a maximum dilatation. This is as opposed to the localised spherical bulging of the saccular type of aneurysm (Figure 1.3) [Macfarlane, Reid and Callander 2000].

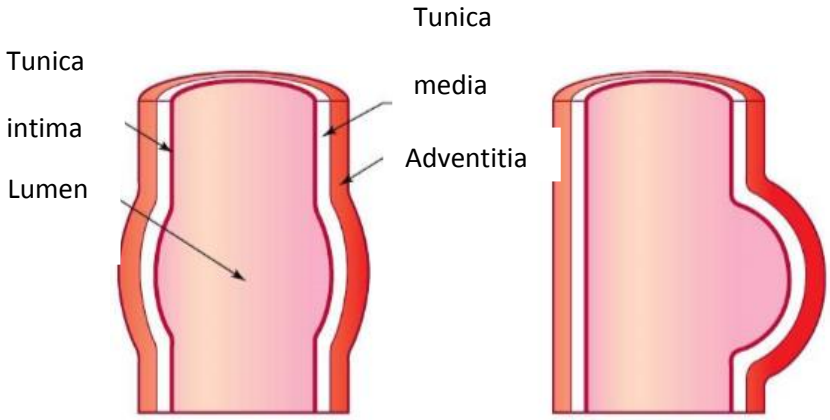

Figure 1.3; Aneurysm Morphology. Schematic of a fusiform aortic aneurysm (right) and a saccular aortic aneurysm (left).

1.2.3.4 Cause

The underlying pathology can be used as a classification, e.g. mycotic aneurysms are due to infection. Where *Treponemapallidum* infection is the cause, the aneurysm is termed a syphilitic aneurysm. Traumatic aneurysms are the result of vessel injury. Congenital aneurysms can be present at birth or due to arterial wall defects that predate the formation of an aneurysm.

1.2.4 Abdominal Aortic aneurysm

Abdominal Aortic Aneurysms (AAA) are abnormal dilatations of the aorta within the abdomen and are commonly located infrarenally, that is the dilatation does not begin until after the origin of the left and right renal arteries from the aorta [Fortner and Johansen 1984]. The dilatation may terminate prior to the aortic bifurcation or it may involve the iliac arteries, known as an aorto-iliac aneurysm.

1.2.4.1 AAA Definition

It is generally accepted that an abdominal aortic diameter of greater than 3 cm is aneurysmal. This 3 cm threshold was accepted by a nationwide randomised control trial (RCT) that investigated the benefit, in terms of mortality, of aneurysm screening programmes [Ashton *et al.* 2002]. However there is evidence that an aortic diameter of greater than 2.5cm, a so called ectasia or an ectatic aorta, is also abnormal. Patients presenting to an aneurysm screening programme at age 65

with an aortic diameter of less than 2.5cm were significantly less likely to have an AAA repair than men with a 2.5cm to 3.0cm aorta within the following 23 years (10.3% vs 15.7%, p = 0.001) [Hafez, Druce and Ashton 2008]. There is on-going debate regarding the status of these men within screening programs, with recent evidence suggesting that over 50% of men presenting to screening with an aortic ectasia progressing to an AAA (with a diameter of 3.0cm or greater) within 5 years [Wild *et al.* 2013]. Clearly the risk of discharging a man with an aortic diameter of between 2.5 and 3.0 cm is that an aneurysm will develop, undergo rapid growth and become at risk of rupture. There is controversy regarding the method and modality of measurement of AAA. An aneurysm can measure from the outer edge to the opposing outer edge (OTO), from inner edge to opposing inner edge (ITI) and also from the anterior leading edge (exterior wall) to the posterior leading edge (internal wall) (LTL). Different screening programmes utilize different methods, the UK NAAASP utilizes ITI whereas the Swedish Screening programme is using LTL. All methods demonstrate inter observer variation, however ITI has been shown to have greater reproducibility [Hartshorne *et al.* 2011]. In comparison of USS to CT measurement of AAA, USS consistently underestimates AAA size, with ITI underestimating to a greater degree than OTO and LTL [Chiu *et al.* 2014].

1.2.5 Diagnosis of AAA

Diagnosis of an aneurysm is usually via ultrasound though it is possible to detect them clinically or as incidental findings in other imaging techniques such as computer tomography (CT). Clinical examination is of limited value in diagnosis

with patient factors, such as obesity, affecting accuracy. The size of aneurysms detected by clinical examination are large, with an average diameter of 6.5cm, and in one observational study 30% of aneurysms were missed [Karkos *et al.* 2000]. The sensitivity of clinical examination is a modest 68% with a specificity of 75%, though sensitivity improves with larger aortic diameter and smaller patient abdominal circumferences [Fink *et al.* 2000]. The incidental discovery of an aneurysm is a well recognised mode of diagnosis, in a recent analysis the rate of incidental AAA diagnosis was 1%, 812 incidental AAAs found in 79,121 scans (USS, CT and MRI) over a 12 year period [van Walraven *et al.* 2010].

1.2.5.1 AAA Screening

Aneurysm screening has recently been implemented in England and utilises an abdominal USS to visualise the aorta and determine its diameter. The Multicentre Aneurysm Screening Study (MASS) trial was a UK based RCT that examined the effect of AAA screening on mortality [Ashton *et al.* 2002]. This trial of 67,800 males aged 65 to 74 randomised into 2 groups of patients, 1 receiving an invitation for a screening ultrasound scan, 1 not. If an AAA (aortic diameter >3.0cm) was detected the patient would enter routine surveillance with the time interval decreasing with increasing diameter until the aorta reached 5.5cm, and the patient was referred for surgery [Ashton *et al.* 2002]. The use of ultrasound as the mode of screening was supported by data from Lindholt *et al.* who successfully demonstrated that inter-observer variability was low and that this test was both highly specific and sensitive [Lindholt *et al.* 1999(a)]. The risk of aneurysm related death was reduced by 50% in the group of patients receiving a USS. The NHS AAA Screening Program (NAAASP) was initiated across England in 2009 [NAAASP]. This programme follows a similar methodology to the MASS trial, men aged 65 are invited for an ultrasound scan, if the aortic diameter is less than 3.0cm they are discharged, men with 3.0 to 4.4 cm diameter aortas are rescanned on a yearly basis, men with 4.5 to 5.4 cm aneurysms are rescanned at an interval of 3 months, men with aneurysms >5.5cm are referred to a consultant vascular surgeon for consideration of surgical repair [NAAASP]. The aim of the NAAASP is to reduce AAA associated mortality by 50% by facilitating AAA repair prior to rupture.

Screening for AAA is not unique to England with Scotland, Wales and Northern Ireland all commencing screening by 2013. Additionally, programmes are also ongoing in Sweden and the USA, though in America the recommendation for screening is only for individuals with a history of smoking in excess of 100 cigarettes in their lifetime and the programme also includes females, whereas the UK programmes currently do not [Stather *et al.* 2013(a)].

The diameter of 5.5 cm was chosen as the diameter where risk of rupture outweighs risk of surgery and is based upon data from 2 large studies, the UK Small aneurysm Trial (UKSAT) and Aneurysm Diagnosis and Management (ADAM) trial [Powell *et al.* 2007, Lederle *et al.* 2002]. UKSAT reported on the outcomes of 1090 patients, who were followed for 12 years. A total of 563 patients were randomised to early surgery whilst the other arm of 527 patients were randomised to enter surveillance and were operated upon when fulfilling given criteria (aortic diameter >5.5cm, aneurysm growth of 1cm in one year or tender/symptomatic AAA) [Powell *et al.* 2007].The ADAM trial was an American study that followed very similar methodology to UKSAT, with similar numbers of patients recruited, 569 having early surgery compared to 567 entering surveillance [Lederle *et al.* 2002]. Mortality analysis in both studies demonstrated no benefit in operating on those with an aneurysm of less the 5.5cm. This evidence is still utilised today to justify the size criteria of an asymptomatic aneurysm. On the basis of these trials there is general acceptance that an asymptomatic aneurysm does not require intervention until reaching a diameter of 5.5cm.

1.2.6 Sequelae of AAA

The presence of an AAA carries significant dangers for an individual. Rupture is the most significant consequence of AAA (RAAA) but there is also a low rate of peripheral limb ischaemia and aorto-enteric fistulation.

1.2.5.1 Rupture of AAA

Rupture occurs when the wall of an aneurysm can no longer resist the force of the blood within its lumen. Rupture is a fatal occurrence in the majority of individuals, with a significant proportion of patients not surviving long enough to reach hospital. Data from the Office of National Statistics for England and Wales reported 3,759 deaths from aortic aneurysms in 2012 (most recent data available), ICD-10 Codes 71.3 (Abdominal Aortic Aneurysm, ruptured) and 71.4 (Abdominal Aortic Aneurysm, without mention of rupture) (Figure 1.4). Within these data 3,302 of these deaths were due to rupture of an AAA. The presenting features of an RAAA include a pulsatile abdominal mass, abdominal/back pain and hypotension [Rosen *et al.* 1984].

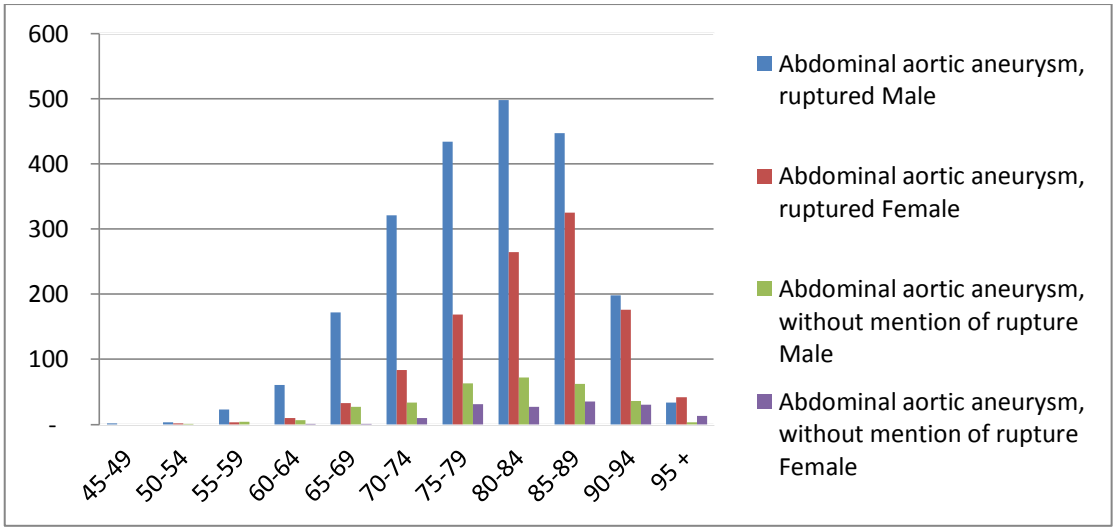

Figure 1.4: 2012 AAA Mortality Data from England and Wales. Office of national statistics, with all causes of aneurysm mortality (Male and Female ICD-10 Codes I71.3: Abdominal Aortic Aneurysm, ruptured and I71.4 Abdominal Aortic Aneurysm, without mention of rupture).

Aneurysm rupture has a poor prognosis; operative mortality approaches 50% of patients and is higher in patients of increasing age [Bown *et al.* 2002]. Data from the UK Small Aneurysm Trial demonstrated that 25% of RAAA patients do not reach hospital alive and a further 51% die prior to surgery [Brown *et al.* 1999].

1.2.5.2 Primary Aortoenteric Fistula

Aortoenteric fistulae occur when there is a persistent connection between the aorta and any part of the enteral tract, the condition was first described by Sir Astley Cooper in 1822 who commented that they were rare but serious sequelae of an AAA [Cooper 1939]. There is a classical triad of presentation with gastrointestinal bleeding, abdominal pain and a pulsatile abdominal mass, though in a larger review of the literature this feature was only present in 11% of 81 reported cases (time period 1994-2003) [Saers and Scheltinga 2005]. Primary aortoenteric fistulae are found in patients prior to any surgical procedure whereas secondary aortoenteric fistulae (iatrogenic fistulae) are seen following procedures such as AAA repair. In total there have been little more than 300 cases of primary aortoenteric fistula reported with the majority being associated with AAA, though certain infections, trauma and ingestion of foreign bodies have also been identified as causes of primary aorto-enteric fistulae [Rhéaume *et al* 2008]. There is significant morbidity associated with this condition, with both open surgical resection and endovascular occlusion of the fistula possible treatments [Saers and Scheltinga 2005].

1.3.1 Incidence of AAA

A general incidence of 21.1 per 100,000 person-years was reported in an American study of 1984, whilst another American series published in the same year reported a similar incidence of 36.5 per 100,000 person-years. However, since this data was published there have been significant changes to rates of smoking and progressively older population that are likely to affect the rate of developing AAA [Bickerstaff *et al.* 1984, Melton *et al.* 1984]. Bickerstaff *et al.* also illustrated a progressive increase in AAA incidence over time from the 1950's to the 1980's, in the period of 1951-1955 the incidence was 12.2 per 100,000 person-years. This effect was also observed in Scotland; in 1971 the incidence was 25.8 per 100,000 person-years whilst in 1984 it had risen to 63.6 per 100,000 person-years [Naylor *et al.* 1988].

A longitudinal study from Malmo in Sweden demonstrated that the incidence of RAAA dramatically rose over time, alongside a rise in the incidence of aneurysm repair [Acosta *et al.* 2006]. The incidence of RAAA in 1971 to 1986 was 5.6 per 100,000 person-years; by 2000 to 2004 this rate was 10.6 per 100,000 person-years. The incidence of elective aneurysm repair rose from 3.4 per 100,000 person-years in 1971 to 1984 to 7.0 per 100,000 person-years in 2000 to 2004. This data was collected from hospital operating codes and autopsy records, the rate of which fell drastically between 1979 and 2004, due to changes in the Swedish legal system. The rupture incidence may be underestimated as the initial data was based upon a greater proportion of autopsies than the more recent cohort. Taken

together this data indicated that there was a steady increase in AAA incidence from the middle of the last century to the beginning of the 21st century, and despite increasing incidence of repairs there is also an increasing incidence of rupture.

1.3.2 Prevalence of AAA

A recent large scale study from Sweden reported a prevalence of 2.2% [Svensjö *et al.* 2011]. This study involved 26,256 65 year old males within 5 Swedish counties and 85% accepted the offer of a USS scan. This prevalence is lower than other published rates, such as the large international study from the CASS (Collaborative Aneurysm Screening Study) group, which published a rate of between 4.5 and 7.7%, (albeit in older men 64 – 83 years) [Collaborative Aneurysm Screening Study Group 2001]. CASS included populations from the United Kingdom (The Multicentre Aneurysm Screening Study: MASS and the Chichester Aneurysm Screening Group), Denmark (Viborg Aneurysm Screening Study) and Australia (Western Australian Abdominal Aortic Aneurysm Program). These datasets arise from large numbers of patients, 46,397, and are multinational, allowing for generalization to most populations, however the rate from the Chichester Aneurysm Screening Group was higher, 7.7%, than the other rates. Rates in the other CASS centres were 3.9% in Denmark, 4.9% in the UK based MASS trial (Multicentre Aneurysm Screening Study) and 7.2% in Western Australia, additionally the cohort in Chichester was the smallest with 2212 individuals screened, whereas the MASS trial screened 27121 people. However, the more

recent data from the NAAASP also has a lower rate, similar to the rate from the Swedish data. Between 2009 and 2010, the first year of the NAAASP, 404 AAA were diagnosed in 23,000 screened individuals in a population of 65 year old males [Earnshaw 2011]. As the project has grown, between 2013 and 2014 235,409 males were screened and 2,941 aneurysms were diagnosed, a prevalence of 1.25%. With the addition of those males older than 65 who have self-referred to the NAAASP, 3,680 aneurysms were diagnosed in 263,088 scanned males in 2013-2014, a prevalence of 1.40%. As the screening program targets the higher risk group of 65 year old males, the prevalence would be lower with the inclusion of younger individuals and females.

1.4 Pathological processes in AAA

There are key pathological features in the microscopic appearance of an AAA, though there is inconsistency there are some hallmark processes that have been identified [Lopez-Candales *et al.* 1997]. These processes are infiltration with inflammatory cells and destruction of the extra cellular matrix (ECM).

1.4.1 Infiltration with inflammatory cells

Within both aneurysmal tissue and the circulation of patients with AAA there are elevated levels of pro-inflammatory cytokines. Circulating levels of tumour necrosis factor alpha (TNFα), interleukin 1beta (IL1β) and interleukin 6 (IL6) are all significantly greater in AAA patients when compared to healthy controls and patients with angiographically proven coronary heart disease (CHD) [Juvonen *et al.* 1997]. The use of patients with CHD as a control demonstrated the different processes taking place in aneurysmal disease to those in atherosclerotic disease. TNFα is able to initiate vascular smooth muscle cell (VSMC) death and induce the release of proteases that can degrade the arterial extra cellular matrix (ECM). In AAA tissue extracts the concentration of TNFα was significantly greater than in control tissue extracts, albeit in a study with relatively small numbers (AAA n= 7, control n=5) [Newman *et al.* 1994(a)]. Analysis of the lysate of AAA (n=8) and controls aortic biopsies (n=3, from redundant aortic tissue post organ transplantation) demonstrated that the cytokines found within aneurysmal tissues are predominantly associated with type 2 T helper cells, interleukins 4, 5 and 10

[Schönbeck *et al.* 2002]. This study compared the cytokine profile seen in stenotic atherosclerotic lesions (carotid plaques that had undergone endarterectomy, n=8) which in contrast to AAA demonstrated strong association with the type 1 T helper cell. The diverse effects of the inflammatory cells found with aneurysmal tissue include secretion of matrix metalloproteinases (MMPs), which are a family of proteases that have repeatedly been associated with AAA due to their ability to degrade elastin and collagen [Pearce and Koch 1996].

1.4.2 Destruction of the extracellular matrix

The ECM is the essential structural component of the arterial wall; it is primarily composed of collagen and elastin. Proteases are the enzymes responsible for protein degradation. Murine animal models have demonstrated that aneurysms can be induced with the infusion of elastases (elastin specific proteases) but interestingly to induce rupture, the addition of collagenase (collagen specific proteases) is required [Daugherty and Cassis 2004]. Proteases that have been found to be active within the aneurysmal wall belong to three protease families, the MMPs, the cysteine proteases and the serine proteases [Jean-Claude *et al.* 1994, Abdul-Hussien *et al.* 2007]. The cysteine protease family includes cathepsins, that possess elastolytic activity. The serine protease family member, plasmin, is secreted as plasminogen which can be activated by a wide range of molecules such a tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Active plasmin has both direct and indirect affects upon the ECM, it has direct proteolytic activity and it can also activate MMPs [Jean-Claude *et al.* 1994].

Important serine proteases in the degradation of ECM include tPA (one of the activators of plasminogen), and plasmin-antiplasmin-complexes (PAPs). PAPs have been shown to correlate with annual expansion rate in AAA; however the mechanism by which this relationship operates has not been elucidated [Lindholt *et al.* 2001].

1.4.3 Pathogenesis of AAA: Summary

The accumulation of inflammatory cells and ECM degradation do not take place in isolation within an aneurysm, rather there is a constant interplay between these 2 processes. As has been briefly highlighted in this section there are multiple biologically active proteins involved in the pathogenesis of AAA. Many of the molecules identified to date have diverse functions and the pathway becomes more complex with the addition of each new molecule, with more inhibitors and promoters are added to this process. What is clear is that as the aneurysm evolves through the stages of initiation to progression and ultimately rupture, reduction in the levels of elastin, increased collagen turnover and the number of smooth muscle cells decrease and the number of inflammatory cells rise.

1.5 Risk Factors for AAA

There are several consistent risk factors identified for patients with AAA in multiple studies. These risks are both environmental and hereditary; therefore not all of them are modifiable.

1.5.1 Smoking

Cigarette smoking is also strongly associated with AAA. In a large observational study of over 70,000 50-79 year old American military veterans within the ADAM trial, where the odds ratio of having an aortic diameter of greater than 3.0cm compared to a diameter of less than 3.0 cm was 2.72 (95% confidence interval 2.37 to 3.11) in patients with a positive smoking history the odds ratio of a 4.0cm or larger AAA compared to a diameter of less than 3.0cm was 5.77 (95% confidence interval 4.24 to 7.31) [Lederle *et al.* 1997]. This data from a large scale population analysis is evidence that smoking strongly associates with AAA and in the latter study smoking was the single most important influence on AAA. More recently data from a longitudinal study of individuals from the Norwegian city of Tromsø has supported the association between cigarette smoking and AAA [Forsdahl *et al.* 2009]. In this study 6892 individuals accepted an invitation to be surveilled and had their abdominal aorta screened with ultrasonography in 1994 and then again in 2001. Removal of patients who had AAA at initial scan, losses to follow up, incomplete data and inaccurate consent resulted in 4345 individuals (2035 males) undergoing a scan in 2001. New AAA was detected in 119 cases in the second

ultrasound scan, and there was a very strong association between current cigarette smoking (of at least 20 cigarettes per day) at the time of the second scan and AAA. Utilising a multiple logistic regression model the impact of smoking 20 cigarettes per day when compared to those who had never smoked produced an odds ratio of 13.72 (95% confidence interval 6.12 to 30.78). Though there was an increased odds ratio with any form of positive smoking history, the degree of association became more modest with reduced number of daily cigarettes smoked and increased period of smoking abstinence. This study had the benefits of being both prospective and population based. Weaknesses included few cases being diagnosed with at the second scan (due to the low incidence of AAA), few women were diagnosed with AAA so that a sex stratified analysis was not possible and the possibility that small AAA were not identified at the first scan due the technical difficulty of detecting an AAA. In a UK based case control study of 210 patients with AAA and 237 healthy controls the relative risk of having an AAA was 1.0 in non-smokers, 4.0 in ex-smokers (95% confidence interval 1.6 to 9.5) and 9.0 in current smokers (95% confidence interval 3.4 to 24.0) [Wilmink *et al.* 1999(a)]. This odds ratio was derived from a logistic regression model that took in to account several variables such as medical history (ischaemic heart disease, peripheral vascular disease and the use of diuretic medication), family history of aneurysms and tissue laxity (which has also been shown to associate with AAA development [Wilmink *et al.* 1999(b)]). In addition, the duration of smoking was a factor that affected relative risk of having an AAA. In those patients that had smoked for less than 20 years duration the OR was 1.4 (95% confidence interval 0.6 to 3.4) whereas in those that had been smoking for 20-40 years the OR was 3.6 (95%

confidence interval 1.6 to 8.2). In patients with greater than 40 years history of smoking the OR was 5.8 (95% confidence interval 2.6 to 13.0), indicating that the effect of smoking is dose dependent and chronic exposure significantly impacts on likelihood of AAA. Despite the authors stating that potential sources of bias were small this study included much smaller numbers than the previous studies and therefore may represent an insufficient sample size.

The evidence for an association between AAA and smoking has been reported in several studies, though the size of this effect varies, smoking consistently increased the risk of suffering an AAA.

1.5.2 Male Gender

The ADAM study also demonstrated that female sex was negatively associated with AAA, OR 0.22 (95% Confidence interval 0.07-0.68), although as this study was of American Military veterans there was a disproportionally large male representation, with men making up 96% of the study population [Lederle *et al.* 1997]. The predominance of AAA in males has also been reported in Europe and Australia. An 8 year study of individuals from Worthing, in the UK, demonstrated the ratio of male to female AAA patients was 3.1:1 [Morris *et al.* 1994]. Also a cross sectional study of over 5000 individuals from the Netherlands demonstrated an AAA prevalence of 2.1% overall, however analysis of males and females separately showed prevalence's of 4.1% and 0.7% respectively [Pleumeekers *et al.* 1994]. The data from the 7 year prospective Tromsø Study (Norway) demonstrated that the relative risk of males having an AAA was 2.66 (95% confidence interval 1.69 to

4.20) when compared to females [Forsdahl *et al.* 2009]. In Australia, a population largely derived from European ancestry, a 10 year study surveyed hospital coding, operating lists and autopsies, generating an incidence of AAA of 377.3 per 100,000 person years in males, over double that of the incidence in females, 125 per 100,000 person years [Castleden *et al.* 1985]. Data from the United Kingdom analysing 65-79 year olds (2203 males and 2832 females) reported a prevalence of 6.8% in males and 1.1% in females [Vardulaki *et al.* 2000]. A cohort of 5140 Swedish women aged 70 accepted an invitation for screening, the prevalence of AAA in this cohort was estimated to be 0.5 (95% confidence interval 0.4 to 0.7), much lower than the prevalence of 2.2% observed in 65 year old males from the same location [Svensjö *et al.* 2013]. Recent evidence has illustrated an interesting facet of this male female dichotomy, in that women with AAA are at a greatly increased risk of rupture when compared to men with AAA [Sweeting *et al.* 2012].

1.5.3 Increasing Age

The risk of dying from an AAA increases with age and prevalence also increases with age. A study from the Netherlands showed that there is also a difference between men and women of different ages, similar to gender influencing AAA, the prevalence of AAA in men aged 65-69 years was 3.8% (women 0.2%), in men aged over 80 years it was 10.3% (women 2.1%) [Pleumeekers *et al.* 1994]. Age was also positively associated with AAA in the ADAM trial, with the prevalence of AAA (defined as 4.0cm or larger in this trial) being highest in men over 70 in both smokers and non-smokers (2.7% and 0.8% respectively) [Lederle *et al.* 1997].

1.5.4 Diabetes

Diabetes mellitus is consistently associated with cardiovascular disease, however there is a negative association to AAA, with diabetes appearing to be protective, OR 0.54 (95% Confidence interval 0.44 to 0.65) [Lederle *et al.* 1997]. Additionally the rate of growth of AAAs are reduced in patients with diabetes, reducing the mean growth rate of 2.21 mm/ year by 0.51 mm / year (total of 15475 individuals) [Sweeting 2012]. A large scale meta-analysis of published data regarding AAA in diabetics has recently been published [De Rango *et al.* 2014]. Combined data from 17 population studies (33407 AAA patients and 3290604 controls) produced an inverse association between diabetes and AAA with an odds ratio of 0.80 (95% confidence interval 0.70 to 0.90, P value = 0.0004. This is compelling evidence of the protective effect diabetes has upon aortic aneurysm formation. Interestingly De Rango *et al.* drew attention to the lack of definition of the type of diabetes (type 1 or type 2) within the included studies, though with the older age of the patients it is likely that Type 2 diabetics will dominate.

1.5.5 Hypertension

Hypertension is defined as the arterial blood pressure being greater than 135/85 mm/Hg (when an average of a single day's measurements is taken [NICE Guidance CG127]). The effect hypertension has on AAA has been inconsistent between studies. Lederle *et al.* reported a positive, albeit small, association between

hypertension and AAA [Lederle *et al.* 1997], however this data arose from patients self-reporting the diagnosis of hypertension which may have led to inaccuracy. In mouse models AAA can be induced by infusing angiotensin subcutaneously into LDL receptor knockout or apoE knockout mice [Daugherty *et al.* 2004]. The blockage of angiotensin production is used as an antihypertensive therapy therefore it is reasonable to associate hypertension to AAA. A recent large scale meta-analysis reported that increases in arterial pressure had no impact on aneurysm growth and the use of medications that treat hypertension lead to non-significant reductions in aneurysm growth; however increased blood pressure did associate with increased risk of AAA rupture [Sweeting *et al.* 2012].

1.5.6 Height

A recent study reported the outcomes of 60 years of mortality and vascular morbidity data from beginning of the 21st century including over 1 million patients and 174374 deaths or serious vascular morbidities, the authors compared outcomes to height [The Emerging Risk Factors Collaboration 2012]. This analysis demonstrated that increasing height was a protective factor for diseases such as coronary disease, stroke, stomach cancer and oral cancer. However there was a positive association between increasing height and ruptured AAA. The hazard ratio for RAAA for every 6.5cm increase in height was 1.12 (95% confidence interval 1.05 to 1.20). Mean follow up 13.7 years. The authors of this meta-analysis suspect that increased height equates to longer arteries and increased arterial length may make an artery more prone to rupture, as has been reported in patients with the

connective tissue disorder, Marfan Syndrome [The Emerging Risk Factors Collaboration 2012]. It is interesting to consider height as a risk factor for AAA as European populations tend to be on average taller than those of Asia and Africa, and European ancestry is associated with increased rates of AAA. Aside from the mechanistic effects of long arteries described it may be associated genetic features that cause this association between height and AAA. This is in contrast to recent data that identified decreasing height as a risk factor for coronary artery disease, one increasing standard deviation in height (6.5 cm) reduced the relative risk of coronary artery disease, 13.5% (95% Confidence Interval 5.4-22.1, P=<0.001) [Nelson *et al.* 2015].

1.5.7 Ethnicity

AAA is a disease that more commonly affects white Europeans. In the American Veterans Administration study of AAA, "Black" race negatively associated with AAA OR 0.49 (95% confidence interval 0.35 to 0.69) [Lederle *et al.* 1997]. In an analysis of a large UK city with an ethnically diverse populous, Asian men, with origins in the Indian subcontinent, were observed to have a greatly reduced AAA prevalence when compared to white Europeans, 0.45% (95% confidence interval 0.054 to 1.161) versus 4.69% (95% confidence interval 4.39 to 5.00) [Salem *et al.* 2009]. In a cohort of 947 Japanese males over the age of 70 years, the prevalence of AAA was 0.84% [Ishikawa 1999]. Aside from white Europeans the other racial group with a significant burden of AAA disease are the New Zealand Maori. Maori individuals are diagnosed with AAA at a younger age, have a larger proportion of

emergency AAA surgery and finally an increased AAA mortality rate, 2.4 times greater, than individuals of European descent [Rossaak *et al.* 2003]. There is good evidence that white Europeans and those with European ancestry are at greatest risk of having an AAA, however other ethnicities can be found to have AAA.

1.5.8 Family History

There is strong evidence that relatives of patients with AAA are at an increased risk of being diagnosed with an AAA themselves. This data comes from both analysis of twins and observational studies. Martin Clifton first proposed a familial effect in AAA when he reported on 3 British brothers who were diagnosed with RAAA [Clifton 1977]. Subsequently the ADAM study reported that family history positively associated with AAA [Lederle *et al.* 1997]. There have been several other studies where the family members of patients with AAA have been screened in a case control series to determine if they have an increased AAA prevalence. The percentage of aneurysms in the siblings of patients with AAA has been reported at between 14.9% to 30% [Bengtsson *et al.* 1989, van der Graaf *et al.* 1998, Ogata *et al.* 2005, Frydman *et al.* 2003]. These studies were all of Europeans or European descendent populations, such as Canadians and Australians. Most of the studies were affected by a significant drop out rate, for instance Ogata *et al.* identified 1703 siblings of 375 AAA patients and only 14.4% (245) of these individuals had a screening ultrasound scan, however the logistical issues such as sibling mortality (42% in the case of Ogata *et al.*) and migration did account for a sizeable proportion of individuals who did not receive a scan. Aside from case-control studies there has been evidence from the Swedish Twin Register, where the

records of all twins born from 1886, was cross referenced a register of in-patient episodes. The results demonstrated that the risk of being diagnosed with an AAA is increased if the patient is the monozygotic twin of a patient with an aneurysm, odds ratio of 71 (95% confidence interval 27-183) whereas if an individual has a dizygotic twin with an AAA, where the genetic relationship is the same as that of any 2 siblings, the odds ratio is only 7.6 (95% confidence interval 3.0-19) [Wahlgren *et al.* 2010]. Taken together this evidence is strongly suggestive that there are genetic factors strongly related to the risk of AAA diagnosis.

1.5.9 Mode of inheritance

There have been several studies that have set out to determine what mode of inheritance is seen in AAA. There does not appear to be a consensus in terms of which inheritance model best fits AAA. An early study reported that both X linked and autosomal dominant inheritance or a combination of both were plausible. This analysis was based upon visual examination of 41 individuals within 16 family pedigrees [Tilson *et al.* 1984]. A multifactorial model, with small effects contributed by several genes and environmental factors, was determined following analysis of 60 consecutive aneurysm repairs, though the authors do admit that their method of collecting data may have introduced error and underestimate prevalence and heritability [Powell *et al.* 1987]. A more detailed analysis, utilising a model testing various modes of inheritance in a sample of 91 families determined that the most likely mode of inheritance was autosomal recessive and not multifactorial, though again this data was from patient histories and did not utilise

ultrasound screening of relatives [Majumder *et al.* 1991]. A mode of autosomal dominance was proposed by Verloes *et al.,* based upon analysis of the families of 313 AAA patients (81 of which were familial [Verloes *et al.* 1995]. Kuivaniemi et al. analysed 233 families and no signal mode of inheritance was found, further supporting a multifactorial model [Kuivaniemi *et al.* 2003]. In all of these studies genetics is implicated in AAA causation; however the mode of inheritance is not clear.

1.6 Medical Treatment of AAA

1.6.1 Medical Treatment

Medical management of AAA is currently inadequate as no medical intervention can cure AAA, though there are some potential therapeutic drugs under investigation, such as propanolol, statins and ACE inhibitors.

1.6.2 Lifestyle

Limiting environmental factors known to causes disease progression is one aspect of medical management. In terms of AAA the cessation of smoking is widely agreed to be beneficial, there are repeated associations between AAA and smoking [Wilmink *et al.* 1999, Lederle *et al.* 1997]. Though to date a randomised control trial has not been performed, recent evidence from English Hospital Admission data attributes a reduction in ruptured aneurysm mortality by 8-11 per 100,000 population due to reduced prevalence of smoking [Anjum *et al.* 2012]. This study utilised nationwide data, however, with all hospital coding data there is a risk of error introduced with inaccurate codes being used and additionally the prevalence of smoking is based upon population estimate and may be underestimating the rate of patients with AAA who smoke. Data from the RESCAN collaborators proves that active smoking is associated with significantly increased rates of aneurysm growth and rupture [Sweeting *et al.* 2012]. The RESCAN collaborators represent several regional aneurysm growth studies, sites that contributed data are outlined in Table 1.1. The collaboration not only analysed growth but also the effect of various factors (smoking, diabetes and drug history) upon growth

Study	No. of patients (diameter measurements)	Mean age at baseline (years)	% men*	% current smokers*
Western Australia	685 (3738)	73·4	100·0	18·4 (0·7)
Bournemouth,UK	677(2959)	72·8	100·0(0·0)	NA
Chichester,UK	1504(9649)	69·6	93·4(0·0)	NA
Edinburgh,UK	1052(5155)	71·4	63·7(0·0)	NA
Gloucestershire,UK	1981(12442)	66·9	100·0(0·0)	NA
Huntingdon,UK	629(3781)	70·1	100·0(0·0)	29·8(34·8)
Leeds,UK	267(1870)	71·8	82·5(0·0)	24·8(0·7)
Leicester,UK	899(4689)	66·8	100·0(0·0)	41·4(1·9)
Manchester,UK	1095(5315)	74·0	76·4(0·0)	NA
MASS,UK	1122(8361)	70·2	100·0(0·0)	31·8(14·8)
Tromso,Norway	224(3817)	67·0	79·9(0·0)	53·4(0·4)
PIVOTAL,USA	715(1753)	70·9	86·6(0·0)	27·5(2·0)
Propranolol,Canada	548(2971)	68·9	83·9(0·0)	36·7(0·0)
Galdakao,Spain	926(3466)	71·6	93·1(0·4)	29·3(0·0)
Stirling,UK	457(1979)	71·7	72·6(0·2)	NA
Gävle,Sweden	243(1114)	70·5	75·7(0·0)	39·1(0·0)
UKSAT,UK	2227(9140)	69·4	78·4(0·0)	38·0(3·0)
Viborg,Denmark	224(912)	68·1	100·0(0·0)	59·9(14·3)

Table 1.1.The RESCAN Collaborators. Outlining region and country of origin, and the various datasets that were complete for each region, percentage of males, current smoking. * =Values in parenthesis are the percentage of patients for whom data are missing. Modified from Sweeting *et al.* 2012.

The meta analysis of the data from the RESCAN collaboration demonstrated that current smoking increased aneurysm growth rate by 0.35 (standard error 0.07) mm/year when compared to ex-smokers and those who had never smoked , p value = <0.001. Analysis of Table 1.1 demonstrates that there are wide variations in cohort size, inclusion of females and the percentage of missing data between the collaborators. This led to heterogeneity in the analysis. Despite the variation the papers methodology is sound and the effect of smoking was uniform between all included studies. Large scale international mortality data has recently shown that in countries with significant decrease in smoking prevalence there is a significant decrease in AAA mortality [Sidloff *et al.* 2014 (a)]. The time period observed was from 1994 to 2010, and smoking prevalence alone may not account for the reduced mortality, for example in the UK there has been progressive centralization of vascular surgery, improved pre and post-operative care and increased rates of Type 2 diabetes, which negatively associates with AAA.

Additionally a randomised trial is on-going to determine the use of physical conditioning as a method of reducing inflammation and reducing haemodynamic stress will affect the progression of AAAs. Initial results have demonstrated that the elderly males involved in the study (mean age of 72) are able to tolerate the exercise without adverse event or progression of AAA, the results of this trial are awaited, despite the one year results being published in 2010 there has been no subsequent publication of data from this trial to date [Myers *et al.* 2010].

1.6.3 Beta Blockers

The use of beta blockade to reduce the progression of AAA growth has been examined in three RCTs. The oldest, a study of 54 patients, was stopped early due to side effects (mainly dyspnea) and a higher death rate in the experimental group, this commenced the debate on the safety of using beta blockade as an AAA treatment [Lindholt *et al.* 1999(b)]. Larger RCTs followed this, Wilmink *et al.* reported a study of 477 patients, of which 256 were randomised to treatment with propranolol [Wilmink *et al.* 2000]. There were issues with compliance, with only 31% of the treatment group remaining on the study medication and the growth rates of those on treatment were not significantly different from controls, 0.24mm per year in treated patient's vs 0.25mm per year in controls. The use of propanolol to reduce aneurysm growth was also not recommended following a Canadian double blinded RCT of 276 patients on treatment and 272 receiving placebo [Propanolol Aneurysm Trial Investigators 2002]. Not only were patients treated with propanolol suffering a poorer quality of life but the average growth rates showed no significant difference from placebo. This evidence indicates beta blockade is not suitable for long term AAA treatment as it is ineffective.

1.6.4 Statins

Though there has not yet been an RCT examining the effect of statins (HMG Co A reductase inhibitors) on AAA the results of 2 large observation studies are available. Thompson *et al.* demonstrated no significant difference in the growth rate of 1231 screening detected AAA who reported regular statin use [Thompson *et al.* 2010].

Another observation study of 652 Australian patients, 52% who were prescribed a statin, did not show a significant difference in growth rates when patients on statins were compared to those who were not [Ferguson *et al.* 2010]. These larger studies are contrary to smaller observational studies that did find a significant reduction in aneurysmal growth in patients receiving statin therapy [Schouten *et al.* 2006, Sukhija *et al.* 2006]. These findings of these studies may represent the inherent problems with observation studies introducing bias, such as poly-pharmacy in patients receiving statins leading to the aggregation of small effects of several medications. The widespread use of statins for cardiovascular disease means that a large proportion of AAA patients are receiving this medication and despite this aneurysms in these patients continue to progress.

1.6.5 Angiotensin Converting Enzyme

The infusion of angiotensin II in to the aorta of mice is a proven method to generate aneurysms in experimental models; therefore it is plausible that ACE (angiotensin converting enzyme) inhibitors and ARBs (angiotensin receptor blockers) may have an effect on AAAs [Daugherty *et al.* 2000]. A large case control study, involving 15,326 patients, observed that there was a reduction in the rate of RAAA in patients who were prescribed ACE inhibitors prior to admission [Hackam *et al.* 2006]. This is countered by a more recent observational study of 4826 patients that showed no effect of ACE inhibitor use on RAAA, though this represented only one third of patients within this large multinational study, as medication history was not recorded in a uniform manner across all of the contributing studies [Sweeting *et al.* 2012]. A trial

entitled “Evaluation of Effect of Angiotensin-converting Enzyme (ACE) Inhibitors on Small Aneurysm Growth Rate (AARDVARK)” began in 2011 in order to determine whether the ACE inhibitor perindopril reduces AAA growth. The trial will not only compare the effect of the ACE inhibitor upon AAA growth but by giving one arm of the trial patients another class of anti-hypertensive medication (amlodipine), the effect of hypertension upon AAA growth will also be determined. Data collection was due to conclude in August 2014 so results should be available during 2015 (ClinicalTrials.gov identifier NCT01118520).

1.6.6 Additional Medications

Other medications that have been of interest are members of the macrolide group of antibiotics, such as azithromycin and roxithromycin though as of yet there is no RCT to support the use of these agents, the smaller of these trials showed a statistically significant reduction in aneurysm growth rate in patients on treatment however the larger and more recent study did not share this finding [Karlsson *et al.* 2009, Vammen *et al.* 2001]. A multinational RCT has been conducted evaluating the use of pemirolast, an novel anti-inflammatory drug, in the treatment of AAA in over 300 medium sized AAA (39 – 49mm). This “Anti-Inflammatory ORal Treatment of AAA” (AORTA trial), evaluated 3 increasing doses of pemirolast (10, 25 and 40mg) against a placebo. The growth rate in the placebo group was 2.04cm per year, while the rates observed in the three increasing treatment arms was 2.58, 2.34 and 2.71 cm per year respectively, there was no statistical difference and this drug does not affect growth in medium sized AAA [Sillesen *et al.* 2015].

1.6.1.6 Medical Treatment of AAA Conclusion

In conclusion, despite a variety of pharmacology agents showing potential to influence aneurysm progression, no single medication has been found to deliver consistent results in observational or case control studies and no large randomised control trial has successfully been conducted in order to determine a suitable agent for the medical management for AAA.

1.7 Surgical Treatment of AAA

Currently the only potential cure for AAA is surgery.

1.7.1 Historical Perspective

Historically Antyllus (Antyllos), a second century Greek surgeon who practiced in Rome, is attributed with being the first to treat aneurysms. However it is in the fifth century surgeon Aëtus whose technique was well described. The aneurysm would be progressively removed from the circulation by ligature of the vessel from proximal to distal [Fortner *et al.* 1984].

Subsequent developments in the management of aneurysms began with the Hunter brothers, William and John, who were able to offer treatment of peripheral aneurysms, using ligation of the vessel. A pupil of the Hunter brothers, Sir Astley Cooper, furthered this technique and treated aneurysms of the common carotid, external iliac and internal iliac arteries, and eventually in 1817, ligating the abdominal aorta in order to treat a leaking left iliac artery aneurysm [Singal *et al.* 2011].

In 1951 the successful management of an AAA using the placement of silver wire in to the aneurysm wall and then electric current applied to the wire in order to cause a thermal output, coagulating the blood, was described [Blakemore 1951]. The case studies presented by Blakemore included a case with 11 years of follow-up, therefore there was certainly success from this method. A case of induced thrombosis within an aneurysm when resection was not possible was reported in 1978 [Berguer *et al.* 1978].

Following bypass of blood flow from the axillary arteries to the femoral arteries, balloon catheters were inserted through the right axillary artery to achieve proximal control and via the iliac vessels in order to obtain distal control. The proximal balloon was inflated below the level of the renal arteries in order to maintain renal perfusion. Thrombin was then introduced in the aneurysm sac, achieving thrombosis and obliteration of the sac.

1.7.2 Professor Oscar Creech

None of these treatments led to universal adoption as the procedure to prevent rupture of an AAA. The open AAA repair pioneered by Professor Oscar Creech Jr of New Orleans Tulane Hospital in 1966 (Figure 5) is carried out through an abdominal incision, with mobilisation of the visceral organs in order to access the retroperitoneal cavity [Creech 1966]. Once the retroperitoneum is opened the aorta is clamped superiorly and the iliac vessels are clamped inferiorly. The aneurysmal sac can then be incised, bleeding branches such as the lumbar arteries are controlled and a prosthetic, Dacron, tube graft is secured (Figure 1.5). The redundant sac is then closed over the graft. The original paper also outlined the use of this approach in patients with aorto iliac aneurysms, where the longitudinal incision into the aneurysm sac must extend on the iliac vessels for a short distance and a bifurcated graft must be utilised in place of a simple tube graft.

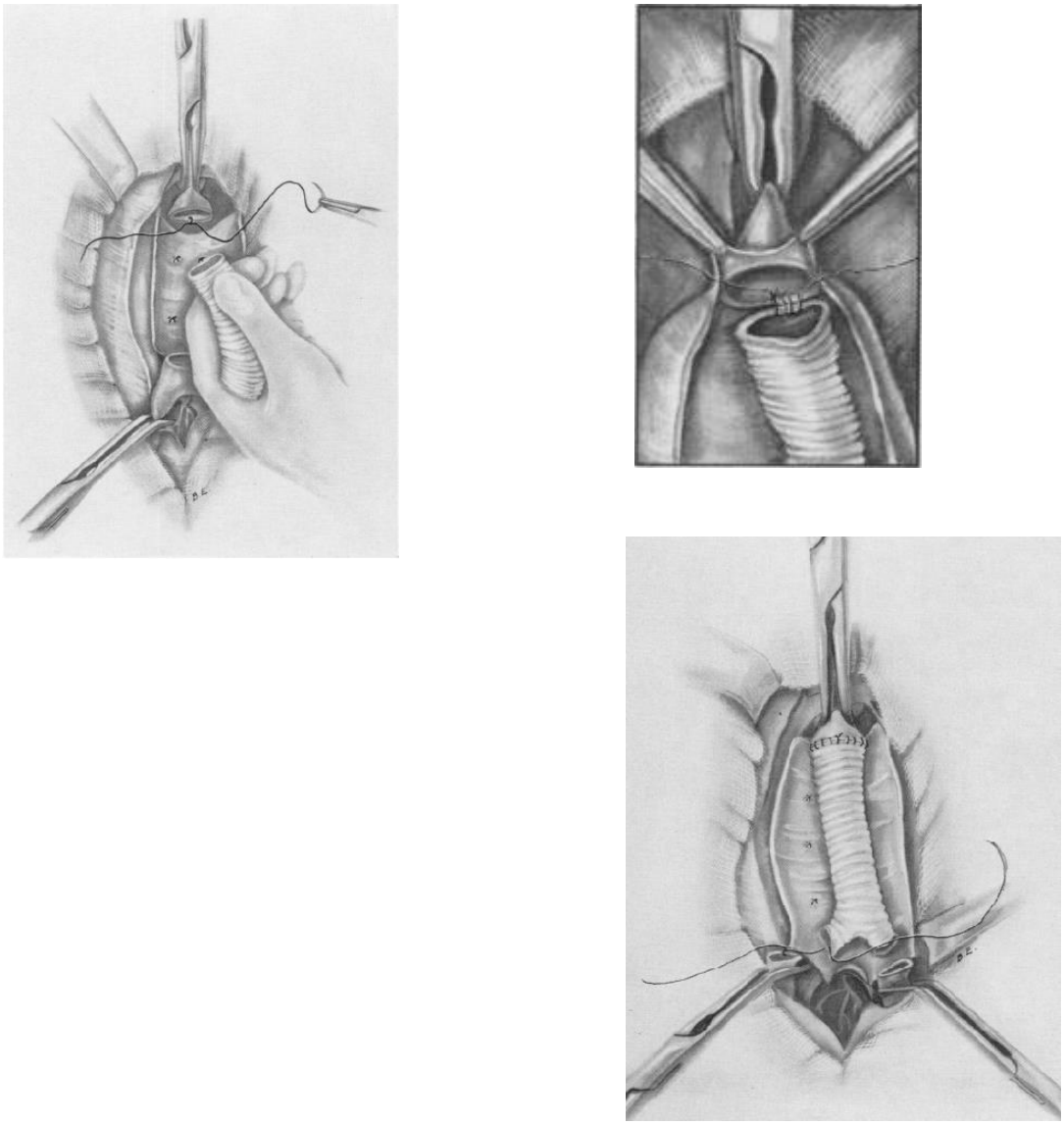

Figure 1.5: Schematic of open repair, from the left. Clamping of the proximal aorta and iliac vessels and arteriotomy, Dissection of aneurysmal sac, aorta with graft in situ following repair in both a pure aortic aneurysm (left) and an aortoiliac aneurysm (right). Taken from Creech 1966.

The procedure of open repair described by Prof. Creech has been adopted worldwide and was the mainstay in both elective and emergency aneurysm repair for many years until the technology for a more minimally invasive approach was developed.

1.7.2.1 Complications of Open Aneurysm Repair

As with any surgical procedure there are risks of bleeding and infection as the skin has been cut. Infection of the graft itself is a more serious but less common complication, 0.2% of cases, and may require long term antibiotic use or even replacing the graft with morality of between 20-40% [Vogel *et al.* 2008]. There are also risks compromising the side branches of the aorta and the iliac arteries. This can lead to loss of blood flow to the bowel, causing ischaemia, damage to the kidneys and ischaemia of the lower limbs. The rate of bowel ischaemia has been reported at 3% of open AAA repairs, however the 30 day mortality rate for those diagnosed with it is around 50% [Becquemin *et al.* 2008]. The rate of significant renal impairment, requiring dialysis, within 1 year following open AAA repair has been reported at 0.7%, whereas the rate of limb amputation due to post-operative critical ischaemia was also 0.7% [Lederle *et al.* 2008]. Despite these and other complications open repair remained to only viable cure for AAA for almost 50 years.

1.7.2.2 Laparoscopic AAA repair

Laparoscopic aneurysm repair was first reported in an animal model (porcine) in 1997, this was followed two years later by a report of the first 20 human cases of

laparoscopic assisted AAA [Chen *et al.* 1996, Kline *et al.* 1998]. The procedure utilised laparoscopic retroperitoneal dissection to prepare the aorta for repair prior to a small (8-11 cm in length) midline laparotomy allowing the placement of a prosthetic graft in the same manner as an open repair. These initial experiences proved the technique was feasible and that there was potential for a reduction in the length of intensive care unit admission duration and inpatient stay. Kline *et al.*, however, did encounter several technical complications, wound cellulitis, calf muscle ischaemia and colon ischaemia leading to colectomy. Subsequently totally laparoscopic AAA repair was reported and now 5 year follow-up of a cohort of 30 cases has been published [Cochennec *et al.* 2012]. The laparoscopic cohort was compared with a matched group of patients who underwent open repair. The rate of incisional hernia was greater in the group of patients undergoing open repair (15.4% v 0%, P= 0.047), the rates of cardiac, renal, pulmonary and distal vascular complications were not significantly different. A striking feature of this study was the small numbers recruited, 30 over an 18 month period, and this reflects the limited availability of laparoscopic AAA repair.

1.7.3 Endovascular Aneurysm Repair

Endovascular aneurysm repair (EVAR) treats AAA by means of insertion of a prosthetic graft into an aneurysm without open surgery. The graft is inserted via the placement of a guide-wire in to a femoral vessel, generally after the vessel has been surgically exposed. An endograft is passed over the guide-wire, and once correct position has

been confirmed with fluroscopy a prosthetic stent is deployed into the aneurysm with balloon catheters (Figure 1.6).

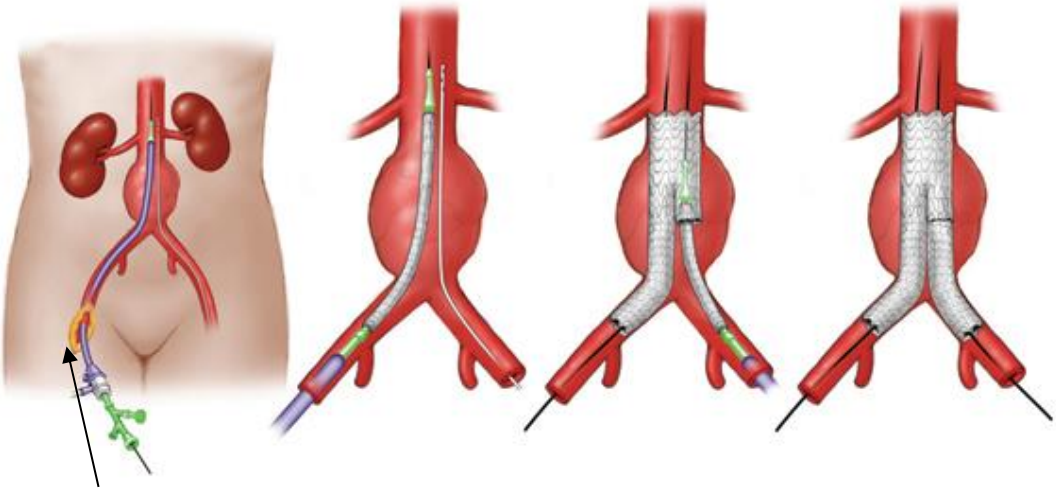

Figure 1.6: Schematic of endovascular repair of an aneurysm (EVAR), from the left. First a guide wire introduced in to aorta via the femoral artery, then a stent graft being introduced in to the aneurysm, balloon dilation of stent graft to secure it in position and finally insertion and inflation of the contralateral side. Modified from http:www.mayoclinic.org

The technique offers benefits by avoiding the need for a laparotomy and with the reduced surgical exposure allows for the use of regional anaesthesia and permitting repair in patients who have poor surgical fitness and multiple comorbid medical conditions. The EVAR 2 trial analysed 404 patients deemed unfit for conventional open repair, those 197 undergoing EVAR had a significantly reduced mortality from AAA [United Kingdom EVAR Trial Investigators 2010(a)]

The technique was first described by Parodi and colleagues in the early nineties [Parodi *et al.* 1991]. The initial experience reported by Parodi originated in work with both an animal model (canine) and 5 human cases (Figure 7). The 5 cases were all unsuitable for standard open repair due to medical comorbidities such as chronic

obstructive airway disease and untreatable coronary artery disease and recent stroke. The stents were custom made for each patient and in one case measurement was inaccurate leading to conversion to an open repair, however the follow up of the other 4 patients was satisfactory and the concept was proven to be valid. In parallel to the work of Parodi was the work of Prof. Nikolai Volodos, a Russian surgeon who was the first to develop a self-expanding and self-fixating stent graft in 1983 [Svetlikov 2014]. Prof. Volodos subsequently utilized this stent graft design for iliac artery stenting in occlusive disease, abdominal aortic aneurysm repair and treatment of thoracic aortic aneurysms. It is possible that the work of Volodos is less well accessed as he published in Russian journals.

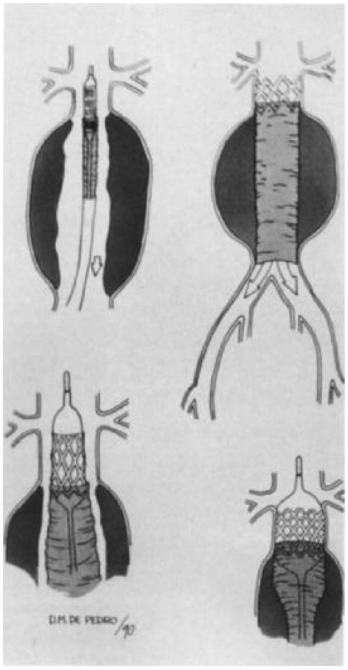

Figure 1.7: Diagram from the initial report on EVAR, modified from Parodi 1991.

Following on from this initial experience, large clinical trials were performed to determine the feasibility of EVAR. The EVAR 1 RCT was a UK based trial of 1252 patients (626 had EVAR, 626 had open repair) comparing open repair and EVAR. This

RCT demonstrated reduced 30 day mortality in EVAR patients when compared to open repairs, although there is greater expense due to increased post procedural intervention rates [United Kingdom EVAR Trial Investigators 2010(b)] and a meta-analysis has also shown EVAR can be used to treat AAA rupture [Rayt *et al.* 2008]. The "The Immediate Management of Patients with Rupture: Open Versus Endovascular Repair –IMPROVE" trial, over the 46 month trial period 623 patients were randomized, 316 to endovascular treatment and 297 to open repair [IMPROVE trial investigators 2014]. This trial showed that patients treated with EVAR did not have significantly increased 30 day mortality when compared to open repair, but they would be more likely to return to their own home and also had a significant reduction in cost. The EUROSTAR (European Collaborators on Stent/graft techniques for aortic aneurysm repair) registry commenced recruitment of patients undergoing EVAR from 1996 [Harris *et al.* 2000]. A report of the collated EUROSTAR data in the year 2000, when 2464 patients had been registered, demonstrated that there was an association between and endovascular repair and late device failure, around 3% per year. A recent meta-analysis has been published which analysed 25078 patients who underwent endovascular repair and 27142 who underwent open repair [Stather *et al.* 2013(b)]. This analysis confirmed the reduction in 30 day mortality in patients who underwent EVAR, 1·3% compared to 4·7% for open repair (odds ratio 0·36, 95% confidence interval 0·21 to 0·61, P value = <0·001). This improved mortality is often quoted as one of the main benefits of EVAR. This analysis, however, demonstrated that by 2 years this benefit was no longer present, 14.3% mortality in the EVAR group and 15.2% in open repair group (odds ratio 0·87, 95% confidence interval 0·72 to 1·06; P value = 0·17). Re-intervention was more common in the EVAR group, (odds ratio 2.08, 95%

confidence interval 1.27 to 3.39; P value = 0·003) and postoperative rupture was much more common in the EVAR cohort (odds ratio 5.94, 95% confidence interval 2.33 to 14.14, P value = <0.001). It is likely that these re-interventions and aneurysm ruptures are increasing the mortality rate of the EVAR patients, leading to the loss of benefit from EVAR. However the counter argument will be that with increasing experience of EVAR and newer and better stent grafts, over time the benefits of EVAR will return.

1.7.3.1 Fenestrated EVAR

An early limitation of endovascular repair was that the treated aneurysm could not be juxtarenal, as satisfactory proximal fixation of the graft would occlude the origins of the aortic branch arteries such as the left and right renal arteries, the superior mesenteric artery and the coeliac axis. This has now been addressed with fenestrated EVAR, where the stent graft has predetermined side holes *in situ*, side branch stents or fenestrations are then placed from within the stent graft in order to maintain blood flow to the bowel or kidneys whilst still excluding the aneurysm from the systemic circulation (Figure 1.8) [Browne *et al.* 1999].

1.7.3.2 Branched EVAR

An alternative to FEVAR is a custom made graft where there are individual endo stents present, preloaded in the main body of the graft, a branched EVAR (BEVAR). These branched endografts have a directional bias, either cranially or caudally. There is less experience with these grafts and they are mainly utilised in the treatment of

thoracoabdominal aneurysms. Initial data demonstrates that BEVAR has favorable 30 day mortality when compared to elective open repair, 6.7 % vs 22.3% [Verhoeven *et al.* 2009].

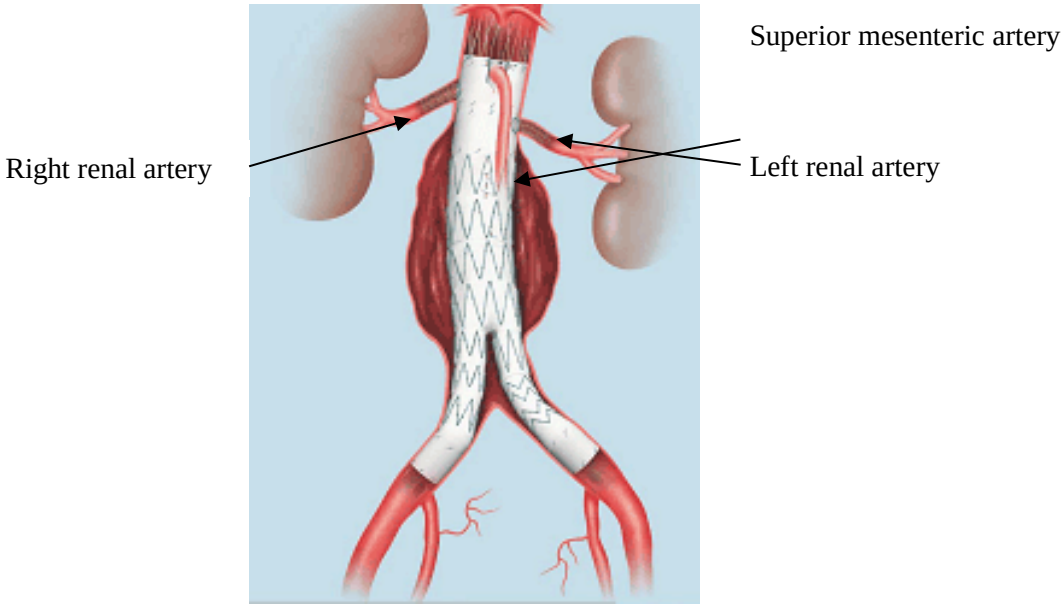

Figure 1.8: Schematic of fenestrated endovascular repair of an aneurysm (EVAR), this is the Zenith fenestrated device from Cook (Bloomington, Illinois, USA). Fenestrations from the main graft are seen within the left and right renal arteries. Image modified from www.fda.gov.

1.7.3.3 Complications of EVAR

There are several complications associated with EVAR, ranging from problems in the femoral vessels, damage to the kidneys, ischaemic injury and issues with the stent itself such as endoleak. The passage of large gauge access sheaths can lead to thrombosis, dissection and pseudoaneurysm of the femoral and iliac arteries. As intraoperative fluoroscopy is required to ensure correct placement of the graft and to perform post procedure angiography, intravenous contrast is utilized and contrast is nephrotoxic. Contrast induced nephropathy has been reported in 6.7% of cases [Wald *et al.* 2006]. Additionally renal function can be damaged by the occlusion of the renal

artery by the stent of by aneurysm thromboemboli during manipulation of the stent during deployment. Renal failure requiring dialysis within 1 year of EVAR has been reported at 1.1%, not significantly greater than in open repair in an RCT [Lederle *et al.* 2008]. Distal limb ischemia leading to amputation within 1 year of EVAR was reported at a rate of 0.2%, not significantly different to the rate observed in the open repair group, however the rate of new or worsening claudication was greater in the EVAR group when compared to the open group (8.3 vs 4.6%, P =0.02) [Lederle *et al.* 2008]. Endoleak is a term used to describe persistent blood flow within an aneurysmal sac post procedure, it was first proposed by White *et al.* in 1996 and further subdivided in to types depending upon the source of the bleeding [White, Yu and May 1996, White *et al.* 1998]. Type 2 are the most common type of endoleak, where side branches of the aorta, such as the lumbar arteries, are the source of bleeding can usually safely be managed without intervention [Sidloff *et al.* 2014 (b)]. Type 1 endoleaks result from inadequate seal of the graft to the aorta either proximally or distally, and due the persistent blood flow within the sac Type 1 endoleaks require urgent treatment with stents or stent graft extensions [Stavropoulos *et al.* 2007]. Type 3 endoleaks are less common and are the result of structural failure of the graft, fractures, holes in the graft fabric, they are considered to be dangerous due to rapid re-pressurisation of the sac. They are often treated with stent graft extensions to cover the area of defect [Stavropoulos *et al.* 2007]. Type 4 endoleaks are due to graft porosity, they are self-limiting, the leak stops once the patients coagulation system returns to normal (patients are anticoagulated with intra venous heparin intra-operatively) [Stavropoulos *et al.* 2007].

1.7.3.4 Endovascular Aneurysm Sealing

A further development in the technique of EVAR is a the Nellix system (Endologix Inc., Irvine, CA, USA), whereby the aneurysm sac is occluded with expanding endobags that fill with a polyethylene glycol polymer aside from two stents that facilitate the continuing flow of blood. This technique expands the range of aneurysm neck anatomy that can be treated endovascularly. Initial experience was positive with a perioperative mortality rate of 2.9%, albeit in a small series of 34 patients [Krievins *et al.* 2011].

1.7.4 Surgical Treatment Conclusion

Therefore surgical options of treatment are diversifying; with a shift to endovascular methods from the traditional open repair. Both open repair and EVAR have risks and benefits, and only by presenting a patient with these risks can an informed consent be achieved. Data from the USA suggests that the use of EVAR in elective aneurysm repair was more common than that of open techniques by the year 2006, with EVAR used in excess of 70% of patients [Schwarze *et al.* 2009]. This is supported by the recent data from the National Vascular Database (NVD) of the British Vascular Society, between January 1st 2010 and December 31st 2012, 13413 elective repairs were carried out and 8712 (65.0%) of these were endovascular repairs [Waton *et al.* 2013]. This can be compared to data from the NVD from between 2006 and 2008, in this time frame

6854 AAA repairs were recorded, 2552 being EVAR (37.2%), demonstrating a progressive move toward EVAR from open repair.

1.8 Chapter 1 Conclusion

AAA are a significant burden upon worldwide health services, affecting predominantly older males. If left untreated rupture of an AAA has a 100% mortality. National screening has been established as it was proven to be beneficial in preventing death in patients diagnosed with AAA. The pathogenesis of the disease involves progressive reduction in the mural extracellular matrix of the artery, the extra cellular matrix, together with an accumulation of inflammatory within the arterial wall and a reduction in the number of vascular smooth muscle cells. This progressive weakening leads to progressive dilatation to the point where the mechanical strength of the wall is insufficient to resist the pressure of blood within it, leading the aneurysm rupture. AAA are associated with male gender, positive family history and smoking. The only effective treatment is surgical, using a prosthetic graft to replace the diseased segment of artery. Open repair has over 50 years of clinical use and was until relatively recently the only option, however EVAR is now a proven, safe and more popular treatment.

Chapter 2: Genes and Abdominal Aortic Aneurysms

2.1 AAA Genetics

There is a diverse collection of genes that have been implicated in aortic aneurysmal disease. One method of identifying these candidate genes is to relate the gene function to one of the pathological features of AAA. There are several hallmarks of aneurysmal disease, such as degradation of the extracellular matrix and chronic inflammation and the genes of the proteins involved in these processes have been analysed for association with AAA. Aside from loci involved in these pathological stages there have also been analyses of genes that are related to other cardiovascular diseases, which share risk factors with AAA.

2.1.1 Single Nucleotide Polymorphisms

The commonly studied genetic polymorphisms are SNPs (single nucleotide polymorphisms). That is at a given position within the genome there is variation in the nucleotide at that position. For instance in a population 90% of individuals may have a cytosine nucleotide at position 100, whereas a thymine nucleotide is seen at position 100 in the other 10%. SNPs nomenclature is based upon the description of the polymorphism, the gene name followed by the position and nature of the polymorphism. SNPs can be identified with a unique rs (refSeq) number (e.g. rs10757278) which each polymorphism is given upon entry of the NCBIs (National Centre for Biotechnology Information) SNP database (dbSNP) [Yu *et al.* 2009]. The alternative is the descriptive format, an example is a cytosine (C) to thymine (T) polymorphism at the 1562 base position in the matrix metalloproteinase 9 gene would be shown as MMP9 1562 C>T. Variation in SNP frequency between those with and

without the disease can be assessed in case control studies, leading to the determination of whether there is a statistical association between genotype and disease [Clark *et al.* 2010].

It is rare that a SNP is directly responsible for a disease as the majority of the polymorphisms do not occur in exons and are therefore not expressed and even those within exons may not be expressed. SNPs within intronic regions can affect gene expression via altering the binding of transcription factors or sites for alternative splicing of genes. SNPs within gene promoter regions, which are also non coding, have also been shown to regulate gene expression [Kim *et al.* 2008].

The statistical analysis of the frequencies of SNPs in cases and controls can be used, generating P values and odds ratios (OR). Where a SNP has been analysed in several distinct cohorts the combination of analyses is achieved with a meta-analysis. The method I utilised was to use the metagen function on "R", calculating a combined association using ORs [Ihaka and Gentleman, 1996].

SNPs are a valuable tool in the genetic analysis of disease and there have been many genes implicated in AAA due to variations in SNP frequency of cases and controls.

In order to present data regarding genes implicated in AAA the reported data has been reviewed and grouped based upon the pathological process that the gene affects, extracellular matrix degradation, accumulation of inflammatory cells and inflammation and genes involved cardiovascular disease.

2.2 Extracellular matrix degradation

As extracellular matrix (ECM) degradation is a key stage in aneurysm development and progression genes involved in the synthesis of matrix proteins and enzymes that degrade these essential structural proteins have been extensively examined for association with AAA.

2.2.1 Elastin

Elastin is an essential structural protein within the ECM, and contributes to the elastic capacity of the arterial wall [Dobrin 1984]. A polymorphism within the *ELN* (Elastin) gene, 1355G>A (rs2071307) which causes a glycine to serine substitution at amino acid position 422, was studied in an Italian population and no significant association was found [Massart *et al.* 2004]. Ogata *et al.* subsequently analysed the same polymorphism in Canadian and Belgian cohorts, again there was no significant association [Ogata *et al.* 2005]. A separate Italian study analysed the same SNP in 846 individuals [Saracini *et al.* 2012]. There was a significant difference in the minor allele frequencies observed in the cases and controls, 38.2% v 42.8% P value = 0.22. Saracini *et al.* further analysed rs2071307 as part of a logistic regression along with age, gender, hypertension, diabetes mellitus, COPD, smoking history and hyperlipidaema. The results of this regression were that rs2071307 was an *independent* protective factor for AAA, odds ratio = 0.64 (95% confidence interval 0.41 to 0.99, P value = 0.046). Due to this positive association Saracini *et al.* undertook a meta-analysis of all published data pertaining to rs2071307 (AAA patients n = 904, controls n = 1069). The resulting analysis was non-significant with a P value =0.25 with an odds ratio = 0.79

(95% confidence interval 0.53 to 1.18, I^2= 72%). The findings of Saracini *et al.* were interesting as the direction of effect was the opposite to the other published data, where the minor allele tended toward association with AAA as opposed to being protective (Table 2.1). Therefore this *ELN* polymorphism is not significantly associated with AAA and no other SNP within *ELN* has been demonstrated to associate with AAA.

2.2.2 Collagen

Collagen fibres are prominent in the ECM. Though type 1 collagen contributes two thirds of the total collagen content of the aortic wall, type 3 collagen (encoded by *COL3A1*) makes up the remaining one third and it is this type of fibre that provides collagens elastic recoil to the systemic blood pressure [Powell *et al.* 1993]. A polymorphism within the *COL3A1*gene, 581C>T, was analysed in a Canadian cohort and a Belgian cohort, there was no association between this polymorphism to AAA [Ogata *et al.* 2005] (Table 2.1).

2.2.3 ECM protein Summary

Genes that encode the proteins of the ECM, such as elastin (*ELN*) and type 3 collagen (*COL3AI*) contain polymorphisms that have been associated with AAA in case-control studies. These associations have been demonstrated in studies with small numbers of patients. The *ELN* SNP rs2071307 has more published data than any other ECM protein SNP, meta-analysis of all the available data (1917 individuals) failed to report a significant association with AAA, however analysis of the data with an Armitage Trend test did reveal a significanr association in the Belgian cohort analysed by Ogata *et al.* and the Italian study from Saracini *et al* (Table 2.1) [Ogata *et al.* 2005, Saracini *et al.* 2013]. However polymorphisms within these genes are yet to conclusively associate with AAA as meta-analysis of published data was did not show a significant association odds ratio 0.72 (95% confidence interval 0.94 to 1.10, P value 0.72).

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
***ELN* (Elastin)**	1355G>A (rs2071307)	i)99:225 ii)207:269 *iii)207:156* iv)423:423	0.95, 1.00 (0.91-1.10) 0.83, 0.95 (0.62-1.47) *0.05, 0.76 (0.59-1.00)* 0.05, 1.19 (1.00-1.41)	Massart 2004 Ogata 2005 (Canada) *Ogata 2005 (Belgium)* Saracini 2012
	Meta-analysis*	**904:1069**	**0.72, 1.01, (0.94-1.10)**	**Wild**
***COL3AI* (Type 3 Collagen)**	581C>T	i)207:269 ii)207:156	0.67, 0.94 (0.71-1.25) 0.69, 0.92 (0.61-1.39)	Ogata 2005 (Canada) Ogata 2005 (Belgium)
	Meta-analysis*	**414:425**	**0.56, 0.93 (0.74-1.18)**	**Wild**

Table 2.1. Polymorphisms within ECM protein genes and associations with AAA (analysed with Armitage Trend Test). Significant results italicized, *= Inverse Variance method, Fixed Effects Model.

2.3 Matrix Metalloproteinases

The MMP (matrix metalloproteinase) gene family are proteases that degrade several essential structural proteins of the arterial ECM, including elastin and collagen. In a small scale study comparing the protein content of aneurysmal and control aortic tissue high levels of MMPs were observed [Yoon *et al.* 1999]. Though MMPs 1, 2, 3, 9 and 12 were all found to have increased levels in aneurysmal tissue it was levels of MMP9 that are most consistently reported to be elevated in AAA

2.3.1 MMP9

MMP9 (synonyms: 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B) is a gelatinase that has been found within macrophages in the aortic wall in AAA patients and also found in elevated levels in the serum of AAA patients [Norman 1994, Hovsepian 2000]. Yoon *et al.* were the first to investigate an association between AAA and an MMP9 polymorphism, a dinucleotide repeat (CA), found within the promoter region of the *MMP9* gene on chromosome 20q11.2-q13.1 [Yoon *et al.* 1999]. However there was no association between this MMP9 polymorphism and AAA. Armani *et al.* also found no significance in the CA dinucleotide repeat and AAA [Armani *et al.* 2007]. Subsequently other polymorphisms in *MMP9* have also been analysed, the MMP9-1811A>T SNP (rs3918241) was genotyped in an Australian study, there was not a significant association between this polymorphism and AAA [Smallwood *et al.* 2008]. rs3918242 was analysed for association with AAA in eight cohorts, only one found a significant association, Jones *et al.* (odds ratio = 2.41, 95% confidence interval 1.44 to 4.02, P value = 0.0008) [Smallwood *et al.* 2008, Ogata *et al.* 2005, Saracini *et al.* 2012,

Jones *et al.* 2008, Saratzis *et al.* 2015, Armani *et al.* 2007]. A UK study also demonstrated that rs3918242 did not influence AAA growth [Eriksson *et al.* 2005].

Polymorphisms with the *MMP9* gene have received more investigative attention than most other genes associated with AAA. Despite the relatively large numbers of individuals analysed a recent meta-analysis of the published data (2191 cases and 2013 controls) conducted by Morris *et al.*, failed to demonstrate a positive association between the MMP9 SNP, rs3918242, and AAA in either the dominant, recessive or additive models in inheritance [Morris *et al.* 2014].However this meta-analysis did not include the data from Saratzis *et al.* As the analysis by Morris *et al.* did not inlcude the data of Saratzis *et al.* I performed a new meta analysis including these data. The addition of this data did not demonstrate a significnat associaiton between rs3918242 and AAA, odds ratio = 0.97 (95% confidence interval 0.94-1.01, P value = 0.11) (Table 2.2). A model proposed by Duellman *et al.* is that a combination of SNPs are required to associate with AAA, this may be a more accurate way of approaching the problem [Duellman *et al.* 2012].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
MMP9	CA Nucleotide repeat	i)47:165 ii)144:154	0.82[#], 0.23[#]	Yoon 1999 Armani 2007
	rs3918241	i)678:659	0.24, 1.21 (0.93-1.36)	Smallwood 2008
	rs3918242	i)678:659	0.22, 1.14 (0.94-1.41)	Smallwood 2008
		ii)207:269	0.70, 0.91 (0.57-1.45)	Ogata 2005 (Canada)
		iii)207:156	0.66, 0.92 (0.62-1.35)	Ogata 2005 (Belgium)
		iv)423:423	0.63, 1.07 (0.78-1.08)	Saracini 2012
		v)144:154	0.88, 1.00 (0.96-1.04)	Armani 2007
		vi)414:203	*0.0008, 0.62 (0.43-0.89)*	*Jones 2003*
		vii)340:389	0.4, 0.85 (0.56-1.28)	Saratzis 2015 (Greece)
		viii)394:389	0.12, 1.35 (0.91-1.98)	Saratzis 2015 (Britain)
	Meta-analysis*	**2807:2642**	**0.11, 0.97 (0.94 – 1.01)**	**Wild**

Table 2.2: MMP9 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test, # = Fishers Exact Test), significant associations italicised (*= inverse variance, fixed effects model meta-analysis)

2.3.2 MMP2

Another member of this protease family that has been implicated in AAA is MMP2 (synonyms: gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase). Polymorphisms within the *MMP2* gene, located on chromosome 16q13-q21, have also been screened for association with AAA. The MMP2-1306C>T (rs243865) polymorphism did not have a significant effect on aneurysm growth [Eriksson *et al.* 2005]. 955A>C, did not associate with AAA [Ogata *et al.* 2005]. A German study genotyped 18 SNPs in MMP2 and none of these SNPs reached a statistically significant association with AAA [Hinterseher *et al.* 2006]. rs243865 (1306C>T) and rs1053605 (649C>T) did not associate with AAA after multivariate analysis, however in an Armitage trend test there was an association between rs1053605 and AAA (OR 1.38 (95% confidence interval 1.06-1.80, P value = 0.02) however AAA cases in this series the genotypes of the AAA cases did deviate from Hardy Weinberg Equilibrium [Smallwood *et al.* 2009]. rs2285053 and rs243866 did not associate with AAA, however

rs243865 did positively associate with AAA (OR = 0.55 (95% confidence interval 0.34 to 0.85, P value = 0.007)) [Saracini *et al.* 2012]. This SNP did have a significant association with AAA following meta-analysis of the published data (OR of 1.23 (95% confidence interval 1.05 to 1.45, P value = 0.01) (Table 2.3). The 1306C>T SNP affects the function of a promoter region within MMP2, with impairment of the activity of the SP1 promoter and the oestrogen receptor binding, this creates a plausible explanation for this SNP to directly affect AAA [Harendza *et al.* 2003].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
MMP2	rs243865	*i)423:423* ii)678:659	*0.007, 1.36 (1.09-1.70)* 0.34, 1.12 (0.88-1.41)	*Saracini 2012* Smallwood 2009
	Meta-analysis*	***1090:1077***	***0.01, 1.23 (1.05-1.45)***	***Wild***
	955A/C	i)207:269 *ii)207:156*	0.87, 1.03 (0.73-1.45) *0.05, 1.40 (1.00-1.96)*	Ogata 2005 (Canada) *Ogata 2005 (Belgium)*
	Meta-analysis*	**414:425**	**0.13, 1.20 (0.95-1.53)**	**Wild**
	rs1053605	*678:659*	*0.02, 1.38 (1.06-1.80)*	*Smallwood 2009*
	rs2285053	423:423	0.37, 0.91 (0.76-1.11)	Saracini 2012
	rs243866	423:423	0.08, 1.23 (0.97-1.58)	Saracini 2012

Table 2.3: MMP2 Polymorphisms screened for association with AAA (Armitage Trend Test), significant associations italicised (*= inverse variance, fixed effects model meta-analysis)

2.3.3 MMP3

Aside from gelatinases, other members of the MMP family have been screened for association with AAA. MMP3 (synonyms: stromelysin 1, pro-gelatinase) is capable of degrading several types of collagen. The *MMP3* gene is located on chromosome 11q22.3. MMP3-1171 5A>6A did not affect aneurysm growth rate [Eriksson *et al.* 2005]. Additionally rs3025058 has been tested for association with AAA in 5 studies, Ogata *et al.* and Yoon *et al.* did not show a significant association with this SNP and AAA [Ogata *et al.* 2005, Yoon *et al.* 1999]. However in 2 further studies the rs3025058

polymorphism demonstrated a significant association between -1612 5A>6A and aneurysms, odds ratio = 1.61 (95% confidence interval 1.14 to 2.28, P value = 0.007) and odds ratio = 1.32 (95% confidence interval 1.09 to 1.60, P value = 0.005) respectively [Saracini *et al.* 2012, Deguara *et al.* 2007]. A meta-analysis of all these studies and 2 additional cohorts was performed (Table 2.4). The result is that rs3025058 does significantly associate with AAA, OR = 1.21 (95% confidence interval 1.11 to 1.32, P Value = $1.26x10^{-5}$). rs3025058 is located within a promoter region of the MMP3 gene. MMP3 polymorphisms have been contradictory in relation to AAA and despite significance in moderately sized studies; the meta-analysed data proves that there is a relationship with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% Confidence Interval)	Reference
MMP3	rs3025058	i) 207:269	0.19, 1.22 (0.91-1.65)	Ogata 2005 (Canada)
		ii)207:156	0.96, 0.98 (0.50-1.93)	Ogata 2005 (Belgium)
		iii)47:174	0.07, 1.12 (0.99-1.26)	Yoon 1999
		iv)423:423	*0.007, 1.61 (1.14-2.28)*	Saracini 2012
		v) 405:405	*0.005, 1.32 (1.09-1.60)*	Deguara 2007
		vi)351:391	0.29, 1.18 (0.87-1.60)	Saratzis 2015 (Greece)
		vii)392:368	*0.04, 1.42 (1.01-1.98)*	Saratzis 2015 (Britain)
	Meta-analysis	***2005:2086***	***$1.26x10^{-5}$, 1.21 (1.11-1.32)***	***Wild***

Table 2.4: MMP3 Polymorphisms screened for association with AAA (Armitage Trend Test), significant associations italicised (*= inverse variance, fixed effects model meta-analysis)

2.3.4 MMP13

MMP13 (synonyms: collagenase 3, CLG3, MANDP1), degrades collagen types 1, 2 and 3. MMP13 is located on chromosome 11q22.3, and an MMP13 SNP, rs2252070, has been associated with AAA by Saracini *et al.* odds ratio of 1.29 (95% confidence interval 1.06 to 1.58, P value = 0.01) [Saracini *et al.* 2012]. However Ogata *et al.* found no

significant association between the same SNP and AAA [Ogata *et al.* 2006]. The combination of these results via meta-analysis determined that the association between MMP13 and AAA remained significant with an odds ratio of 1.37 (95% confidence interval 1.04 to 1.82, P value = 0.027) [Saracini *et al.* 2012]. This is in contrast to the meta-analysis performed by Morris *et al.*, where no significance was seen when the same data was meta-analysed in a variety of models based upon potential modes of inheritance (additive, dominant or recessive). This brings doubt to the significance reported by Saracini *et al*. Therefore using the published data, I calculated the common odds ratio with an Armitage trend test and then performed a meta-analysis with metagen on "R" [R Core Team 2013] (Table 2.5). Meta-analysing the data from Ogata *et al.* and Saracini *et al.* does lead to a positive association between AAA and MMP13 when a fixed but not random effects model is utilized (odds ratio = 1.18 (95% confidence interval 1.02 to 1.34, P value = 0.03)). This analysis demonstrates that MMP13 does associate with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% Confidence Interval)	Reference
MMP13	rs2252070	i)207:269	0.40, 1.31 (0.85-1.51)	Ogata 2005 (Canada)
		ii)207:156	0.82, 0.96 (0.68-1.35)	Ogata 2005 (Belgium)
		iii)423:423	*0.01, 1.29 (1.06-1.58)*	*Saracini 2012*
	Meta-analysis	***837:848***	***0.03, 1.18 (1.02 – 1.34)***	***Wild***

Table 2.5: MMP13 Polymorphisms screened for association with AAA (Armitage Trend Test), significant associations italicised (*= inverse variance meta-analysis)

2.3.5 MMP1

MMP1 (interstitial collagenase) is a gene located on chromosome 11q22.3 (Figure 2.1), it encodes an enzyme that degrades collagen types 1, 2 and 3, and due to alternative splicing multiple transcripts of the gene are possible. This gene is one of several

members of the MMP family located on chromosome 11q22.3. rs1799750 has been analysed for association with AAA in 3 cohorts no significant association with AAA was demonstrated [Saracini *et al.* 2012, Ogata *et al.* 2005] (Table 2.6). Despite biological plausibility for causality in AAA, MMP1 studies to date have failed to prove an association with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% Confidence Interval)	Reference
MMP1	rs1799750	i) 207:269	0.63, 1.09 (0.77-1.55)	Ogata 2005 (Canada)
		ii)207:156	0.58, 0.93 (0.73-1.19)	Ogata 2005 (Belgium)
		iii)423:423	0.96, 1.00 (0.86-1.18)	Saracini 2012
	Meta-analysis*	**837:848**	**0.90, 0.99 (0.87-1.13)**	**Wild**

Table 2.6: MMP1 Polymorphisms screened for association with AAA (Armitage Trend Test).(* = Inverse variance, fixed effects model meta-analysis)

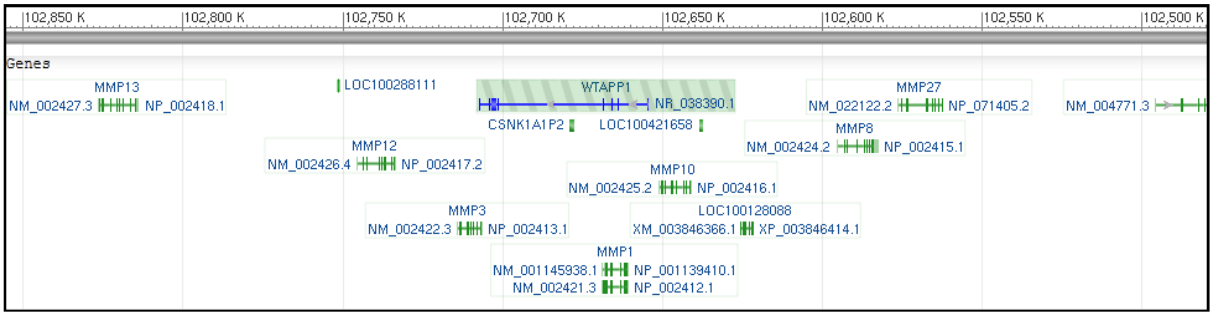

Figure 2.1: This is a screen image from the NCBI: Gene (ncbi.nlm.nih.gov/gene/) database, it illustrates the position of several genes located at chromosome 11q22.3.

2.3.6 MMP12

Another member of the MMP gene family located on chromosome 11q22.3 is MMP12 (synonym: macrophage elastase). The active MMP12 enzyme degrades elastin in both its soluble and insoluble forms. The MMP12 polymorphism rs2276109 has been genotyped in 3 cohorts and there was not a significant association with AAA [Saracini

et al. 2012, Ogata *et al.* 2005] (Table 2.7). Saratzis *et al.* genotyped rs2276109 in a Greek and a British cohort, there was still a significant association with AAA in the British cohort, OR 1.44 (95% Confidence Interval 1.06 to 1.96, P value = 0.02) [Saratzis *et al.* 2015]. The same SNP was analysed in a British cohort where it was determined that the polymorphism does not affect aneurysm growth rates [Eriksson *et al.* 2005]. Therefor these SNPs within MMP12 lack sufficient evidence to be associated with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% Confidence Interval)	Reference
MMP12	rs2276109	i) 207:269	0.97, 0.96 (0.14-6.43)	Ogata 2005 (Canada)
		ii)207:156	0.97, 0.96 (0.11-8.08)	Ogata 2005 (Belgium)
		iii)423:423	0.99, 0.97 (0.01-143.19)	Saracini 2012
		iv)348:393	0.62, 1.07 (0.82-1.41)	Saratzis 2015 (Greece)
		v)379:370	*0.02, 1.44 (1.06-1.96)*	*Saratzis 2015 (Britain)*
	Meta-analysis*	**1564:1611**	**0.09, 1.21 (0.99-1.49**	**Wild**

Table 2.7: MMP12 Polymorphisms screened for association with AAA (Armitage Trend Test), significant associations italicised. Significant results italicised, *= Inverse variance, fixed effects meta-analysis

2.3.7 MMP10

MMP10 (synonyms: stromelysin 2, SL-2; STMY2) is an enzyme that can degrade fibronectin and proteoglycans, both ECM component proteins, and is another gene found in the MMP cluster on chromosome 11q22.3. rs486055 leads to a arginine to lysine substitution at amino acid 53 in the chain that makes up the MMP10 protein, it has been analysed in the same 2 studies, there was no significant association [Saracini *et al.* 2012, Ogata *et al.* 2005] (Table 2.8). MMP10 is not associated with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% Confidence Interval)	Reference
MMP10	rs486055	i) 207:269	0.34, 0.82 (0.55-1.23)	Ogata 2005 (Canada)
		ii)207:156	0.32, 1.30 (0.78-2.16)	Ogata 2005 (Belgium)
		iii)423:423	0.76, 1.09 (0.63-1.87)	Saracini 2012

	Meta-analysis	**837:848**	**0.95, 1.01 (0.77-1.32)**	**Wild**

Table 2.8: MMP10 Polymorphisms screened for association with AAA (Armitage Trend Test), significant associations italicised. *= Inverse variance, fixed effects meta-analysis

2.3.8 1 Tissue Inhibitors of Matrix Metalloproteinases

The TIMP (Tissue Inhibitor of Matrix Metalloproteinase) family are 4 protease inhibitors that regulate MMPs by inhibiting them, therefore these proteins have the potential to affect aneurysm formation via reduction of MMP mediated degradation of the extracellular matrix.

2.3.8.2 TIMP1

In 1999 Wang *et al.* analysed an American cohort for the association of a TIMP1 polymorphism, 434C>T (rs4898) and AAA [Wang *et al.* 1990]. In this instance there was no significant association between this SNP and AAA. No association between rs4898 and AAA was found by Ogata *et al.* however Saracini *et al.* did report a significant difference in these frequencies, P value = <0.0001 [Ogata *et al.* 2005, Saracini *et al.* 2012]. Available data was meta-analysed and no significance was found in the association between rs4898 and AAA [Morris *et al.* 2014]. This analysis utilised the available data (141 AAA and 340 controls) on female patients as the TIMP1 gene resides on the X chromosome (Table 2.9). Wang *et al.* also genotyped another polymorphism in the *TIMP1* gene, the 323C>T SNP, there was no significant difference in the frequencies of this polymorphism between male cases or controls [Wang *et al.* 1999]. Ogata *et al.* also screened their study group for rs2070584 [Ogata *et al.* 2005]. In an unadjusted analysis this SNP did not associated with AAA, however when males without a family history were analysed there was a significant association between rs2070584 and AAA.

The TIMP1 polymorphism, -372C>T does not affect growth rates of AAA [Powell *et al.* 2006]. Three TIMP1 polymorphisms have been analysed in case control studies for potential association with AAA and none of these have proved to be associated with AAA.

Gene	Polymorphism	Participants (case:control)	P value	Reference
TIMP1	rs4898	i)29:64 male	1.00, 1.37 (0.14-13.83)	Wang 1990
		22:20 female	0.29, 0.55 (0.19-1.65)	Wang 1990
		ii)*152:32 male*	*0.03, 0.40 (0.18-0.88)*	*Ogata 2005 (Canada)*
		50:123 female	0.80, 1.08 (0.59-1.97)	Ogata 2005 (Canada)
		iii)157:178 male	0.44, 0.83 (0.54-1.28)	Ogata 2005 (Belgium)
		85:18 female	0.84, 0.92 (0.38-2.18)	Ogata 2005 (Belgium)
		iv)44:9 male£	1.00, OR n/a	Hinterseher 2007
		6:19 female£	1.00, OR n/a	Hinterseher 2007
		v)423:423$	*0.0002, 0.20 (0.09-0.46)*	*Saracini 2012*
	Meta-analysis*	**338:274 Male**	**0.77, 0.71 (0.49-1.04)**	**Wild**
		157:161 Female	**0.73, 0.92 (0.59-1.45)**	**Wild**
	323C>T	i)29:64 male	0.50, 1.47 (0.61-3.54)	Wang 1990
		22:20 female	0.002, 4.44 (1.77-11.17)	Wang 1990
		ii)44:20 male	0.029, 4.38 (1.14-20.47)#	Hinterseher 2007
		12:48 female	0.10, 0.27 (0.05-1.23)#	Hinterseher 2007
	rs2070584	i) *152:32 male*	*0.03, 0.40 (0.18-0.88)*	*Ogata 2005 (Canada)*
		51:123 female	0.91, 1.06 (0.40-2.80)	Ogata 2005 (Canada)
		ii) 157:178 male	0.44, 0.83 (0.54-1.28)	Ogata 2005 (Belgium)
		86:19 female	0.75, 1.14 (0.53-2.45)	Ogata 2005 (Belgium)
	Meta-analysis*	**309:210**	**0.07, 0.70 (0.48-1.02)**	**Wild**
		137:142	***0.01, 1.07 (1.02-1.13)***	***Wild***

Table 2.9: TIMP1 Polymorphisms screened for association with AAA (Armitage Trend Test, # = Fishers Exact Test). Significant results italicised (* = inverse variance meta-analysis, £ = excluded due to statistical abnormality, $ = excluded as results presented for whole cohort, not as male and female)

2.3.8.3 TIMP2

TIMP2 is located on chromosome 17q25 (synonyms: metallopeptidase inhibitor 2, CSC-21K) and like the other members of the family it affects activity of MMPs via inhibition. Wang *et al.* examined two *TIMP2* polymorphisms, 306C>T did not associate with AAA, however 573G>A, did have a statistical association with AAA when males were

analysed in isolation, the G allele was significantly more common in the cases than in the controls (G frequency cases = 0.91, controls = 0.80, P = 0.03) [Wang *et al.* 1999]. Hinterseher *et al.* analysed six TIMP2 SNPs but none were associated with AAA, additionally this paper was published in German and has been inaccessible [Hinterseher *et al.* 2008]. Similarly rs2009196 did not associate with AAA [Ogata *et al.* 2005]. TIMP2 lacks association with AAA but no SNP has been investigated in adequate numbers to make a reasoned decision on its involvement in AAA pathogenesis (table 2.10).

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
TIMP2	306C>T	84:51	0.58, 0.71 (0.21-2.39)	Wang 1999
	573G>A	*84:82*	*0.03, 2.15 (1.08-4.29)*	*Wang 1999*
	rs2009196	i) 207:269	0.59, 1.14 (0.71-1.84)	Ogata 2005 (Canada)
		ii)207:156	0.34, 0.92 (0.78-1.09)	Ogata 2005 (Belgium)
	Meta-analysis*	**414:425**	**0.47, 0.94 (0.80-1.11)**	**Wild**

Table 2.10: TIMP2 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.3.8.4 TIMP3

TIMP3 (synonyms: SFD; K222; K222TA2; HSMRK222) is located in chromosome 12q12.3 and is also a regulator of MMP activity. The 1296C>T (rs9619311) polymorphism did not associate with AAA in an unadjusted analysis from Ogata *et al.*, nor in the analysis from Saracini *et al.* [Ogata *et al.* 2005,Saracini *et al.* 2012]. Morris *et al.* meta-analysed all published data regarding rs9619311 and AAA (803 AAA, 845 controls) and no significance was determined (Table 2.11) [Morris *et al.* 2014]. TIMP3 does not associate with AAA.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
TIMP3	rs9619311	i) 207:269	0.04, 1.36 (1.02-1.82)	Ogata 2005 (Canada)
		ii)207:156	0.11, 0.77 (0.56-1.06)	Ogata 2005 (Belgium)
		iii)423:423	0.07 , 0.82 (0.66-1.02)	Saracini 2012
	Meta-analysis	**803:845**	**0.35, 0.93 (0.80-1.08)**	**Wild**

Table 2.11: TIMP3 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.3.9 Other MMP regulators

Aside from members of the TIMP family, MMP activity is also controlled by plasmin. Plasmin, a serine protease, activates MMPs. Plasmin levels are in turn regulated by plasminogen activator inhibitors (PAIs) which prevent the conversion of plasmin precursor, plasminogen, to active plasmin. PAIs achieve this by inhibiting plasminogen activators, either tPA (tissue plasminogen activator) or uPA (urokinase plasminogen activator). There is a polymorphism within the promoter of the PAI gene, a 4G/5G insertion/deletion. The 5G allele is associated with reduced transcription as it enhances the binding of a repressor [Rossaak *et al.* 2000]. However this same 4G/5G polymorphism did not associate with AAA in a Finnish population and also it did not influence the growth rates of small AAA [Yoon *et al.* 1999, Jones *et al.* 2002] (Table 2.12). The evidence for association of AAA with the PAI 4G/5G insertion/deletion arose from a small study and subsequent small studies have not shown a causal relationship.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
PAIs	4G/5G	i)190:163	0.49, 0.88 (0.62-1.23)	Rossaak 2000
	insertion/deletion	ii)47:174	0.92, 1.03 (0.64-1.64)	Yoon 1999
	Meta-analysis	**237:337**	**0.59, 0.93 (0.71-1.22)**	**Wild**

Table 2.12: PAI Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.3.10 MMP and AAA Summary

There is a wide variety of polymorphisms in the MMP genes, their natural inhibitors (TIMPs) and activators (Plasmin), and these polymorphisms have repeatedly been implicated in aneurysmal disease. The studies that have examined this relationship have been varied in both size and physical location though there have been discrepancies in the effect size. Some SNPs have shown inconsistent effects. These heterogeneous results may be due to geographical genetic variation, or may represent the small effect sizes not being accurately measured in small populations. Despite this there is strong evidence, from meta-analyses, that some MMP polymorphisms do associate with AAA.

2.4 Other Proteolytic enzymes that affect the ECM

The cathepsin gene family, like the MMP genes, are a group of proteases that degrade the ECM. There are cathepsins that degrade both elastin and collagen and therefore they are a plausible candidate for association with AAA. Some authors have identified the cathepsins as having the most potent elastolytic activity aside from the MMPs [Sukhova *et al.* 2006]. Initial evidence for the involvement of cathepsins in aneurysm pathology arose from the comparative analysis of normal aorta and aneurysmal tissue, where RNA from the aneurysmal tissue had a 30 fold increase in cathepsin H (CTSH) [Tung *et al.* 2001]. Histological analysis with specific stains of 32 aneurysmal aortas and 10 normal aortas (from organ donors) revealed increased levels of several members of the Cathepsin family (B, D, K, L and S) [Lohoefer *et al.* 2012]. In a genetic linkage analysis conducted in the Netherlands, several variable number tandem repeats within the *Cathepsin H* (*CTSH*) gene were analysed but did not reach a significant level of association [van Vlijmen-van Keulen *et al.* 2003]. In animal models of AAA the absence, by way of breeding knock-out species, of both cathepsin S and K has been shown to attenuate the development of induced AAA, whereas in mice lacking Cathepsin L, perfusion induction of AAA proved impossible [Sun *et al.* 2012, Qin *et al.* 2012, Sun *et al.* 2011]. In much the same way that MMP activity is regulated by TIMPs the activity of cathepsins are regulated by Cystatin C (CST3). CST3 has been found to be reduced in the aneurysmal tissue. Shi *et al.* demonstrated that serum levels of Cystatin C negatively correlated with increasing aortic diameter [Shi *et al.* 1999]. As with the MMP family, there are small studies with early positive findings that

lack validation. There is yet to be a SNP associated with AAA and until there is a significant investigation these genes will remain on the periphery of interest in AAA.

2.5 ECM degradation and genetic polymorphisms: Conclusion

Although ECM degradation is widely accepted as a pathological step in AAA formation there are few consistently associated polymorphisms in genes responsible for this degradation. Significant associations between AAA and MMP SNPs have been demonstrated in a variety of studies, though there is a tendency for the inclusion of relatively small numbers of patients. With no agreement in a single gene of interest for the ECM pathway it is plausible that the combined effect of several genes, as reported by Duellman *et al.*, and environmental factors will be ultimately responsible for this stage on aneurysm formation [Duellman *et al.*2012].

2.6. Inflammation and AAA

Inflammation is a key stage in aneurysm formation, with chronic transmural infiltration by macrophages and inflammatory mediated collagen degradation observed in AAA tissue analysis [Thompson *et al.* 2008]. Polymorphisms in various inflammatory mediators have been analysed, including the interleukin family, C reactive protein (CRP), human leukocyte antigen locus (HLA) genes, chemokines and several others.

2.6.1 Interleukins

Interleukins are a group of cytokines originally isolated from white blood cells however subsequently interleukins have been isolated from a variety of cell types. Association between AAA and interleukin gene family members 1, 6 and 10 have been investigated.

2.6.1.1 Interleukin 1

Interleukin 1 comprises a family of genes, including the interleukin 1 α (*IL1α*) gene 2q14 , interleukin 1 β (*IL1β*) and interleukin 1 receptor antagonist (*IL1RA*) all located on chromosome 2q14. Marculescu *et al.* analysed 6 SNPs in all, 2 in IL1α, 3 in IL1β and a further 1 in IL1RA, there was no significant association between these polymorphisms and AAA (Table 2.13) [Marculescu *et al.* 2005]. One of the IL1β

polymorphisms, 3953C>T was also studied in a UK population; again there was no significant association [Bown *et al.* 2003].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
Il1ß	−511 C>T	135:270	0.31, 1.22 (0.83-1.77)	Marculescu 2005
	−31 C>T	135:270	0.22, 1.24 (0.88-1.75)	Marculescu 2005
	3954 C>T	i) 135;270	0.21, 0.76 (0.50-1.16)	Marculescu 2005
		ii) 100:100	0.11, 1.79 (0.88-3.63)	Bown 2003
	Meta-analysis*	**235:370**	**0.79, 0.95 (0.66-1.37)**	**Wild**
IL1RA	−889 C>T	135:270	0.76, 0.93 (0.59-1.47)	Marculescu 2005
	4845 G>T	135:270	0.46, 0.89 (0.64-1.22)	Marculescu 2005

Table 2.13: Interleukin 1 gene family Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), * = inverse variance, fixed effects model meta-analysis

2.6.1.2 Interleukin 6

Interleukin 6 (*IL6*) is another member of the interleukin family that has been analysed for association with AAA. IL6 is located on 7p21. Levels of circulating IL6 have been reported to be greater in patients with AAA when compared to controls [Smallwood *et al.* 2008]. The IL6 -174G>C (rs1800795) SNP was analysed in patients with small aneurysms from the UK, the frequency of the C allele in this AAA population did not differ from a healthy population and there was no effect on growth rate [Jones *et al.* 2001]. Jones *et al.* also determined IL6 levels are higher in the iliac artery than the brachial artery in patients with large AAA. rs1800795 was also analysed in a separate UK population there was no difference in the frequency of the C allele [Bown *et al.*

2003]. The same polymorphism was genotyped in a large Australian study there was no difference in genotype frequency between cases and controls [Smallwood *et al.* 2008]. This study also determined the association of 2 other SNPs with AAA, the first, rs1800797 did not associate with AAA however the second, rs1800796, did [Smallwood *et al.* 2008]. The analysis of the rs1800796 genotypes did not differ between cases and controls, however in a multivariate analysis where a recessive model was assumed this SNP was an independent risk factor for AAA, OR 5.78 (95% confidence interval 1.18 - 28.42, P = 0.031) (Table 2.14) [Smallwood *et al.* 2008]. Despite the significance of this result the authors of this paper highlight that the rare nature of this polymorphism makes it unlikely to be involved in the majority of cases of AAA. This appears to be the only analysis of this polymorphism in AAA. The overriding concern with IL6 polymorphisms associating with AAA is that the relationship is not clear, is the aneurysm the result of the inflammation or the inflammation due to the presence of the aneurysm? This question has yet to be answered.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
Il6 (Interleukin 6)	rs1800795	i) 677:656 ii)100:100	0.38, 1.08 (0.91-1.28) 0.92, 1.00, (0.92-1.07)	Smallwood 2008 Bown 2003
	Meta-analysis*	**777:756**	**0.80, 1.01 (0.94-1.08)**	**Wild**
	rs1800796	677:656	0.57, 0.88 (0.56-1.38)	Smallwood 2008
	rs1800797	677:656	0.78, 1.02 (0.87-1.20)	Smallwood 2008

Table 2.14: IL6 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.6.1.3 Interleukin 10

Interleukin 10 (IL10) is located in chromosome 1(q31-q32). The -592C>A SNP demonstrated no significant difference in the frequency of the rare A allele (cases = 27%, controls = 21.5%). The -1082G>A allele did have significantly different frequencies, P = 0.03 (A allele frequencies in cases = 58.5%, in controls = 47%). As a risk factor for AAA, -1082 A has an OR 1.8 (95% confidence interval 0.9 - 3.6, P value = 0.1) (Table 2.15) [Bown 2003]. In a larger the A allele was more common in the AAA group, 53% in cases 45% in controls P = 0.014 [Bown *et al.* 2007]. Multiple logistic regression determined that this SNP did not have independent association with AAA if other factors, such as tobacco smoking and history of ischaemic heart disease, were taken in to account [Bown *et al.* 2007].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
IL10	-592C>A	100:100	0.21, 0.74, (0.46-1.19)	Bown 2003
	-1082G>A	*i)100:100* *ii)389:404*	*0.02, 0.63 (0.43-0.94)* *0.004, 0.74 (0.61-0.91)*	*Bown 2003* *Bown 2007*
	Meta-analysis*	***489:504***	***0.0003, 0.72 (0.60-0.86)***	***Wild***

Table 2.15: IL10 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.6.2.1 Tumour Necrosis Factor

Tumour necrosis factor (TNF) is another inflammatory cytokine. The *TNF* gene is located on chromosome 6p21.3 and the association between -308G>A SNP and AAA has been analysed in a UK cohort, the frequencies of the rarer A allele were the same in cases and controls, 21% (Table 2.16) [Bown *et al.* 2003].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
TNF	-308G>A	100:100	1.00, 0.98 (0.54-1.08)	Bown 2003

Table 2.16: TNF Polymorphisms screened for association with AAA (analysed with Armitage Trend Test)

2.6.2.2 C reactive Protein

C reactive protein (CRP) is produced in response to a multitude of stimuli such as infection, tumours and wounds and is used clinically to monitor the resolution of these injuries. CRP is produced in hepatocytes and lymphocytes. The *CRP* gene is located on chromosome 1q21-q23. rs3091244 is a triallelic polymorphism (A/C/T), was profiled in a Belfast cohort, there was no significant difference in the distribution of the alleles in cases and controls, though serum CRP did rise in line with aortic diameter (Table 2.17) [Badger *et al.* 2009]. Recently Saratzis *et al.*, analysed the same SNP in Greek and British cohorts, the minor T and A alleles were more common in AAA patients [Saratzis *et al.* 2014]. In the Greek cohort the odds ratio = 2.15 (95% confidence interval 1.73 to 2.67, P value =1.6 x 10^{-11}). In the British cohort the minor allele were also associated with AAA, odds ratio = 1.47 (95% confidence interval 1.19 to 1.81, P value = 0.0003). Saratzis *et al.* then performed meta-analysis of their own data along with the data from Badger *et al.*, concluding with a positive association between the rate rs3091244 genotypes and AAA, odds ratio = 1.47 (95% confidence interval 1.01 to 2.14, P value = 0.05).

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
CRP	rs3091244	i)227:278 *ii)351:391* *iii)371:362*	0.84, 1.03 (0.79-1.34) *1.6 x 10^{-11}, 2.15 (1.55-2.99)* *0.0003, 1.47 (1.17-1.84)*	Badger 2009 *Saratzis 2014 (Greece)* *Saratzis 2014 (Britain)*
	Meta-analysis	***949:1031***	***0.047, 1.47 (1.01 - 2.14)***	***Saratzis 2014***

Table 2.17: CRP Polymorphisms screened for association with AAA. Univariate analysis of three groups of CRP genotypes, 1 (TT, TA, AA), 2 (CT, CA) and 3 (CC).

2.6.2.3 Human Leukocyte Antigen

The human leukocyte antigen (HLA) system is the human major histocompatibility complex, a family of cell surface proteins that mediate leukocyte interactions; they are a gene family found on chromosome 6. HLA genes are, by their nature, highly variable and therefore there is a wide range of potential polymorphisms to study (Table 2.8). The HLA DR B1 locus was analysed in an American cohort (AAA = 102, controls = 118), of the 12 alleles analysed HLA B1 02 and B1 04 were associated with AAA [Rasmussen *et al.* 2001]. This group later analysed 96 aortic tissue biopsies and graded the level of inflammation and determined that the HLA B1 02 and B1 04 doubled the association with AAA, odds ratio = 2.5 (95% confidence interval 1.4 to 4.3) and 2.1 (95% confidence interval 1.2 to 3.7) respectively [Rasmussen *et al.* 2002]. In two cohorts of Japanese individuals, HLA DR2 15 positively associated with AAA (present in 58.7% of cases and 28.0% of controls, P = 0.003) and HLA DQ3 allele negatively associated with AAA (present in 22.2% of AAA cases and 41.0% of controls, P = 0.015 [Hirose *et al.* 1999 and Hirose *et al.* 2001]. As the prevalence of AAA varies between different ethnic groups and the findings of this Japanese study may not be generalizable to a predominantly European disease. In a separate, slightly larger cohort of Japanese patients 78 HLA alleles were analysed HLA A2 and HLA B61 significantly associated with AAA [Sugimoto *et al.* 2003]. There was a significant association with AAA and HLA DR2 15 when data from Sugimoto and Hirose were meta-analysed, odds ratio 0.44 (95% Confidence Interval 0.26 to 0.77, P value 0.004) [Hirose *et al.* 1999, Sugimoto *et al.* 2003]. In a Spanish cohort reported a negative association between AAA and HLA DR B1 [Monux *et al.* 2003]. Subtype analysis of this HLA allele (B1 0101, 0102 and

0103) did not reveal any one of the three subtypes reached statistical significance. In a two cohorts, one Belgian and one Canadian, the HLA DQA1 locus 0102 subtype was statistically more common in AAA cases (20.8% in cases, 12.4% in controls, P = 0.019) in the Belgian cohort, but not there was not a significant association in the Canadian cohort [Ogata *et al.* 2006]. There has been some agreement that the HLA DRB1 allele subtypes are associated with AAA moreover this finding was significant in the largest cohort analysed to date [Ogata *et al.* 2006].

Gene	**Polymorphism**	**Participants (case:control)**	**P value, OR (95% confidence interval)**	**Reference**
HLA DR B1	B1 02	*142:118*	*0.03, 2.2 (1.20-4.00)@*	*Rasmussen 2001*
	B1 04	142:118	0.08, 2.0 (1.10-3.70)@	Rasmussen 2001
	B1 01	72:380	0.09, 0.50 (0.23- 1.15)χ	Monux 2003
HLA DR2 15		*i)46:50*	*0.003, 0.27 (0.12-0.64)χ*	*Hirose 1999*
		ii)49:237	0.19, 0.63 (0.32-1.28) χ	Sugimoto 2003
		Meta-analysis*	***0.04, 0.45 (0.26-0.77)***	***Wild***
HLA DQ3		*36:39*	*0.002, 5.71 (2.00-16.32)χ*	*Hirose 2001*
HLA A2		*49:237*	*0.04, 2.06 (1.03-4.11)χ*	*Sugimoto 2003*
HLA B61		*49:237*	*0.002, 3.40 (1.56-7.39)χ*	*Sugimoto 2003*
HLA DQA1	01 02	i)207:157	0.20, 0.76 (0.51-1.13) χ	Ogata 2006 (Canada)
		ii)180:269	*0.006, 0.59 (0.42-0.85)χ*	*Ogata 2006 (Belgium)*
		Meta-analysis*	***0.003, 0.66 (0.51-0.87)***	***Wild***

Table 2.18: HLA Loci Polymorphisms screened for association with AAA (analysed by χ = Chi Square Test, @ = Fishers Exact Test). Significant results italicised, * = Inverse Variance, fixed effects model meta-analysis>

2.6.3 Other inflammatory mediators

The *CCR5* (Chemokine {C-C motif} receptor 5) gene is located on chromosome 3p21.31 and the expressed protein is found on the surface of leukocytes where it binds to chemokines, cytokines responsible for chemotaxis. CCR5 Δ 32 is a 32 base pair deletion in CCR5 that shortens the protein and prevents the formation of the receptor (Table 2.19) [Ghilardi *et al.* 2004]. In an Italian study the frequency of the Δ 32 allele in was greater in cases (0.14 v 0.06) odds ratio = 2.7 (95% confidence interval 1.41 to 5.15,P value = 0.002) [Ghilardi *et al.* 2004]. The same polymorphism was analysed in a UK, however in this study the frequency of the Δ 32 allele in cases was not significantly different [Sandford *et al.* 2009].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
CCR5	Δ 32	*i)70:172* ii)253:233	*0.002, 0.36 (0.15-0.64)* 0.18, 1.26 (0.90-1.78)	*Ghilardi 2004* Sandford 2009
	Meta-analysis*		**0.93, 0.99(0.72-1.35)**	**Wild**

Table 2.19: CCR5 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.6.4 Inflammatory Polymorphism Conclusion

Although the inflammatory system appears to be involved in the pathogenesis of aneurysmal disease it is as yet unclear what the consequences of polymorphisms within inflammatory genes are, there have been associations within modest studies however large scale analyses have not been conducted and are required in order to determine how much effect these genes are having an aneurysmal disease. Current data has also yet to determine if the inflammatory process observed cause and aneurysm or are the result of an aneurysm.

2.7 Cardiovascular system

With AAA sharing several features consistent with other cardiovascular diseases experimental investigations have been undertaken in order to determine if SNPs that associate with cardiovascular disease also associated with AAA.

2.7.1.1 Angiotensin Converting Enzyme

Angiotensin converting Enzyme (ACE) is a key regulating enzyme in the renin-angiotensin-aldosterone system which is a major determinant of systemic blood pressure. ACE converts inactive angiotensin I in to the highly biologically active angiotensin II. Although there is inconsistent evidence for the effect of blood pressure upon AAA, a polymorphism within the *ACE* gene have been analysed for association with AAA. The polymorphism, rs1799752, is the I/D (insertion/deletion) polymorphism, where a 287 bp sequence from within the 16th exon of this gene on chromosome 17q23.3 is either present (insertion) or absent (deletion). There have been 12 cohorts analysed for association between the I/D polymorphism and AAA, a positive association has been reported in 2 of these, Fatini *et al.* and Lucarini *et al.* [Fatini *et al.* 2005, Lucarini *et al.* 2008, Hamano *et al.* 1999, Korcz *et al.* 2009, Pola *et al.* 2001, Jones *et al.* 2008, Obukofe *et al.* 2010, Saratzis *et al.* 2013] Saratzis *et al.* combined their data from their cohorts along with the data from Obufoke *et al.*, Jones *et al.*, Fatini *et al.*, Korcz *et al.* and Pola *et al.*. This meta-analysis (2858 AAA, 2675 controls) approached statistical significance, odds ratio = 1.09 (95% confidence interval 0.99 – 1.21 , P value = 0.08). The inclusion of the data from Lucarini *et al.* and

Hamano *et al.* yielded a significant association between rs1799752 and AAA, odds ratio = 1.11 (95% confidence interval 1.02 – 1.21 , P value = 0.02). Another case control study has examined the effect of the ACE D genotype on aortic wall [Ljundberg *et al.* 2011]. Men in this study with the ACE D polymorphism had increased aortic wall stiffness compared to those who did not; the authors postulate that this sign of altered wall mechanics may be an early stage of aneurysm formation. There has been significant interest in rs1799752 and its association with AAA, however there has been inconsistency in the results from the various studies. The meta-analysis of available data did show a significant association with the I/D polymorphism and AAA, indicating that this association cannot be excluded from future consideration.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
ACE	rs1799752	i)125:153	0.97, 1.01 (0.68-1.78)	Hamano 1999
		ii)124:152	0.13, 1.90 (0.83-4.33)	Korcz 2009
		iii)201:252	*0.002, 1.9 (1.30-2.90)*	*Lucarini 2008*
		iv)205:250	*0.0001 ,1.9 (1.35-2.69)*	*Fatini 2005*
		v)124:112	*0.001, 3.29 (1.59-6.77)*	*Pola 2001*
		vi)1173:1612	0.70, 1.07 (0.77-1.48)	Jones 2008
		vii) 1115:996	0.63, 0.97 (0.86-1.09)	Obukofe 2010
		ix) 348:393	0.31, 1.12 (0.90-1.39)	Saratzis 2013 (Greece)
		x) 400:394	0.93, 0.98 (0.65-1.47)	Saratzis 2013 (Britain)
	Meta-analysis*	***3815:4314***	***0.02, 1.11 (1.02-1.21)***	***Wild***

Table 2.20: ACE Polymorphisms screened for association with AAA (Armitage Trend Test), Significant results italicised, *=Inverse Variance, Fixed Effects Model meta-analysis.

2.7.1.2 Angiotensinogen

The precursor to angiotensin 1 is angiotensinogen (AGT), which is converted via the action of renin. This was analysed in a combined UK, New Zealand and Australian cohort [Jones *et al.* 2008]. The *AGT* gene is located at chromosome 1q42.2 and a C>T polymorphism at position 704 (rs699), within the second exon of the gene, leads to

the substitution of a methionine to a threonine residue at position 268 of the amino acid chain [Jones *et al.* 2008]. This polymorphism had a statistically significant association with AAA in the New Zealand cohort only (odds ratio 0.75, 95% Confidence Interval 0.62 to 0.91, P = 0.005) however there was no such association in either the UK or Australia cohorts, nor when all three cohorts data were combined (odds ratio = 0.96, 95% Confidence interval 0.89 to 1.04, P value = 0.33) (Table 2.21). This appears to be the only investigation into an association between *AGT* and AAA and therefore it is difficult to draw a conclusion as to whether larger cohorts should be analysed.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
AGT	rs699	i)297:897	0.09266, 1.323	Jones 2008 (Britain)
		ii)525:375	*0.00418, 0.750*	*Jones 2008 (New Zealand)*
		iii)351:340	0.81505, 0.989	Jones 2008 (Australia)
	Meta-analysis*	**1226:1723**	**0.33, 0.96 (0.89-1.04)**	**Wild**

Table 2.21: AGT Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse variance, fixed effects model meta-analysis

2.7.1.3 Receptors related to angiotensin

Angiotensinogen II receptor type 1 (AGTR1) and bradykinin receptor 2 (BDKRB2) have been analysed for association with AAA [Jones *et al.* 2008]. *BDKRB2* encodes a receptor for bradykinin, which is an antagonist of the action of angiotensin II. rs5223 is an insertion/deletion polymorphism of 9 bases within the first exon of *BDKRB2* however this SNP did not associate with AAA. A polymorphism in *AGTR1*, rs5186 (an A>C substitution at the 1166 position) did associate with AAA, OR = 0.80 (95% confidence interval 0.73 to 0.89, P value = 0.009) (Table 2.22). Though this is the only

report of an *AGTR1* SNP associating with AAA the level of significance achieved from this well designed multinational study is encouraging.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
BDKRB2	rs5223	i)297:897	0.35, 0.97 (0.76-1.10)	Jones 2008 (Britain)
		ii)351:340	0.97, 1.01 (0.77-1.32)	Jones 2008 (Australia)
		iii)525:375	0.88, 0.99 (0.85-1.15)	Jones 2008 (New Zealand)
	Meta-analysis	**1226:1723**	0.53, 0.97 (0.87-1.07)	**Wild**
AGTR1	rs5186 (C Carrier)	*i)297:897*	*0.001, 0.74 (0.61-0.88)*	*Jones 2008 (Britain)*
		ii)351:340	*0.001, 0.71 (0.57-0.88)*	*Jones 2008 (Australia)*
		iii)525:375	0.12, 0.89 (0.77-1.03)	Jones 2008 (New Zealand)
	Meta-analysis	***1226:1723***	***0.009, 0.80 (0.73-0.89)***	***Wild***

Table 2.22: BDKRB2 and AGTR1 Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse varaiance, fixed effects model meta-analysis

2.7.2 Homocysteine Metabolism

Homocysteine (Hcy) is an amino acid that is synthesised from methionine, elevated levels of Hcy are a risk factor for cardiovascular disease, with endothelial damage and thrombogenicity both increased. A large scale clinical trial has examined the use of Hcy reducing medications in reducing cardiovascular disease [Lonn *et al.* 2006]. There was lack of an association between Hcy reduction and mortality benefit, myocardial infarction whereas there were reduced numbers of strokes and admissions for unstable angina in the treatment group. Genes encoding several enzymes involved in Hcy metabolism have been analysed for association with AAA.

2.7.2.1 Methylenetetrahydrofolate Reductase

Methylenetetrahydrofolate Reductase (MTHFR) is an enzyme that catalyses a reaction in the remethylation of Hcy to methionine, reducing the levels of Hcy. The *MTHFR* gene is located at 1p36.3 and the +677C>T (rs1801133) SNP has been analysed for association with AAA. Ferrera *et al.* reported a significant increase in the T allele in cases [Ferrara 2006]. This positive association was replicated in further studies [Strauss *et al.* 2003, Sofi *et al.* 2005, Brunelli *et al.* 2000] (Table 2.23). However not all studies have found that this association is consistent [Jones *et al.* 2005, Giusti *et al.* 2008]. Meta-analysis of the available data regarding rs1801133 did demonstrate a significant association with AAA, the odds ratio = 1.06 (95% confidence interval 1.01 to 1.12, P value = 0.01). Other SNPs in *MTHFR* gene, rs1801131, rs2274976 and rs4846049 did not associate with AAA [Giustu *et al.* 2008].

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
MTHFR	rs1801133	*i)88:46*	*0.001, 1.9 (1.30-2.78)*	*Ferrara 2006*
		ii)63:75	*0.007, 1.46 (1.11-1.92)*	*Strauss 2003*
		iii)438:438	*0.03, 1.11 (1.01-1.22)*	*Sofi 2005*
		iv)58:60	*0.05, 1.21 (1.00-1.46)*	*Brunelli 2000*
		v)428:282	0.78, 1.01 (0.94-1.08)	Jones 2005
		vi)423:423	0.06, 0.49 (0.23-10.3)	Giusti 2008
		vii)454:362	0.24, 0.74 (0.45-1.22)	Obukofe 2008
		viii)319:3930	0.81, 0.97 (0.77-1.22)	Wong 2013
		ix)288:393	0.29, 1.28 (0.81-2.02)	Saratzis 2014 (Greece)
		x)393:399	0.32, 0.80 (0.52-1.24)	Saratzis 2014 (Britain)
	Meta-analysis*	***2952:6408***	***0.01, 1.06 (1.01–1.12)***	***Wild***
	rs1801131	423:423	0.34, 0.96 (0.79-1.09)	Giusti 2008
	rs2274976	423:423	0.14, 0.69 (0.42-1.13)	Giusti 2008
	rs4846049	423:423	0.69, 0.89 (0.51-1.56)	Giusti 2008

Table 2.23: MTHFR Polymorphisms screened for association with AAA (analysed with Armitage Trend Test), significant results italicised, * = inverse varaiance, fixed effects model meta-analysis

2.7.2.2 Methylhydrofolate Enzymes

Positive associations were seen between AAA and polymorphisms in 5 genes involved in homocysteine metabolism. These were reported by Giusti *et al.* who performed a microarray genotyping experiment, where 56 SNPs were typed [Giusti *et al.* 2008] (Table 2.24). Within *AHCY* (adenosylhomocysteinase) rs819146 positively associated with AAA, OR = 1.68 (95% confidence interval 1.30 to 2.16, P = 0.00006). rs8003379 was the only SNP of the 7 within *MTHFD1* (methylenetetrahydrofolate dehydrogenase 1) that positively associated with AAA, OR 2.33 (95% confidence interval 1.90 to 2.85, P = 2.42×10^{-16}). *MTR* (methyltetrahydrofolate-homocysteine methyltransferase) contains a SNP, rs2853523, that positively associated with AAA, OR 1.86 (95% confidence interval 1.55 to 2.23, P = 1.69×10^{-11}). Of the 7 polymorphisms genotyped in *MTRR* (methionine synthase reductase), only rs326118 significantly associated with AAA (OR 2.21, 95% confidence interval 1.69 to 2.88, P =5.35×10^{-9}). *TYMS* (thymidylate synthase) contains rs16430 which positively associated with AAA, OR 1.498 (95% confidence interval 1.25 to 1.79 P = 0.00001). This study is the only analysis of these SNPs with regard to AAA, there was no replication within the study and there has not been since.

Gene	Polymorphism	Participants (case:control)	P value, OR (95% confidence interval)	Reference
AHCY	*rs819146*	*423:423*	*0.00007, 1.68 (1.30-2.16)*	*Giusti 2008*
	rs7271501	423:423	No variation, all CC	Giusti 2008
MTHFD 1	*rs8003379*	*423:423*	*$2.42x10^{-16}$, 2.33 (1.90-2.85)*	*Giusti 2008*
	rs2357481	423:423	0.32, 1.22 (0.82-1.81)	Giusti 2008
	rs1076991	423:423	0.70, 1.04 (0.86-1.25)	Giusti 2008
	rs3783732	423:423	No variation , all AA	Giusti 2008
	rs1950902	423:423	0.78, 1.00 (0.98-1.01)	Giusti 2008
	rs4902283	423:423	0.32, 1.41 (0.72-2.77)	Giusti 2008
	rs2236225	423:423	0.64, 1.05 (0.85-1.30)	Giusti 2008
MTR	*rs2853523*	*423:423*	*$1.21x10^{-11}$, 1.87 (1.56-2.24)*	*Giusti 2008*
	rs4659725	423:423	0.63, 0.93 (0.70-1.25)	Giusti 2008
	rs1805087	423:423	0.54, 0.88 (0.58-1.33)	Giusti 2008
	rs2275566	423:423	0.36, 0.91 (0.76-1.10)	Giusti 2008
	rs6676866	423:423	0.67, 0.96 (0.80-1.16)	Giusti 2008
MTRR	*rs326118*	*423:423*	*$6.97x10^{-9}$, 2.20 (1.68-2.87)*	*Giusti 2008*
	rs1801394	423:423	0.26, 1.12 (0.92-1.37)	Giusti 2008
	rs1532268	423:423	0.73, 0.97 (0.80-1.17)	Giusti 2008
	rs2303080	423:423	0.32, 1.41 (0.72-2.77)	Giusti 2008
	rs10064631	423:423	0.16, 1.48 (0.86-2.55)	Giusti 2008
	rs16879334	423:423	0.16, 5.71 (0.51-63.53)	Giusti 2008
	rs8659	423:423	0.89, 1.00 (0.95-1.06)	Giusti 2008
TYMS	*rs16430*	*423:423*	*0.00001, 1.50 (0.88-1.22)*	*Giusti 2008*
	rs502396	423:423	0.66, 1.04 (0.88-1.22)	Giusti 2008

Table 2.24: MTHFR Polymorphisms screened for association with AAA (analysed by Armitage Trend Test). Significant results italicised

2.7.3 Cardiovascular System Genes Conclusion

Association has been demonstrated between AAA and cardiovascular disease genes however there is inconsistency in these associations. Some of the published data arises from small cohorts and positive findings have not persisted once larger, better powered, studies have been conducted. The MTHFR and ACE genes have both reached statistical significance in meta-analysis and because of this certainly warrant further study.

2.8 AAA and Genome Wide Association Studies

In 2005 a case control study of patients with age related macular degeneration an approach was taken to genotype all patients for a sample of over 110,000 SNPs from across the entire human genome, not limiting the analysis to previously reported or suspected genes [Klein 2005]. This represented the first Genome Wide Association Study (GWAS), and since this date there have been many of studies utilising this technique, where by an initial group of cases and controls are genotyped and SNPs with a highly significant association are carried forward to large scale replication. The large numbers of SNPs analysed can lead to large numbers of false positives. For instance, if 500,000 independent markers are analysed, 25,000 can be expected to falsely associate with a disease (with a P value of <0.05) by chance alone. Therefore a widely accepted level of association in GWAS is 5×10^{-8} in order to avoid analysing false positive markers [Shi *et al.* 2010]. Despite the popularity of GWAS this type of study is not without problems. There are general issues with GWAS that many studies suffer, these include false positive results, limited environmental information, bias due to case/control selection, the need for large sample sizes and the chance of overlooking a true association when taking positive results from a discovery phase to a replication phase [Pearson 2008]. An example of a systematic issue with GWAS is the paper by Sebastiani *et al.* where extreme long life (living in excess of 100 years) was analysed [Sebastiani *et al.* 2010]. The authors identified 33 SNPs that significantly associated with extreme age. However, shortly after publication the editor of the publishing journal announced that there had been concerns regarding the methodology undertaken and requested re-analysis of the data [Alberts 2010]. The

concern was that there had been more than one genotyping platform utilised and that this had led to false positive SNP detection, and retraction of the original paper, despite the authors maintaining that there were SNPs significantly associated with longevity [Sebastiani *et al.* 2011].

GWAS methodology has been used to investigate AAA on 6 occasions.

2.8.1 Helgadottir *et al.*

Helgadottir *et al.* identified the SNP rs10757278-G on chromosome 9p21 to be associated with AAA through candidate gene study [Helgadottir *et al.* 2008]. The authors utilized 2 methods to genotype the samples, the Illumina 317K Bead chip and Centaurus, covering over 300,000 SNPs. This group initially determined the frequency of this genotype in a cohort of Icelandic individuals (398 AAA and 14259 controls) and found a significant association between G allele and AAA, odds ratio = 1.37 (95% confidence interval 1.18 to 1.58, P value = 2.6×10^{-5}). Replication was conducted in cohorts from Belgium (176 AAA, 267 controls), Canada (206 AAA, 150 controls), America (101 AAA, 447 controls), The Netherlands (476 AAA, 915 controls), Britain (478 AAA, 252 controls) and New Zealand (588 AAA, 442 controls). Positive associations were seen in all replication cohorts except Belgium and Canada and meta-analysis of all data yielded an odds ratio =1.31 (95% confidence interval 1.22 to 1.42, P value = 1.2×10^{-12}). This SNP was also found to be associated with intracranial aneurysm and coronary artery disease but not type 2 diabetes.

2.8.2 Elmore *et al.*

Elmore *et al.* identified 4 SNPs on chromosome 3p12.3, rs7635818 associated with AAA [Elmore *et al.* 2009]. This modestly powered study utilised samples from 2 regions of the USA with predominantly northern European ancestry. The discovery cohort utilised the Affymetrix 500K chip array, genotyping over 500,000 SNPs (Affymetrix Inc, Santa Clara, California, USA). The discovery cohort contained 502 AAA and 736 controls, the frequency of the rare GG genotype was 27.0% in cases, 33.0 % in controls, P value = 0.017. The replication cohort consisted of 448 AAA and 410 controls, and the frequency of the GG genotype was again 27.0% in AAA and 33.0% in controls, P value = 0.013. Combination of the 2 cohorts yielded an odds ratio = 1.33 (95% confidence interval 1.10 to 1.61, P value = 0.0014). This SNP is located upstream of the *CNTN3* (Contactin 3) gene, involved in cell adhesion, however a subsequent analysis of a larger cohort did not validate this association [Gretarsdottir *et al.* 2010]. Additionally this level of significance would not reach the accepted standard for genome wide association, 5×10^{-8}.

2.8.3 Gretarsdottir *et al.*

Gretarsdottir *et al.* identified the A allele of rs7025486 on 9q33 to be associated with AAA [Gretarsdottir *et al.* 2010]. rs7025486 is located within *DAB2IP* (Disabled homolog 2-interacting protein), which is involved in cell signaling (including apoptotic induction). The discovery phase genotyped samples from Iceland and The Netherlands with Illumina SNP Chips (both the 370 and 610 chips) allowing the assessment of 293677 SNPs, following quality control (Illumina, San Diego, California, USA). The Icelandic cohort contained 452 AAA and 27712 controls, The Netherlands cohort 840

AAA and 2791 controls. 22 SNPs (excluding 3 found within the 9p21 locus, previously associated with AAA by Helgasdottir *et al.*) achieved genome wide significance and 19 of these were then successfully genotyped in the first follow up set. rs7025486 was significantly associated with AAA in the discovery set, odds ratio = 1.21 (95% confidence interval 1.11 to 1.32, P value = 2.9×10^{-5}). Replication was then conducted in individuals from Belgium (172 AAA, 266 controls), Canada (196 AAA, 150 controls), New Zealand (848 AAA, 1144 controls) and Britain (455 AAA, 667 controls). Combined significance for this initial replication cohort was odds ratio = 1.28 (95% confidence interval 1.15 to 1.41, P value = 3.6×10^{-6}). A second follow up study was then conducted with samples from Denmark (297 AAA, 4380 controls), a novel cohort from The Netherlands (147 AAA, 301 controls), and 2 American cohorts; Danville (758 AAA, 380 controls) and Pittsburgh (101 AAA, 447 controls), with the resulting odds ratio = 1.11 (95% confidence interval 0.98 to 1.25, P value = 0.081). Despite the lack of significance in this second follow up cohort overall significance was maintained as the direction of effect was the same in all but one population (4559 AAA and 37954 controls), odds ratio = 1.21 (95% confidence interval 1.14 to 1.28, P value = 4.6×10^{-10}). Aside from AAA, rs7025486 also significantly associated with early onset MI, peripheral arterial disease and pulmonary embolism.

2.8.4 Bown *et al.*

Bown *et al.* (2011) studied 1866 patients with AAA and 5435 controls followed by replication of promising SNPs in a further 2871 cases and 32687 controls, and further follow up in 1491 cases and 11060 controls was then performed [Bown *et al.* 2011].

The discovery phase utilised individuals from 8 different UK centres Chichester (58 AAA), Leeds (319 AAA), Leicester (778 AAA), St Georges London (70 AAA), UK Small Aneurysm Trial (262 AAA), Otago in New Zealand (129 AAA) and Western Australia (250 AAA) with controls from the Wellcome Trust Case Control Consortium (5435). Any SNPs with a significant association (P value = $<1 \times 10^{-5}$) were then tested for association in the replication cohort. The replication cohort was made up of cases directly genotyped and those where *in silico* data was obtained from other GWAS. Laboratory genotyping was performed on cases independent from the discovery phase; Chichester (190 AAA), Belfast (228 AAA and 244 controls), Kings College London (210 AAA), Leeds (27 AAA), Leicester (152 AAA and 761 controls), St Georges London (24 AAA), UK Small Aneurysm Trial (90 AAA), Otago (623 AAA and 515 controls) and Western Australia (35 AAA and 664 controls). *In silico* data was obtained from GWAS datasets from Iceland (452 AAA, 27712 controls) and the Netherlands (840 AAA, 2791 controls). Finally SNPs that continued to have genome wide significance were genotyped in a follow up cohort, with individuals from Copenhagen in Denmark (193 AAA and 10180 controls) and Viborg in Denmark (503 AAA and 196 controls), Otago (579 AAA, 430 controls) and Leeds (216 AAA and 254 controls). This indicated an association between rs1466535, located within the second intron of the *LRP1* (Low density lipoprotein receptor related protein 1) gene, on chromosome 12q13-14, and AAA. The meta-analysis of all cohort data yielded an odds ratio = 1.15 (95% confidence interval 1.10 to 1.21, P value = 4.52×10^{-10}). This association between LRP1 and AAA was independent of risk factors for generalized cardiovascular disease, suggesting that the association is specific for AAA. Additionally Bown *et al.* determined that rs1333049 (a proxy SNP for rs10757278 reported by Helgadottir *et al.*) positively associated with

AAA but did not reach genome wide significance (odds ratio = 1.15, 95% confidence interval 1.09 to 1.22, P value = 4.4 x 10^{-7}). However the association between AAA and DAB2IP (via the proxy SNP, rs10818583) showed a greatly reduced level of significance when samples from that GWAS were removed (odds ratio = 1.098, 95% confidence interval 0.89 to 1.35, P value = 0.017).

2.8.5 Jones *et al.*

Jones *et al.* hypothesised that as AAA had been associated with both dyslipidaemia and coronary artery disease (CAD), polymorphism that had been associated with these diseases may associate with AAA [Jones *et al.* 2013]. The initial cohort (New Zealand, 608 AAA and 612 controls) was genotyped for polymorphisms within 44 loci that had previously been associated with lipid dysfunction and CAD. This lead to 15 SNPs with significant association with AAA (P values = <0.05), these were then genotyped in an independent cohort (New Zealand 713 AAA and 1766 controls). This second round of genotyping yielded 3 SNPs that continued to associated with AAA. These 3 SNPs were then genotyped in the WTCCC AAA cohort (1286 AAA, 5605 controls), 2 remained positively associated. One of these was within the same locus described by Gretarsdottir *et al.,* the other was rs599839. Replication of the association between rs599839 and AAA was then conducted in multiple cohorts, Iceland (1163 AAA, 61639 controls), The Netherlands (840 AAA, 2792 controls), Australia (377 AAA, 373 controls), UK (684 AAA, 456 controls), USA (230 AAA, 1000 controls), Belgium (176 AAA, 267 controls) and Canada (198 AAA, 153 controls). Meta-analysis of all cohorts

yielded an odds ratio = 0.81 (95% confidence interval 0.76 to 0.85, P value = 7.2×10^{-14}). There have been other positive associations reported with rs599839, both blood lipid profile and CAD. The region on chromosome 1 (p13.3) where rs599839 resides contains 3 genes, *CELSR2* (Cadherin EGF LAG seven-pass G-type receptor 2), *PSRC1* (Proline/serine-rich coiled-coil protein 1) and *SORT1* (Sortilin 1). Jones *et al.* analysed mRNA extracted from aortic tissue (n = 11) and SORT1 protein (98 AAA and 20 controls) with Western blotting. They found that only *SORT1* was strongly expressed in aneurysmal tissue. This association was independent of dyslipidaemia and a history of coronary artery disease.

2.8.6 Bradley *et al.*

Bradley *et al.* utilised the same discovery data as Bown *et al.*, however utilising a different method of analysis, CRLMM (Corrected Robust Linear Model with Maximum Likelihood Classification), additional markers were detected and analysed [Bradley *et al.* 2013]. Positively associated SNPs were studied with *in silico* data analysis of samples from Iceland (452 AAA, 27712 controls) and The Netherlands (840 AAA, 2791 controls). SNPs that remained associated with AAA were then genotyped in a second validation cohort of samples from the UK (Leeds, Leicester and Belfast 706 AAA, 1063 controls) and Denmark (507 AAA, 199 controls). Any positive associations after this phase were then analysed for association to AAA utilising the *in silico* data from Jones *et al.* (885 AAA and 1000 controls) [Jones *et al.* 2013]. Previously associated loci were not analysed however a novel SNP, rs6511720, found within the Low-density lipoprotein receptor (*LDLR*) gene on chromosome 19(p13.2) was discovered. The level

of association reached genome wide significance when all data was meta-analysed (5138 AAA, 39273 controls) odds ratio = 0.76 (95% confidence interval 0.70 to 0.86, P value = 2.08×10^{-10}). This gene is part of the same gene family as *LRP1* and has previously been associated with levels of LDL cholesterol. Additional support for the role of this gene in AAA is the use of LDLR knockout mice as models for AAA.

2.8.7 AAA GWAS Conclusion

There have been 6 Studies that have utilised genome wide association methodology, the results are summarised in Table 2.25. The results of Elmore *et al.* failed to reach genome wide significance, there were highly significant results obtained in the other studies. The remaining studies provide robust evidence of the involvement of each of the respective genes in AAA. What GWAS fail to answer is how the individual polymorphisms affect the patient in order to lead to the development or propagation of AAA.

Author	**SNP**	***Gene***	***p* value**	**Odds Ratio (95% CI)**	**Sample size**	
					Cases	**Controls**
Helgadottir *et al.*	rs10757287	CDKN2A/B	1.2×10^{-12}	1.31 (1.22-1.41)	2836	16732
Elmore *et al.*	rs7635818	CNTN3	2.8×10^{-3}	1.33 (1.10-1.61)	950	1146
Gretarsdottir *et al.*	rs7025486	DAB2IP	4.6×10^{-10}	1.21 (1.14-1.28)	4559	37954
Bown *et al.*	rs1466535	LRP1	4.5×10^{-10}	1.15 (1.10-1.21)	6228	49182
Bradley *et al.*	rs6511720	LDLR	2.1×10^{-10}	0.76 (0.70-0.83)	3390	32765
Jones *et al.*	rs599839	SORT1	7.2×10^{-14}	0.81 (0.76-0.85)	7048	75976

Table 2.25: Summary of the data from genome wide association studies in AAA. Key CI = Confidence Interval

2.9 Chapter 2 Conclusion

There are a wide variety of SNPs that have demonstrated an association to AAA though very few of these have a statistically significant effect when combined in meta-analysis. There have been consistent associations seen in SNPs within the MMP3, MMP 13, MMP9, ACE and MTHFR when published results have been combined in meta-analysis. However as the investigation of AAA has moved away from the candidate gene approach and toward genome wide association studies polymorphism in genes previously unrelated to AAA have been identified. These genes include a highly significant disease specific association between AAA and LRP1, a cell surface transporter that has several interesting ligands, making it plausible as a candidate for aneurysmogenesis. A large number of polymorphisms fail to maintain association to AAA when analysed by other research groups or when all published data is combined. This underlines the importance of replicating analyses in geographically distinct cohorts once a positive association is discovered. As the SNPs utilised within GWAS are used to represent large areas of the genome it is possible that there may be a SNP with a more significant association within the gene that was not on the discovery array. Fine mapping is the process whereby SNPs in association with a positive GWAS lead SNP are genotyped [Freedman *et al.* 2011].

Chapter 3: LRP1 and Abdominal Aortic Aneurysms

3.1 LRP1 and AAA

Low density lipoprotein receptor related protein 1 (LRP1) has been associated with AAA in a GWAS [Bown *et al.* 2011]. This association between LRP1 and AAA was independent of risk factors for generalized cardiovascular disease, suggesting that the association is specific for AAA. As LRP1 had not previously been proposed as a candidate gene for LRP1 a systematic review of the literature was performed in order to determine how LRP1 may affect aneurysm development (Figure 3.1). Search terms LRP1 OR Low density Lipoprotein receptor related protein 1 AND Aneurysm AND Aorta were utilised on an Ovid search of Medline 1946 –present and Health and Psychosocial Instruments 1985-Present.

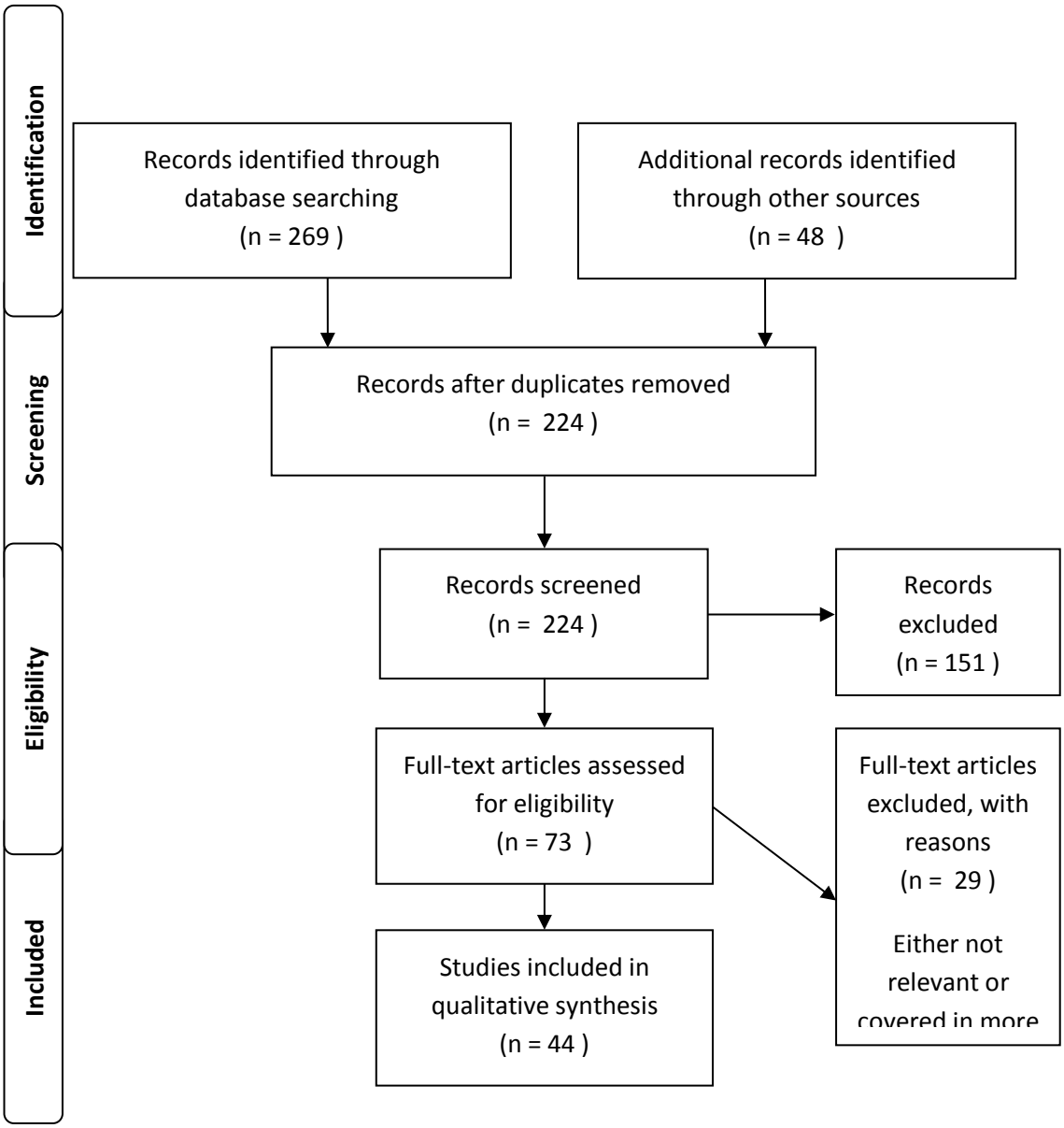

Figure 3.1. Prisma Diagram representing the Systematic Review process.

3.2 LRP1 Structure

The structure of LRP1 permits the binding of a wide variety of ligands (Figure 3.2). It is through structural similarities with the LDL (low density lipoprotein) receptor that LRP1 was classified as a member of the LDL receptor family. In 1988 Herz and colleagues first described a 500kDa liver cell surface protein whose sequence closely resembled LDL receptor and epidermal growth factor (EGF) [Herz *et al.* 1988]. They proposed this protein, LDL-receptor related protein (LRP) functioned not only as a lipoprotein receptor but also had possible functions in modulating cell growth. LRP was located at the cell surface, had high levels of tissue expression in liver, lung and brain and significant levels in intestine and muscle. LRP1, as LRP is now known, is initially synthesised as a 600kDa precursor that then undergoes furin cleavage within the golgi apparatus, resulting in the 85kDa intracellular and intramembrane fragment and the 515kDa extracellular fragment [Herz *et al.* 1990, Van der Deer *et al.* 2001]. These two fragments form a non-covalently bonded heterodimer spanning the cell membrane; with both domains featuring structural motifs capable of binding a diverse range of over 40 ligands [Herz *et al.* 2001, Lillis *et al.* 2007]. LRP1 is a member of a gene family of low density lipoprotein receptors, including LRP1B, LRP5, LRP6, low density lipoprotein receptor (LDLR), very low density lipoprotein receptor (VLDLR) and megalin [Boucher 2004]. Structural homology exists between the different members of the family; however LRP1, LRP1B and megalin are the 3 largest members. All members of the LRP protein family have at least one NPxY motif (asparagine-proline-x-tyrosine, where x can be any amino acid) in their intracellular tail. LRP1 has 2 NPxY motifs within its intracellular region and a variety of ligands are known to bind specifically to these motifs [Trommsdorff *et al.* 1998]. The extracellular domain contains a variety of

binding sites, cysteine-rich complement type binding domains, EGF receptor like cysteine repeats and YWTD (Tyrosine-Tryptofan-Threonine-Aspartic acid) domains. LRP1 acts as a membrane receptor for a wide variety of ligands thanks to the 4 cysteine-rich complement binding domains. As it is capable of binding such a wide variety of ligands LRP1 is involved in multiple processes and several of these have potential to affect the arterial wall. For example, the proteases MMP2, MMP9 and the growth factor PDGF (platelet derived growth factor) can all bind to the extracellular portion of LRP1 [Herz *et al.* 2001, Lillis *et al.* 2007]. LRP1 is essential for embryonic implantation and LRP1 knockout in mice is an early embryonic lethal variant, demonstrating its importance [Herz *et al.* 1992]. In summary, LRP1 has structural similarity to other members of the lipoprotein receptor family and several distinct binding regions that facilitate adhesion to multiple ligands, some of which have potential to affect the arterial wall and lead to aneurysm formation.

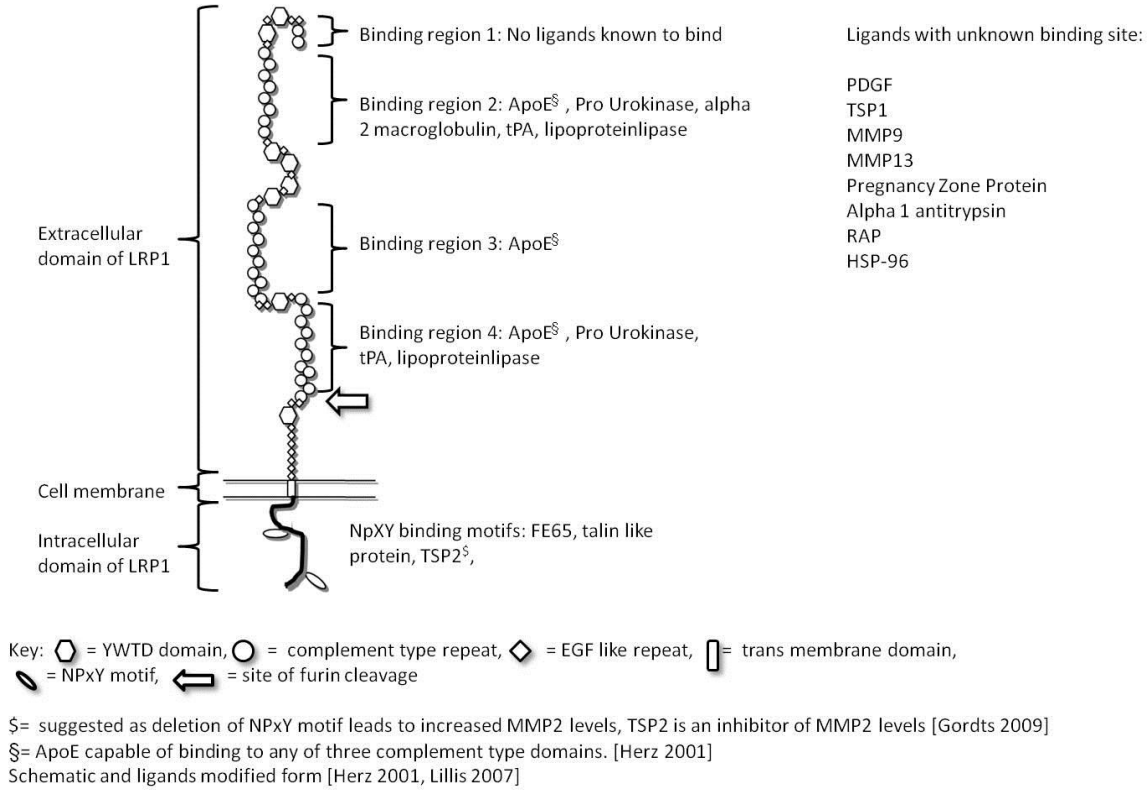

Figure 3.2: Schematic LRP1 molecular structure and some annotated ligands at given binding sites.

3.3 LRP1 and extracellular matrix degradation

The regulation of proteases by LRP1 may impact on aneurysmal development, via degradation of the extracellular matrix (ECM), a key feature of aneurysm development [Thompson *et al.* 1996]. LRP1 does not appear to directly possess any proteolytic activity however it does interact with other molecules which do have degrading properties. MMP9 has been shown to directly bind to LRP1 with high specificity, with LRP1 depleted cell lines having reduced capacity to internalize and degrade MMP9, proving LRP1 is a regulator of active MMP9 levels [Hahn-Dantona *et al.* 2001]. This experimental evidence was achieved in a cell line of mouse embryonic fibroblasts,

where LRP1 activity was depleted by addition of the receptor associated protein, a ligand antagonist, to culture media. LRP1 is essential for MMP9 upregulation, as LRP1 depleted microglial cells have upregulated MMP9 in response to ischaemia; however a further study showed that both LRP1 and MMP9 were upregulated in response to hypoxia in renal cells [Zhang *et al.* 2009, Caron *et al.* 2005]. This conflicting evidence may be due to the interaction between LRP1 and MMP9 being cell dependent or due to a lack of knowledge of the underlying mechanism of LRP1's interaction with MMP9. This interaction may be through transport of MMP9 through the cell membrane via LRP1 or through its interaction with other matrix proteins that regulate MMP9 activity. Despite the relationship between LRP1 and MMPs not being clear, crucial data from aortic tissue and animal models demonstrate LRP1 loss increases levels of MMP and ultimately leads to AAA formation [Boucher *et al.* 2003, Gordts *et al.* 2009].

LRP1 may also regulate ECM degradation and AAA propagation via thrombospondins, TSP1 and TSP2 which both bind to LRP1 [Bein *et al.* 2000, Bornstein *et al.* 2000]. TSPs 1 and 2 inhibit the gelatinases MMPs 2 and 9 by preventing the conversion of inactive precursor into the active form of the molecule. LRP1 is responsible for the endocytosis that leads to degradation of TSPs [Godyna *et al.* 2005]. If a cell had reduced levels of LRP1 then TSP levels would be increased, decreasing the levels of active MMP2 and 9. TSP2 polymorphisms have previously been associated with thoracic aortic aneurysms (TAA) in hypertensive patients, but not AAA [Kato *et al.* 2008]. The NPxY motif within the intracytoplasmic region of LRP1 has been shown to be essential in the regulation in MMP2 levels, with knock out of this region leading to increased MMP2 activity of up to 3 times the normal level in mouse aortic tissue, implicating this motif as a potential binding region for TSP or another MMP regulatory protein [Gordts *et al.* 2009]. In

addition TSP2 knockout mice demonstrate increased blood vessel density, highlighting a regulatory role for TSP2 in angiogenesis, which may also impact on AAA [Kyriakides *et al.* 1998]. Therefore LRP1 depletion may act both directly and indirectly on the activity of MMPs. The interaction between LRP1 and MMPs illustrates the diversity of LRP1 function, with LRP1 affecting levels of MMPs via different pathways in different tissue types. As it has already been shown that members of the MMP family play a role in the development of AAA, the fact that their level of activity is regulated by LRP1 indicates this may be the avenue by which LRP1 modulates aneurysmogenesis.

3.4 LRP1 and vascular smooth muscle cell depletion

Depletion of VSMCs is a pathological hallmark of AAA [Lopez-Candales *et al.* 1997, Dai 2001]. Boucher *et al.* demonstrated that LRP1 regulates smooth muscle cell migration and proliferation via interaction with PDGFRβ (platelet derived growth factor receptor beta) [Boucher *et al.* 2003]. A strain of mice was created that were LDLR knockout with smooth muscle depletion of LRP1 (smLRP1$^-$). The LDLR$^-$smLRP1$^-$ mice had a significantly altered vascular phenotype, although they appeared superficially normal. Macroscopically the mice had aortas that were consistently distended and dilated, a feature that worsened with increasing age, together with progressive thickening of the aorta due to VSMC proliferation and eventually suprarenal aneurysm formation. Microscopically there was gross disruption of the elastic laminae of the aorta. These animals, whether fed a raised cholesterol diet or normal chow, did not have increased serum triglycerides or cholesterol, however there was an increase in the activity of MMPs 2 and 9 in the smLRP1$^-$ animals suggesting that LRP1 has an overall negative regulatory effect on MMPs. Furthermore smLRP1$^-$ arterial smooth muscle cells were α

actin depleted when compared to wild type cells of the same nature, reducing the ability of the vessels to contract [Basford *et al.* 2009]. Boucher postulates that the mechanism by which LRP1 regulates smooth muscle cell proliferation and migration is via PDGF dependent phosphorylation of the LRP1 tail leading to Shc binding and activation of the Ras pathway. Tyrosine kinase inhibitors (TKI) not only suppress LRP1 and PDGFRβ (PDGF receptor β) phosphorylation in bovine smooth muscle cells but also reduce aortic thickness and improve elastic layer stability in $LDLR^{-}smLRP1^{-}$ mice. PDGFRβ co-precipitates with LRP1 and comparison of LRP1 deficient and LRP1 expressing mouse aortas demonstrated that $LRP1^{+}$ mice have decreased levels of PDGFRβ, with PDGFRβ signaling known to precede atherosclerotic lesion formation. This demonstrates that LRP1 can suppress pro-atherosclerotic PDGFRβ activation. As TKIs can rescue the LRP1 knockout to a degree, reducing aortic thickness and improving elastic layer stability, this may have potential as a future therapeutic strategy in AAA. These key experiments demonstrate that LRP1 depletion is a stage in AAA development. This work, and subsequent experiments, produced invaluable insight in to the importance of LRP1 to arterial wall structure, however the mainstay of these publications all arise from the same group of researchers [Boucher *et al.* 2003 & 2007, Basford *et al.* 2009]. LRP1 knockout not only raised MMP levels but impaired smooth muscle cell proliferation and migration. Finally it is apparent that the deleterious effects of LRP1 depletion can be improved with the addition of a tyrosine kinase inhibitor, which may prove to be a future treatment for AAA.

If medications are going to be used to stabilise the expansion of small AAA, then utilising medications that are already in use for the treatment of other diseases will be desirable in order to reduce time and developmental costs. Aside from TKI treated

LRP1 knockout mice demonstrating a reduction in arterial wall destruction, the addition of rosiglitazone to smLRP1^{-}, one of the thiazolidinedione class of insulin sensitizers, reduced atherosclerosis and features such as elastic layer disruption and smooth muscle proliferation [Boucher *et al.* 2007]. With partial correction of effects of LRP1 knockout, rosiglitazone may also have clinical potential in modulating AAA progression. Rosiglitazone has been withdrawn due to negative effects seen in patients with heart failure, acute coronary syndrome, ischaemic heart disease and peripheral vascular disease; however another thiazolidinedione, pioglitazone, has not been withdrawn. Therefore the use of drugs from the thiazolidinedione class may be of use in reducing the impact of LRP1 loss on arterial structure. This experiment also demonstrated the potential importance of TGFβ (transforming growth factor beta) in AAA pathogenesis [Boucher *et al.* 2007]. TGFβ is a cytokine and an LRP1 ligand, it has also been implicated in AAA pathogenesis, the *TGFβR2* (TGFβ receptor 2) gene is found to have significant deletions in AAA wall biopsies with a proposed reduction of the antiproliferative effect of TGFβ [Biros *et al.* 2012].

Data from LRP1 smooth muscle knockout mouse experiments suggest that loss of normal LRP1 results in destruction of the arterial wall associated with increased MMP levels which may have been responsible for the tissue damage. The use of tyrosine kinase inhibitors and an insulin sensitizer, rosiglitazone, were able to ameliorate some of the negative feature of LRP1 depletion and may prove to have potential for treatment.

3.5 LRP1 and Inflammation

Several polymorphisms in inflammatory mediators have been implicated in AAA pathogenesis, including Interleukins 6 and 10 [Smallwood *et al.* 2008, Bown *et al.* 2003]. The role of LRP1 in inflammation is unknown with evidence demonstrating pleiotropic effects. The association between AAA and local chronic inflammation is well established, with transmural infiltration of the arterial wall in AAA by macrophages and lymphocytes a prominent histological feature [Ailawadi *et al.* 2003], additionally mast cells, regulators of inflammation, are key to AAA development with mast cell deficient mice resistant to AAA formation [Sun *et al.* 2007]. Mast cells contain granules that store a wide variety of proteases, cytokines and growth factors including PDGF and TGFβ, known LRP1 ligands [Krishnaswamy *et al.* 2006]. Macrophages are known to express LRP1 and after adipocytes, monocytes show the highest level of expression [Su *et al.* 2004]. Experimental work from a rat microglial cell model has demonstrated that LRP1 functions as a pro-inflammatory molecule. 2'-hydroxycinnamaldehyde is an anti-angiogenic, anti-proliferative and pro-apoptotic compound that binds to LRP1. When LRP1 is bound to 2'-hydroxycinnamaldehyde it induces an anti-inflammatory state in microglial cells, by the inhibition of cell signaling pathways suggesting LRP1 is pro-inflammatory [Hwang *et al.* 2011]. This study utilised novel and established cell lines and defined protein level and activity under a variety of experimental conditions to from a robust argument for their findings. However, LRP1 depletion has been shown to increase the levels of MMP9 and tissue necrosis factor α, INOS (inducible nitric oxide synthase), activated complement proteases and IL-6 (interleukin-6), indicating that LRP1 may have innate anti-inflammatory properties [Overton *et al.* 2007, Gaultier *et al.* 2008]. The article from Overtan *et al.* utilised a

mouse model with LRP1 depleted macrophages [Overton *et al.* 2007]. Through Western blotting, real time PCR and gel electrophoresis they demonstrated that MMP activity was increased [Gaultier *et al.* 2008]. However Gaultier *et al.* utilised an LRP1 deficient mouse fibroblast cell line and similar experimental techniques to prove increased expression of several inflammatory mediators takes place when LRP1 is absent. Therefore it has been shown that LRP1 can exhibit both pro and anti-inflammatory behaviour, however with greater understanding of the specific ligand binding regions of the molecule it may be possible to inhibit the pro-inflammatory effects whilst maintaining the anti-inflammatory potential of LRP1.

3.6 LRP1 Regulation

In understanding the mechanisms regulating LRP1 function it may be possible to identify potential therapeutic targets for LRP1 that could be used to slow the progression of small AAA. LRP1 is regulated by several factors; one of these is angiotensin II (AngII). It is well characterised that continuous infusion of AngII leads to the development of aneurysms in apolipoprotein-E deficient mice [Daugherty *et al.* 2000]. AngII induces overexpression of LRP1 in the arterial wall and also increased its activity, resulting in increased uptake of aggregated LDL in cultured human VSMC [Sendra *et al.* 2008]. These effects can be negated with administration of the angiotensin receptor blocker, losartan. Additionally in mouse models of TAA, the thoracic aorta of mice treated with losartan were found to have improved structural integrity and elastic fibre organisation and reduced levels of MMP 2 and MMP 9 activation when compared to untreated mice [Yang *et al.* 2009]. These data suggests that reduction of LRP1 expression is beneficial in aneurysms, which conflicts with the

other data presented. The exact mechanisms are still unclear but from these data, it is likely that AngII related development of aneurysms is LRP1 independent or any aneurysmogenic effects of AngII induction override the protection achieved from increased LRP1 expression.

3.7 Implications for the treatment of small AAA

Aside from inducing LRP1 expression with known medications in order to halt small AAA progression it may be possible to modulate AAA growth via novel methods involving the LRP1 pathways. LRP1 is regulated by systemic lipoprotein levels; in response to aggregated LDL (agLDL) in VSMC LRP1 is upregulated. This upregulation is coexistent with down regulation of SREBP (sterol regulatory binding protein) [Costales *et al.* 2010]. Experimental silencing of SREBP isoforms increased LRP1 levels whereas SREBP overexpression reduced LRP1 expression. Furthermore, agLDL prevent the SREBP-2 isoform from efficiently binding to the promoter region of *LRP1* gene. This demonstrates that silencing of specific SREBP isoforms may be utilised to increase LRP1 expression in AAA patients, without having to increase systemic lipoprotein levels which would be associated with other deleterious effects. Recent hepatocyte cell culture experimental evidence indicates that LRP1 is upregulated in the presence of the cholesterol lowering medication, hydroxymethylglutaryl-coenzyme A (HMG co-A) reductase inhibitor, Atorvastatin [Moon *et al.* 2011]. This is coupled with data that the aortic diameter of AAA patients treated with a HMG co-A reductase inhibitor expand at a reduced rate when compared to patients not receiving this group of medications [Karrowni *et al.* 2011].

Possible methods of regulating LRP1 at the level of transcription is via miR-205, a microRNA, which down-regulates the expression of LRP1 mRNA, therefore miR-205 blockade may increase LRP1 activity [Song *et al.* 2009]. Similarly a short interfering RNA has been shown as an effective silencer of LRP1 expression in smooth muscle cells leading to inhibition of VSMC migration [Li *et al.* 2003]. Modulation of LRP1 expression may be achieved by the selective inhibition of both these negative regulatory elements; however this form of molecular medicine is a long way from mainstream practice. If high LRP1 levels reduce AAA growth then this may form the basis of small AAA treatment. Modulation of LRP1 expression may be achievable in a laboratory setting, however these examples show that although theoretically possible the systemic administration of an agent that would interfere with one of these regulatory elements would need rigorous investigation in order to determine safety before a treatment could be utilised.

3.8 Chapter 3 Conclusion

LRP1 and its associated pathways are biologically plausible candidates that may have a role in the development of AAA. These effects go beyond the ability of LRP1 to bind a wide variety of ligands that interact with the arterial wall such as the matrix metalloproteinases. LRP1 knockout studies in mice implicate the importance of the regulatory relationship between LRP1 and PDGFRβ, with loss of LRP1 leading to a pro atherosclerotic state, extracellular matrix disruption and even aneurysmal formation.

LRP1 has already been implicated in several diverse diseases, notably Alzheimer's disease [Bates 2009] obesity [Hofmann *et al.* 2007], myocardial infarction [Gonzalez *et al.* 2002], bicuspid aortic valve [Folkersen *et al.* 2011] and in cancers such as

medulloblastoma [Annabi *et al.* 2010], although modulation of LRP1 activity is yet to be utilised as a therapeutic target in clinical practice. Further investigation is required to fully elucidate the LRP1 pathway and the exact mechanism by which it plays a role in the pathogenesis of AAA. LRP1 mutation is likely to part of a collaboration of genetic and environmental factors. LRP1 and its associated pathways appear to be a potential candidate target for the treatment of AAA, but further work is required to define the specific role of LRP1 in aneurysmogenesis.

Chapter 4: Aims

4.1 Aims of this thesis

AAA is a life threatening condition, when rupture occurs out of hospital survival rates are low. Cure is possible with surgical correction of the diseased portion of the aorta however this not without significant risk to the patient in terms of post-operative mortality. It is also not yet clear what processes are causing patients to develop AAA. There are some well documented risk factors such as male gender, cigarette smoking and positive family history. As family history is consistently associated with AAA this has led to attempts to identify genetic associations with AAA in order to better understand the underlying pathology. Multiple SNPs with reported association to AAA have subsequently been found to lack significance when analysed in new cohorts. Therefore the associations reported by Bown *et al.* and Rayt *et al.* will be analysed [Bown *et al.* 2011, Rayt *et al.* 2012]. The SNPs will be genotyped in new samples in order to test the association with AAA. Once the SNP within a gene with the most significant association to AAA has been identified the next step will be to sequence the exons within the region of the gene contiguous to this polymorphism (Figure 4.1). The aim of this targeted sequencing is to identify any novel polymorphism within the coding regions of the gene that may be causal [Freedman *et al.* 2011].

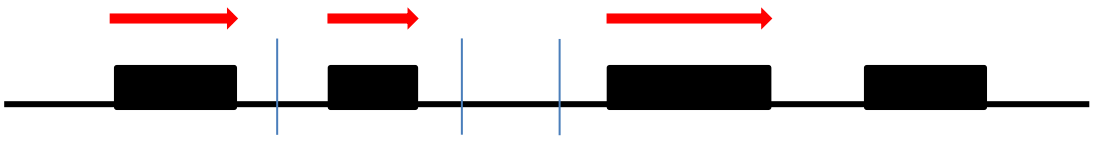

Figure 4.1: Schematic of a gene, the alleles are represented by black squares, the SNPs of interest are the blue lines and the red arrows represent the sequencing of these alleles.

Having determined which polymorphism has the most significant association with AAA an enzyme linked immunsorbant assay (ELISA) will be conducted on a cohort of the same samples in order to determine if the polymorphism affects gene expression. Finally aortic aneurysm tissue biopsies will be obtained. These samples will be processed and stained in order to determine the location of the protein of interest within the arterial wall.

4.2 Hypotheses

1. SNP genotyping
    a. There is a significant association between *CTSC* and AAA
    b. rs1466535 is not the SNP in LRP1 with the most significant association to AAA
2. There is a variation within the exons of LRP1 associated with the lead SNP from the re-sequencing experiment
3. The concentration of sLRP1 will be different in AAA cases and screened controls
4. The distribution of LRP1 will be predominantly found within one of the three arterial wall layers

Chapter 5: Sample collection

5.1 Collection of samples

Samples were collected from individuals in order to analyze genotypes, protein levels and where possible aortic tissue biopsies were collected for subsequent immunohistochemical analysis. Samples were obtained in accordance with ICH GCP (International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use Good Clinical Practice) guidelines. The ethical approval within Leicester was REC (regional ethics committee) study 6819, titled "An investigation into candidate genes and protein profiling for abdominal aortic aneurysms". Patients were approached to provide a blood sample in clinics and on the wards, prior to repair and when attending aneurysm screening and surveillance. Full informed consent was obtained. Controls were defined as patients with radiological evidence of an aortic diameter of less than 25mm. Aneurysm cases were patients who had a radiologically proven aortic diameter of greater than 30mm. Blood was obtained using 10 ml collection tubes containing Ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer; Franklin Lakes, New Jersey, USA). Patients with aortic diameters of between 25 and 29 mm were deliberately not included. An aortic diameter of 25-29 mm is termed an ectatic aorta, recent evidence has demonstrated a significant proportion of patients with an ectatic aorta will progress to an having an AAA within 5 years, 67.7% [Wild *et al.* 2013]. A total of 409 samples (266 cases) were collected in Leicester for DNA extraction, the majority processed by myself. Within this cohort 192 (96 cases) were utilised for DNA sequencing and serum was utilised for an ELISA in 70 patients (44 cases). Aoritc tissue biopsies were obtained from 6 cases, biopsies would not be possible in controls. DNA samples from cases and controls were also obtained

from 3 other geographical regions Leeds (n = 468), Belfast (n=473) and Viborg (n= 668). These were all collected in accordance with local ethical approval.

5.2 Collection of Plasma and Buffy Coat

A patient's blood sample was used to both extract the DNA required for subsequent genotyping and sequencing experiments and to obtain protein for ELISA. In order to obtain DNA blood samples were processed following collection. As blood was collected in the presence of EDTA, an anticoagulant, the sample would remain liquid. The initial processing step was a 10 minute centrifuge at 2000G at room temperature (RT). This would allow the cellular and non-cellular constituents of the sample to separate (Figure 5.1). Erythrocytes are the red cells of blood. Plasma is mostly water that also contains proteins (albumin, globulins and fibrinogen). The buffy coat is the layer of containing the white blood cells and platlets.

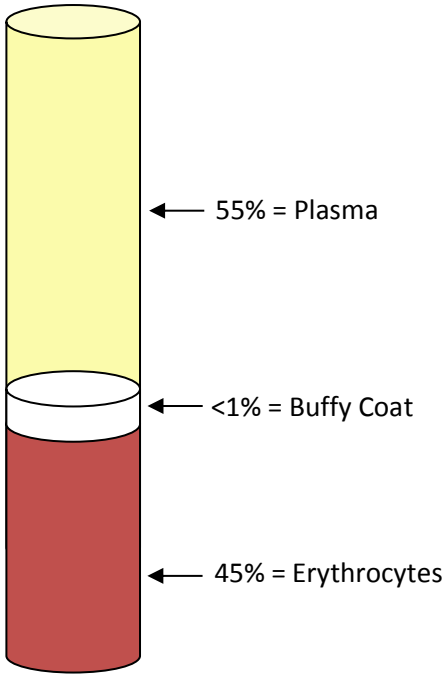

Figure 5.1: Schematic of a blood sample, following centrifugation. Plasma forms a supernatant and the cellular layers precipitate at the bottom of the tube. Erythrocytes are inferior to the buffy coat (containing Leukocytes and Platelets).

The buffy coat contains the nucleated cells that are required for extraction of DNA, erythrocytes are anuclear and plasma is acelluar so these fractions are not required for DNA extraction. Plasma was aliquoted in to 1.5ml eppendorf tubes and placed in a microcentrifuge for 10 minutes at 10,000G, this allowed for precipitation of any platelets from Plasma that were then disposed of. Plasma aliquots were subsequently stored at -80^{o}C following flash freezing in liquid Nitrogen (-196 ^{o}C), this allowed for use in protein concentration assays such as ELISA. Once the buffy coat is extracted from the sample it was flash frozen and stored at -80^{o}C until required.

5.3 DNA extraction from buffy coat.

The buffy coat of a blood sample contains the majority of the nucleated cells, the white blood cells, and therefore this is the best source for subsequent DNA extraction. DNA extraction is possible from whole blood however the laboratory in Leicester has utilized DNA extraction from the buffy coat for several years therefore there was familiarity with this technique and as all samples collected prior to the start of this research had been processed in this way the technique was continued to prevent a potential bias. A Buffy coat was removed from frozen storage when required and the sample was rapidly thawed in a 37^{o}C water bath. Once thawed the buffy coat was added to 4.5ml RBC Lysis Solution (Qiagen; Venlo, Netherlands) in a 15ml centrifuge tube and repeated inversions were used to mix before a 10 minute incubation at RT. The sample was then centrifuged at speed of 2000G for 10 minutes at a temperature of 21^{o}C, allowing the leukocytes to from a pellet. This pellet was preserved while the supernatant was discarded. The leukocyte pellet was then resuspended in 4ml of Cell Lysis Solution (Qiagen) and mixed with a vortex mixer before the solution was

incubated in 37^{o}C water bath for 2 hours. Following removal from the water bath the reaction was left to homogenise for 4 days at RT. Once the solution had homogenised 1ml of Protein Precipitation Solution was added to it before mixing with a vortex and 10 minute centrifuge at 2000G at RT. This centrifugation precipitated out any residual protein as a pellet, that was discarded, the supernatant was then mixed with 3ml propan-2-ol in a 15ml centrifuge tube. Addition of the supernatant to the propan-2-ol created a white band at the top of the mixture. The sample was mixed with fifty inversions before it was centrifuged for 3 minutes at 2000G at RT. A DNA pellet was then preserved and the supernatant disposed of. The DNA was washed in 3ml of 70% ethanol by inverting the solution ten times, the DNA was then precipitated again with 1 minute of centrifugation at 2000G at RT, and the supernatant was then discarded. The pellet was air dried for 15 minutes and prior to quantification the DNA was re-suspended with the addition of 250ul DNA Hydration Solution and incubation for 1 hour in a 65^{o}C water bath.

5.4 Assessment of DNA Quality

The quality of DNA samples was assessed using a Nanodrop 1000 (NanoDrop Technologies, Inc; Wilmington, Delaware, USA) spectrophotometer. The passage of monochromic light through 1µl of the sample is measured, different substances have specific absorbance maxima and this will affect the amount of light absorbed and subsequently detected. Prior to sample analysis it was necessary to clean the aperture of the Nanodrop of any recently analysed sample with pure H2O and then a blank sample was measured. A blank sample was 1µl of DNA hydration solution, this allowed the machine to calibrate. The wavelength of the absorbance maxima of nucleic acid is 260nm whereas protein is 280nm, the ration of these values is used to determine the purity of a sample. A pure sample of DNA has a 260/280 ration of 1.8, whereas the pure ratio for RNA is 2.0. When sampling DNA low 260/280, <1.8, ratios may be due to protein contamination, low sample DNA concentration (>10ng/µl) or residual ethanol (from the process of DNA extraction). Conversely high (>1.8) 260/280 ratios may be observed if a sample is contaminated with RNA. Only DNA samples with a 260/280 ratio ranging from 1.7 to 1.9 were accepted for use in further analysis (mean ratio of samples used was 1.85) (Figures 5.1 & 5.2).

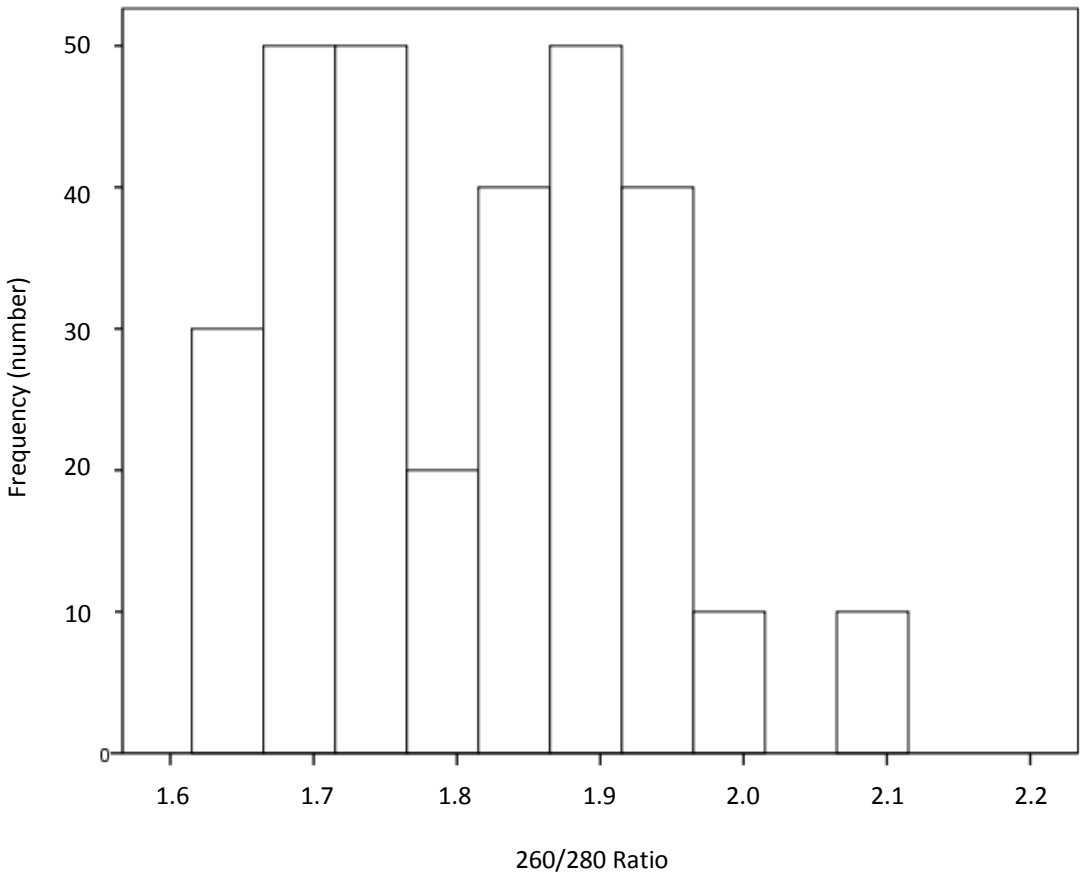

Figure 5.1. Histogram displaying the number patient samples and the respective 260/280 ratio of the DNA samples following processing.

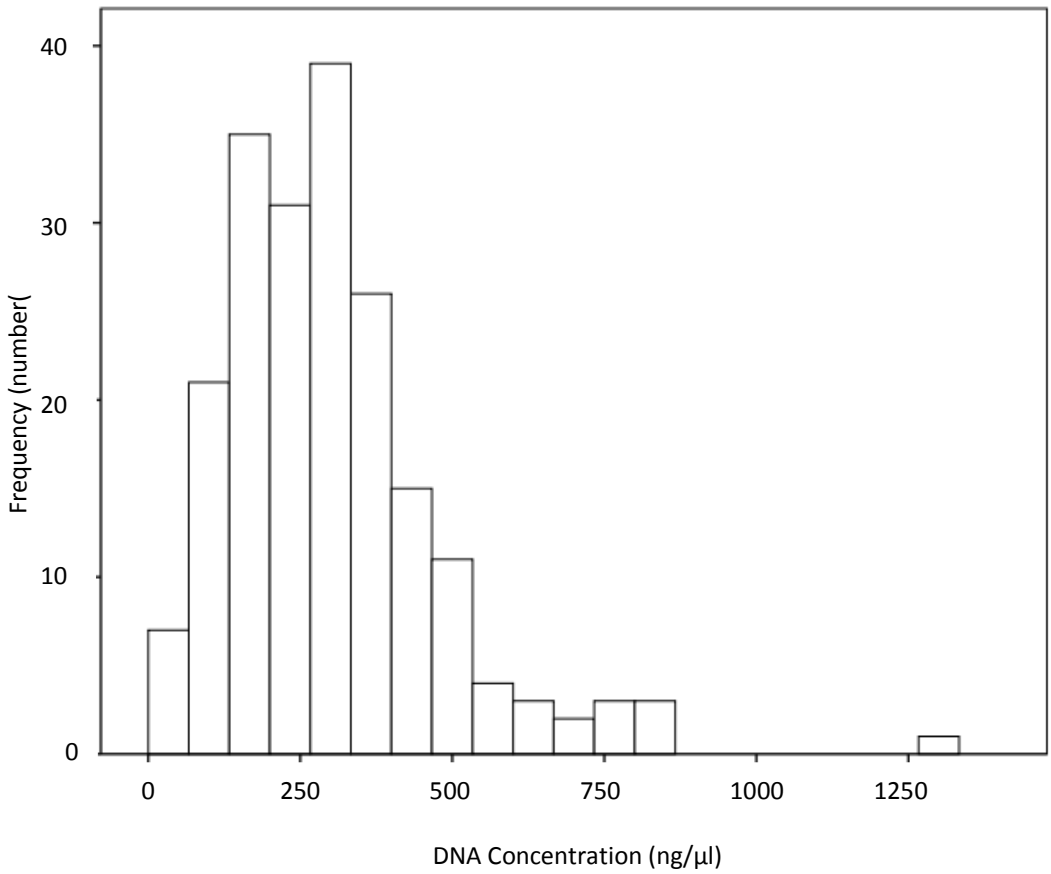

Figure 5.2. Histogram displaying the number patient samples and the respective concentration (ng/µl) of DNA samples processed.

5.5 Chapter 5 Conclusion

All samples will be processed in the same manner using the above methodology. Quality control was conducted with a nano drop in order to ensure only pure DNA is used in experiments.

Chapter 6: SNP genotyping

6.1 SNP Genotyping Background

Multiple studies have reported an association between AAA and specific genes (as discussed in Chapter 2). However many of these associations do not demonstrate association when tested by other research groups. A replication study aims to assess the validity of a previously reported association, usually in a population that has not been used in the initial study. A common theme seen in association studies is that the initial association has a strongly significant association and subsequent replication studies report either weaker levels of association, or no association at all [Ioannidis *et al.* 2001]. Replication studies are however essential in order to ensure that the initial discovery is not a variant limited to the initial population alone or a false association.

6.2 SNP genotyping Methods

There are a wide variety of assays available for SNP genotyping, the method utilised in this project was the TaqMan® assay (Applied Biosystems; Foster City, California, USA). TaqMan® utilises the 5^1 (5 prime) to 3^1 (3 prime) endonuclease activity of *ThermusAquaticus* (Taq) Ploymerase to displace a specific probe from a DNA sequence. The two asymmetric termini of a DNA strand are the 5^1 end, with a terminal phosphate group and the 3^1 end has a terminal hydroxyl group. The assay was first described by Holland *et al.* in 1991, the name TaqMan® derived from the action of the Taq polymerase (Figure 6.1) [Holland *et al.* 1991].

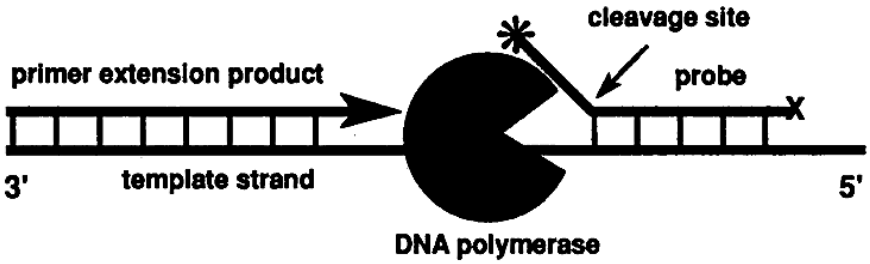

Figure 6.1: Reproduction of the figure from the initial report of the TaqMan assay [Holland *et al.* 1991].*= 5^1 labelled probe, X= 3^1 phosphate block. Reproduced with permission.

The assay has altered little over time and the current process begins with the production of 2 probes, corresponding to the 2 possible SNP alleles. Each Probe has a distinct flurophore at the 5^1 end and a quencher molecule at the 3^1 end. The flurophores used are VIC (a proprietary dye owned by Applies Biosystems, emission wavelength 554nm) and FAM (Fluorescein amidite, emission wavelength 520nm). The PCR reaction is then undertaken with probes, template and TaqMan® mastermix (master mix contains AmpliTaq Gold® DNA polymerase, ultra pure dNTPs, Glycerin, 1,3-Propanediol 2-amino-2-(hydroxymethyl)-hydrochloride, Sodium azide). Hybridization of either probe to template DNA will then take place. The 5^1 endonuclease action of Taq polymerase will cleave the fluorphore of whichever probe is hybridized. When a flurophore is separated from its quencher it can then be detected following laser excitation. Each cycle of PCR will release further fluorophore, increasing ease of detection. Following completion of the PCR reaction samples are analysed using a Plate Reader (Light Cycler 480; Roche; Penzberg, Upper Bavaria,

Germany), which displays the fluorescent signal of each sample, generating a scatter plot for each plate with both 96 and 384 well plate options suitable (Figure 6.2). Each point on the scatter corresponds to the fluorescence of a given sample.

The use of TaqMan® for SNP genotyping is widespread as it offers several advantages over other methodologies [De La Vega *et al.* 2005].

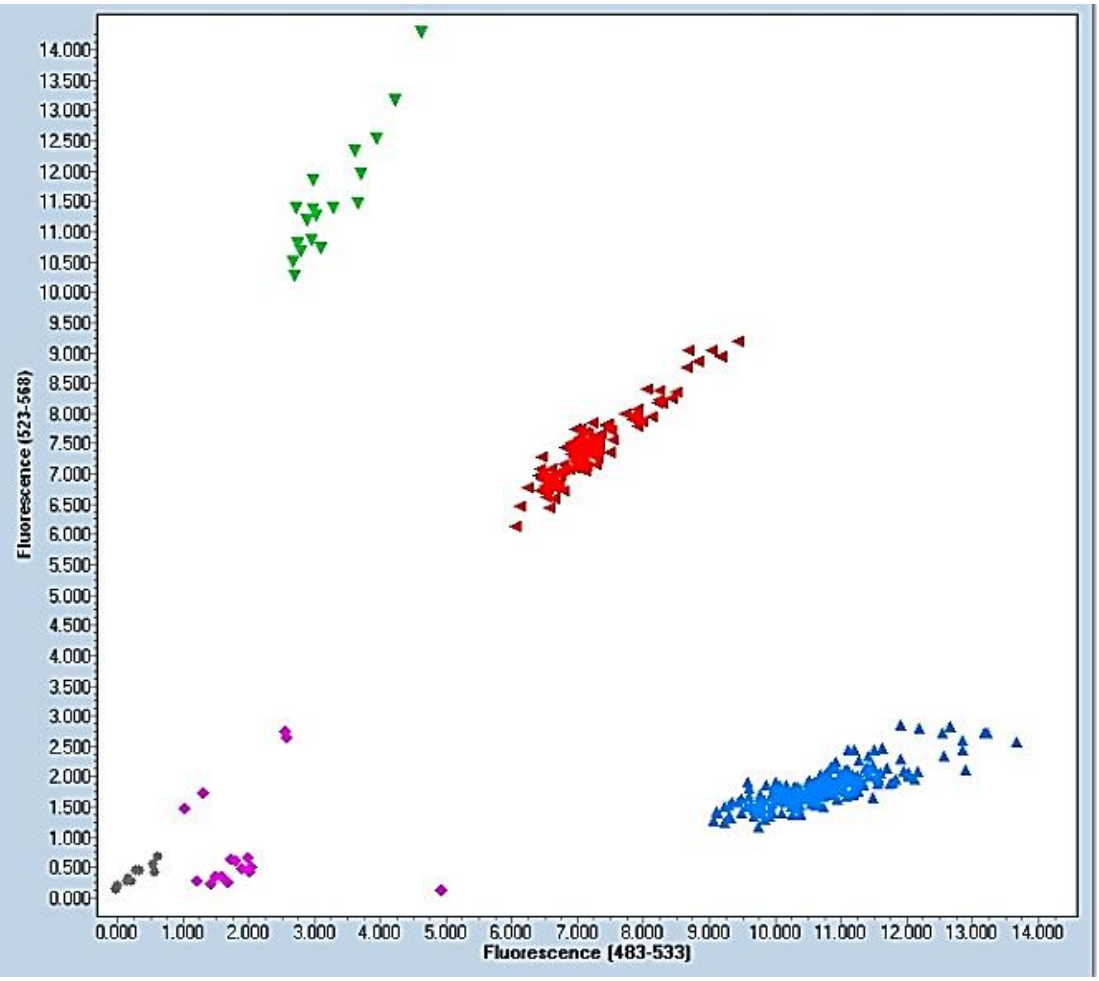

Figure 6.2: Example output from TaqMan® assay. Fluorescence of 2 wavelengths displayed (483-533nm on x axis, 523-568nm on the y axis. ▲ = common homozygote, = heterozygote, = rare homozygote, & = insufficient fluorescence for analysis.

These advantages include:

- Single step enzymatic reaction
- Universal reaction conditions, unaffected by probes or template
- Flexibility of probe location, with wide range of “off the shelf” and custom probes available
- No post PCR processing required
- Capable of genotyping insertion/deletion polymorphisms
- Master mix is universal, simplifying reaction set up

These features made TaqMan® ideal for use in this project as both time and resources were limited however TaqMan® reagents come at a significant cost.

6.3 Quality Control

The scatter plot analysis allows for the identification of clusters of common or rare homozygotes and heterozygotes depending upon the relative fluorescence detected by the plate reader. The software can occasionally attribute a result to a cluster that in reality it is unlikely to represent. Reviewing individual data points allows for more accurate calling of results and the removal of spurious calls. In the plot in Figure 6.2, those samples represented as a purple diamond were initially called as either heterozygotes or homozygotes. This does however increase the failure rate of the assay. Additionally each cohort was analysed to ensure that it was in Hardy-Weinberg Equilibrium (HWE), if any cohort significantly deviated from the expected frequencies then it would be unreliable. The Hardy-Weinberg equation can be applied to a population in order to determine that observed genotype frequencies differ from the expected frequencies based upon the equation. In a case control study like this one, if the genotype frequencies do differ from than those expected the sampled cohort may not be a true representation of the population.

6.4 Validation of the association of rs217120 with AAA

Initial SNP genotyping was conducted in order to confirm or refute the association between AAA and rs217120. The initial association between this SNP and AAA arose from a Golden Gate Assay analysing 466 AAA cases and 466 controls, the major allele frequency was 0.77 (genotypes CC 222, CT 211, TT 7) in cases and 0.71 (genotypes CC 197, CT 192, TT 22) in controls, a Cochrane-Armitage trend test demonstrated a significant difference in these frequencies, P=0.005 OR (odds ratio) = 1.55 (95%

Confidence Interval 1.14-2.10) [Rayt *et al.* 2012]. It must be noted that both the cases and controls deviated significantly from Hardy Weinberg Equilibrium. The rs217120 SNP is a C to T substitution at the 88036839 position on chromosome 11 (Genome Build GRCh37.p13), within the CathepsinC (CTSC) gene. The samples available for this experiment were from Leicester, Belfast and Leeds in the UK and Viborg in Denmark. Demographics that were available are shown in Table 6.1.

	Leicester (409)			Viborg (668)			Leeds (463)			Belfast (473)		
	AAA (266)	Controls (143)	P value	AAA (214)	Controls (249)	P Value	AAA (473)	Controls (195)	P Value	AAA (211)	Controls (262)	P Value
Median Age (range)	71 years (55-91)	65 years (51-78)		76 years (56-92)	69 years (59-90)		70 years (56-75)	69 years (65-75)		72 years (50-92)	69 years (50-79)	
Smoking	222	92	>0.0001	186	194	0.02	203	34	>0.0001	143	157	0.1
MI	60	15	0.004	62	37	0.0003	100	N/A	--	54	52	0.17
HTN	172	57	>0.0001	158	147	0.001	250	86	0.05	94	113	0.83
T2DM	32	12	0.33	19	45	0.006	52	28	0.28	19	39	0.0015

Table 6.1 Demographic data of the Leicester, Viborg, Leeds and Belfast cohorts utilised in the genotyping of rs217120.
Key: Smoking = active or former cigarette smoking, MI = Myocardial Infarction,
HTN = Hypertension, T2DM = Type 2 Diabetes Mellitus

The TaqMan probeAssay used was C____623925_10 at a concentration of 20X along with TaqMan master mix, and individual sample DNA, the probe sequence is shown in Table 6.2. Following SNP genotyping frequencies were tested for association with an Armitage trend test, deviation from Hardy Weinberg Equilibrium was also tested with Pearsons Chi squared test.

Allele	Flurophore	Probe sequence
C	VIC	GCACCCTGTCCTTGTGAGCCTTATA**C**GGGGACTTCATCAAATAGGCACAAC
T	FAM	GCACCCTGTCCTTGTGAGCCTTATA**T**GGGGACTTCATCAAATAGGCACAAC

Table 6.2: The TaqMan® probes for the SNP rs217120

6.4.1 rs217120 Genotyping Results

Samples of DNA were obtained from Leicester (n= 409, samples that had not previously been analysed) in the UK, Belfast (n=473) and Leeds (n = 468) in the UK and Viborg, Denmark (n= 668). The results of the genotyping can be seen in Table 6.3.

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=266	n=143	1.07 (0.80-1.43)	0.658
	CC	123	63		
	CT	112	62		
	TT	31	18		
Viborg		n=473	n=195	0.77 (0.59-1.00)	0.047
	CC	201	98		
	CT	214	80		
	TT	58	17		
Leeds		n=219	n=249	1.01 (0.95-1.07)	0.742
	CC	92	118		
	CT	108	101		
	TT	19	30		
Belfast		n=211	n=262	1.93 (1.39-2.69)	0.0001
	CC	121	108		
	CT	84	132		
	TT	6	22		

Table 6.3.Results of Validation of rs217120 Genotyping on cohorts from Leicester, Leeds and Viborg

6.4.2 Discussion of the validation of rs217120

A significant association was seen in the cohort from Belfast, however the samples from Leeds and Leicester did not significantly associate the rs217120 polymorphism with AAA the samples from Viborg demonstrated the opposite association to the initial data from Leicester and Belfast, with an odds ratio of 0.77 of having an AAA in individuals with the minor frequency, T, allele. It must be noted that in the Belfast cohort genotype distribution within the controls lay outside of Hardy Weinberg Equilibrium (HWE) (Pearson Chi Squared P value =0.036). None of the other cohorts demonstrated deviation from HWE. It is clear that in all cohorts there was significant variation seen in the comorbidities of the cases and controls.

6.4.3 Meta-analysis of rs217120 data

The results of the different geographic cohorts were then combined using and inverse variance meta-analysis (Metagen on "R", Ihaka 1996). The inverse variance meta-analysis demonstrated that overall the odds ratio for having an AAA with the minor allele of rs217120 was 1.17 (95% Confidence Interval 0.98-1.39), P value = 0.08 (Table 6.4). This association was therefore not statistically significant.

Location	Odds Ratio (95% Confidence Interval)	P value
Discovery Study	1.55 (1.14-2.10)	0.005
Leicester	1.07 (0.80-1.43)	0.658
Viborg	0.77 (0.59-1.00)	0.047
Leeds	1.01 (0.95-1.07)	0.742
Belfast	1.93 (1.39-2.69)	0.0001
Meta-Analysis	**1.17 (0.98-1.39)**	**0.08**

Table 6.4: The results of the cohort genotyping for association between rs217120 and AAA and meta-analysis (inverse variance, fixed effects model).

6.5 Analysis of SNPs within LRP1

A recent genome wide association study has identified a significant association between a SNP, rs1466535 and abdominal aortic aneurysms [Bown 2011].This SNP is located at 12q13.3 within the low density lipoprotein receptor related protein (LRP1) gene. The rs1466535 polymorphism is a C to T substitution at position 57534470 on chromosome 12 (Genome Build GRCh37.p13).

This SNP lies between the first and second exons of the LRP1 gene, analysis of this region of the LRP1 gene was undertaken in order to determine if a more significant association could be identified between a regional SNP and AAA.

One approach to Fine Map this region would then be to genotype all SNPs that are in high linkage disequilibrium (LD) with rs1466535. LD refers to the correlation between SNPs, as the distribution of SNPs is not random some SNPs are always found in association together. The r^2 value is a numerical representation of LD, with values ranging from 0 when there is no relation and 1 when 2 SNPs are always found together. The list of SNPs in high LD (r^2 >0.2) with rs1466535 is in Table 6.5 and Figure 6.3. As this would require 39 SNPs to be genotyped a strategy was undertaken to reduce the number of SNPs in the analysis to reduce cost and experimental time. This was achieved in 2 stages. The first was to utilise the Imputed data from the GWAS to determine the SNPs in high LD with rs1466535 that were not directly genotyped but were inferred to have the most significant association with AAA. Secondly SNPs associated to rs1466535 were screened for the potential of residing within regulatory elements as expressed as being within areas of relatively high DNase hypersensitivity.

SNP	Distance from rs1466535	r^2
rs4759277	780	1
rs1385526	1721	1
rs4367982	2838	1
rs10876965	3020	1
rs10876964	3656	0.963
rs79770071	442	0.926
rs11172114	5027	0.855
rs4257011	6273	0.821
rs4759044	3800	0.722
rs4759276	7824	0.675
rs10467124	14776	0.586
rs12309413	20591	0.542
rs11172113	7187	0.541
rs12368672	22000	0.532
rs4759275	8714	0.518
rs12298170	19107	0.518
rs12322902	20604	0.518
rs3001425	24901	0.495
rs3122929	25368	0.495
rs3001427	25821	0.495
rs11172106	21595	0.448
rs12312693	22736	0.448
rs324014	24161	0.41
rs167769	30695	0.404
rs324011	32288	0.382
rs7968719	6281	0.381
rs703817	44642	0.353
rs324013	23809	0.338
rs2122692	23959	0.324
rs324020	48437	0.294
rs4759042	157123	0.272
rs715948	1488	0.259
rs841718	41474	0.253
rs1843314	172048	0.228
rs1044931	84660	0.227
rs324021	88625	0.227
rs7959725	249380	0.211
rs7967940	254514	0.211

Table 6.5: All SNPs in high LD with rs1466535

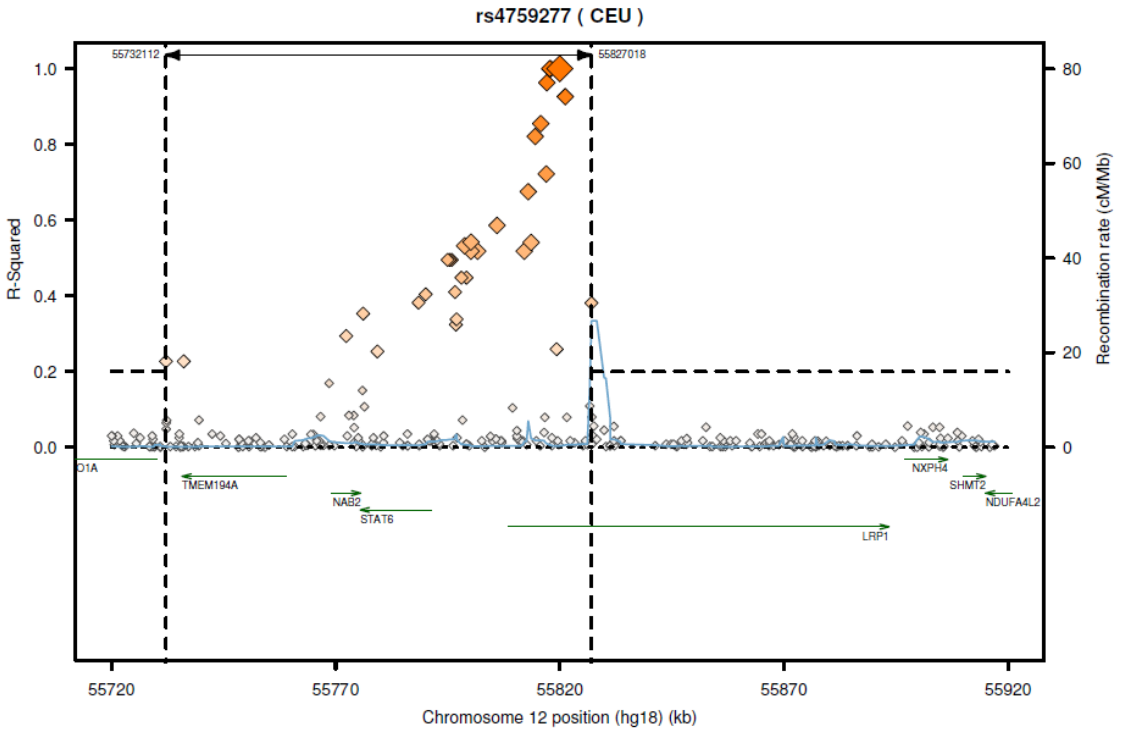

Figure 6.3. Regional Association Plot of all the SNPs associated with rs1466535, created by SNAP from the Broad Institute, USA [Johnson *et al.* 2008].

6.5.1 Imputation

Imputation is the process where SNPs that were not directly genotyped but are in association can have their frequency estimated or Imputed based upon the correlation (LD or linkage disequilibrium) between markers [Marchini 2011]. This process utilises the frequencies determined from the GWAS data and the reported LD of SNPs from the International HapMap project and other published studies to statistically derive the likely frequencies of many more SNPs than can be found in the genotyping arrays used for GWAS. An illustration of the ability of Imputation to increase the data from a GWAS is shown in Figure 6.4. Focused regional imputation of the discovery phase of a

GWAS (1866 AAA cases and 5435 controls) was conducted in order to infer genotypes of SNPs that had not been directly typed [Bown 2011]. Analysis of polymorphisms in high LD (r^2>0.2) with rs1466535 revealed 2 imputed SNPs had a more significant association with AAA than the GWAS lead SNP (Table 6.6). These 2 SNPs were therefore selected for laboratory genotyping on novel samples than had not been used in the GWAS discovery phase.

SNP	Location on Chromosome 12	R^2 to rs1466535	P Value from imputed GWAS data
rs4257011	*55814464*	*0.821*	*5.028E-08*
rs11172114	*55815710*	*0.855*	*5.373E-08*
rs1466535	55820737	1	1.247E-07
rs4759277	55819957	1	1.247E-07
rs4367982	55817899	1	1.289E-07
rs1385526	55819016	1	1.604E-07
rs10876965	55817717	1	2.042E-07
rs10876964	55817081	0.963	3.662E-07
rs4759276	55812913	0.675	0.000001277
rs4759044	55816937	0.722	0.000001596
rs11172113	55813550	0.541	0.000007905
rs10467124	55805961	0.586	0.000009795
rs4759275	55812023	0.518	0.00005736
rs3001427	55794916	0.495	0.0003332
rs12368672	55798737	0.532	0.0003456
rs3001425	55795836	0.495	0.0003557
rs3122929	55795369	0.495	0.0003884
rs12309413	55800146	0.542	0.0004323
rs167769	55790042	0.404	0.0005802
rs12298170	55801630	0.518	0.0009492
rs324011	55788449	0.382	0.0009987
rs12322902	55800133	0.518	0.001076
rs703817	55776095	0.353	0.004353
rs7968719	55827018	0.381	0.004837
rs11172106	55799142	0.448	0.006215
rs12312693	55798001	0.448	0.008597
rs324014	55796576	0.41	0.01071
rs841718	55779263	0.253	0.01753
rs715948	55819249	0.259	0.01879
rs324020	55772300	0.294	0.02277
rs1843314	55648689	0.228	0.03388
rs4759042	55663614	0.272	0.05561
rs1044931	55736077	0.227	0.09116
rs324021	55732112	0.227	0.09116
rs324013	55796928	0.338	0.1356
rs2122692	55796778	0.324	0.2057
rs7959725	55571357	0.211	0.3057
rs7967940	55566223	0.211	0.3423

Table 6.6: SNPs chosen for genotyping based upon imputation results with alleles, chromosomal location and LD to rs1466535, chosen SNPs italicized.

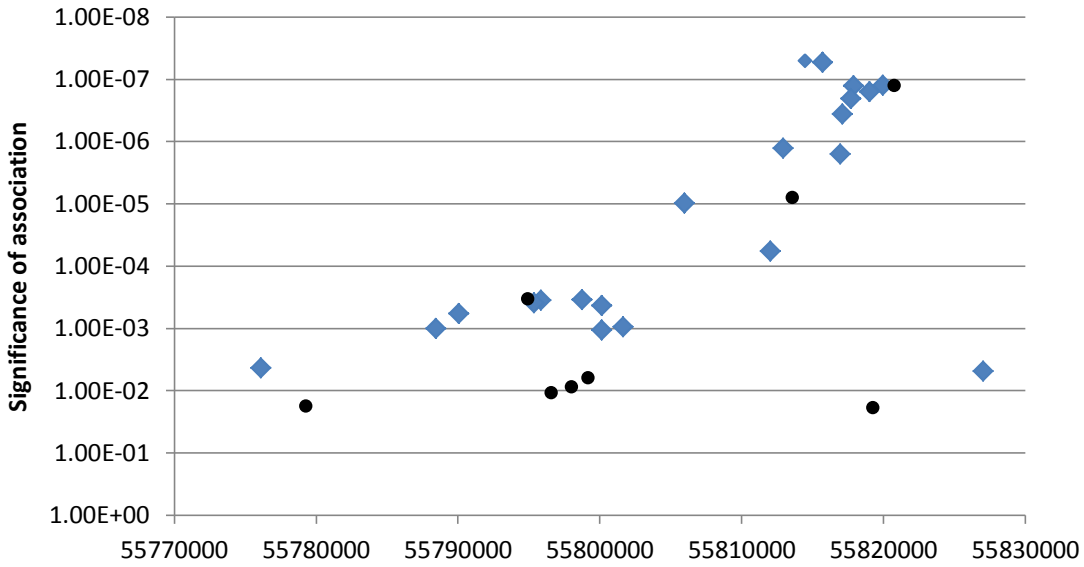

Figure 6.4. This is a schematic representation of the results of GWAS with imputation. Each point represents a SNP, the Y axis corresponds to the degree of significance of association between the SNP and the disease, the X axis represents the position of a SNP upon Chromosome 12. The black circles are those SNPs directly genotyped, the blue diamonds represent the SNP data available after imputation.

6.5.2 DNase Hypesensitivity

Within genes there are a variety of regions that are affect gene transcription, known as the *cis*-regulatory elements. These regions can have a variety of functions related to gene expression and include promoters, enhancers, insulators and silencers. These elements are found in areas of the genome that are accessible to transcription factors, regions that are not bound in tight chromatin. These regions of open chromatin are detected via their relative hypersensitivity to DNase 1 digestion [Thurman 2012]. The ENCODE (Encyclopedia of DNA elements) project published a map of these regions (accessible via the UCSC Genome Browser application, Figure 6.5) and it was possible to determine regions within the LRP1 gene that exhibit DNase 1 hypersensitivity [Rosenbloom 2013]. Any SNPs found within these regions were cross referenced with SNPs associated with rs1466535 (LD r^2>0.2). This process yielded 3 further SNPs from the list of SNPs in high LD with rs1466535 that were selected for genotyping (Table 6.7).

SNP	SNP alleles	Location on Chromosome 12	R^2 to rs1466535	Imputed P-value
rs4759044	C/T	55816937	0.72	1.60×10^{-6}
rs11172113	C/T	55813550	0.54	7.91×10^{-6}
rs715948	A/G	55819249	0.26	0.019

Table 6.6: SNPs chosen for genotyping based upon DNase 1 hypersensitivity analysis with alleles, chromosomal location and LD to rs1466535.

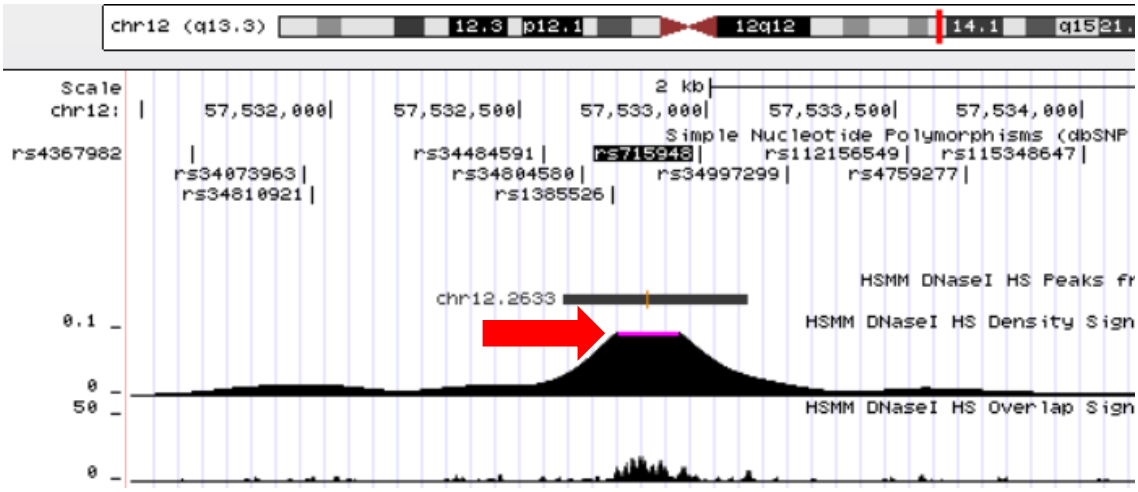

Figure 6.5: Image from Genome Browser, the red arrow represents an area within Chromosome 12 where there is relative DNase hypersensitivity with the peaks corresponding to areas of relative DNase 1 Hypersensitivity. There are several SNPs associated with this region however it is rs715948 that is a SNP in LD with rs1466535 (r^2 = 0.26), therefore it was this SNP that was chosen for genotyping.

6.5.3 Genotyping of samples for SNPs within LRP1

Samples of DNA were obtained from Leicester (n= 331, fewer control samples were available as they had been utilized in the Discovery Phase of the GWAS), Leeds (n = 468) in the UK and Viborg, Denmark (n= 668). The reaction mixture was 5ul in total, 1ul of individual patient DNA (concentration 10ng/ul), 2.5ulTaqMan® master mix at 2X, 1.25µl Ultra pure H_2O and 0.25µl of specific probe at 20X concentration. The list of probes utilised are in Table 6.7. The PCR programme was 10 minutes at 95^0c in order to denature the genomic DNA followed by 40 cycles of 15 seconds of denaturing at 92^0c and 1 minute of annealing and extension at 60^0c. Plates were analysed on the Roche Lightcycler 480, a q (real time) PCR machine. Following SNP genotyping frequencies were tested for association with an Armitage trend test, deviation from Hardy Weinberg Equilibrium was also tested with Pearsons Chi squared test. Data combination was achieved with meta-analysis with metagen on "R" [R Core Team 2013].

SNP	Allele	Assay reference number	Flurophore	Probe sequence
rs1466535	A	C___7499211_10	VIC	GAAGGAGTTTTTCTTTATCATTGGA**A**TTTCTCTGCCATGGACCCTGCTTCC
	G		FAM	GAAGGAGTTTTTCTTTATCATTGGA**G**TTTCTCTGCCATGGACCCTGCTTCC
rs4257011	A	Custom	VIC	CAGGGAGAACG**T**TGTCGT
	G		FAM	AGGGAGAACG**C**TGTCGT
rs11172114	C	Custom	VIC	TGGCCCCCT**C**CCCCGG
	T		FAM	TGGCCCCCT**T**CCCCGG
rs4759044	C	C___27995834_10	VIC	GTCATACTGTGGAGGGAATTAAATA**C**GATGATACATGGAAAAAACTGCTTT
	T		FAM	GTCATACTGTGGAGGGAATTAAATA**T**GATGATACATGGAAAAAACTGCTTT
rs11172113	C	C___1955075_10	VIC	TGTCTGAGCCTCAGGAAAGAGCCAC**C**GGGCAACACCCAAAATACAAAAAAT
	T		FAM	TGTCTGAGCCTCAGGAAAGAGCCAC**T**GGGCAACACCCAAAATACAAAAAAT
rs715948	A	C___1955075_10	VIC	GCTGGCACAGTCCTGGTGGCTCCAC**C**TTTAGCCTCTCCCCTGCTTGGTTAA
	G		FAM	GCTGGCACAGTCCTGGTGGCTCCAC**T**TTTAGCCTCTCCCCTGCTTGGTTAA

Table 6.7: SNPs in high LD to rs1466535 selected for genotyping with alleles, flurophores and probe sequence.

6.5.4 rs1466535 genotyping results

The results from genotyping of all samples from Leicester, Leeds and Viborg are presented in Table 6.8 (as some Leicester samples had been used in the discovery GWAS fewer samples were available, n = 331), the demographics for the Leicester cohort is shown in Table 6.9, the demographics for Viborg and Leeds are the same as earlier. These results show that rs1466535 did not reach a significant association to AAA in any cohort. These data from Leeds and Viborg were presented in the GWAS [Bown 2011]. The data from Leicester was not utilised in the GWAS. No cohort deviated from HWE. This is presented with the data from the focused regional imputation of the discovery data from Bown *et al.* and the resulting Meta-analysis. Overall rs14466535 is significantly associated with AAA, resulting OR 1.15 (95% confidence interval 1.09 to 1.22, $P = 7.43 \times 10^{-7}$).

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	CC	96	54		
	CT	81	60		
	TT	26	14	1.03 (0.88-1.21)	0.674
Leeds		n=216	n=254		
	CC	97	104		
	CT	95	117		
	TT	24	33	1.12 (0.96-1.31)	0.14
Viborg		n=503	n=196		
	CC	222	131		
	CT	221	90		
	TT	60	25	1.07 (0.94-1.21)	0.3
Imputed				1.23 (1.14-1.33)	$1.25x^{10-7}$
Combined				1.15 (1.09-1.22)	$7.43\ x^{10-7}$

Table 6.8: Data combining laboratory genotypes for rs1466535 in Leeds and Viborg (from Bown *et al.*) and Leicester with Imputed results and meta-analysis of all data.

	Leicester (331)		
	AAA (203)	Controls (128)	P value
Median Age (range)	74 years (55-91)	68 years (63-90)	
Smoking	151	91	0.6
MI	30	16	0.67
HTN	103	52	0.09
T2DM	24	12	0.60

Table 6.9 Demographic data of the Leicester samples utilised in the genotyping of rs1466535.Key: Smoking = active or former cigarette smoking, MI = Myocardial Infarction,HTN = Hypertension, T2DM = Type 2 Diabetes Mellitus

6.5.5 rs11172114 genotyping results

rs11172114 was selected for genotyping on the basis that it had a greater degree of association to AAA than rs1466535 in the Imputed data. The genotyping results from Leicester, Leeds and Viborg are presented in Table 6.10. There was no association demonstrated between rs11172114 and AAA in any cohort, P = >0.05. There was no deviation from HWE in any cohort. Additionally the Imputed data from the discovery phase of the GWAS are presented, this SNP was not directly genotyped in the GWAS, the association is inferred upon the basis of rs11172114 being in LD with SNPs that were genotyped. Combination of the genotyping data from all 3 sites and the imputed GWAS discovery data yields a significant association between rs11172114 Odds Ratio = 1.23 (95% confidence interval 1.14 to 1.31, P = 3.87×10^{-8}). This association is of greater significance than that of rs1466535.

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	CC	92	51		
	CT	67	48		
	TT	17	14	1.23 (0.88-1.72)	0.22
Leeds		n=216	n=254		
	CC	92	103		
	CT	81	106		
	TT	21	30	1.14 (0.87-1.49)	0.35
Viborg		n=503	n=196		
	CC	208	83		
	CT	204	84		
	TT	54	27	1.15(0.84-1.45)	0.47
Imputed				1.24 (1.14-1.34)	$5.37x^{10-8}$
Combined				1.23 (1.14-1.31)	$3.87x^{10-8}$

Table 6.10: Data combining laboratory genotypes for rs11172114 with Imputed results and meta-analysis of all data.

6.5.6 rs4257011 genotyping results

rs4257011 was selected for genotyping on the basis that it had a greater degree of association to AAA than rs1466535 in the imputed data. The SNP genotyping results from Leicester, Leeds and Viborg are presented in Table 6.11. There was no association demonstrated between rs4257011 and AAA in any cohort, P = >0.05. There was no deviation from HWE in any cohort. Additionally the Imputed data from the discovery phase of the GWAS are presented; this SNP was not directly genotyped in the GWAS. Combination of the genotyping data from all 3 sites and the imputed GWAS discovery data yields a significant association between rs4257011 Odds Ratio = 1.22 (95% confidence interval 1.13 to 1.31, P = $1.98x^{10-7}$).

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	GG	97	57		
	GA	78	55		
	AA	25	16	1.07 (0.82-1.39)	0.61
Leeds		n=216	n=254		
	GG	90	98		
	GA	81	97		
	AA	22	29	1.10 (0.83-1.46)	0.51
Viborg		n=503	n=196		
	GG	200	82		
	GA	205	82		
	AA	56	27	1.07(0.79-1.45)	0.68
Imputed				1.24 (1.14-1.34)	$5.03x^{10-8}$
Combined				1.22 (1.13-1.31)	$1.98x^{10-7}$

Table 6.11: Data combining laboratory genotypes for rs4257011 with Imputed results and meta-analysis of all data.

6.5.7 rs11172113 genotyping results

rs11172113 was selected for genotyping as it is in high LD (r2 = 0.54) with rs1466535 and is within a region of DNase hypersensitivity. The SNP genotyping results from Leicester, Leeds and Viborg are presented in Table 6.12. There was no association demonstrated between rs11172113 and AAA in any cohort, all P = >0.05. There was no deviation from HWE in any cohort. Additionally the Imputed data from the discovery phase of the GWAS are presented; this SNP was not directly genotyped in the GWAS. Combination of the genotyping data from all 3 sites and the imputed GWAS discovery data yields a significant association between rs11172113 Odds Ratio = 1.23 (95% confidence interval 1.14 to 1.31, P = 2.25 $x^{10\text{-}5}$).

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	TT	87	43		
	TC	25	61		
	CC	17	21	0.99 (0.88-1.12)	0.90
Leeds		n=216	n=254		
	TT	77	91		
	TC	88	110		
	CC	44	39	1.12 (0.89-1.41)	0.33
Viborg		n=503	n=196		
	TT	155	60		
	TC	203	81		
	CC	71	28	1.12(0.88-1.43)	0.35
Imputed				1.20 (1.11-1.29)	$7.91x^{10-6}$
Combined				1.23 (1.14-1.31)	$2.25\ x^{10-5}$

Table 6.12: Data combining laboratory genotypes for rs11172113 with Imputed results and meta-analysis of all data.

6.5.8 rs4759044 genotyping results

rs4759044 was selected for genotyping as it is in high LD (r2 = 0.76) with rs1466535 and is within a region of DNase hypersensitivity. The SNP genotyping results from Leicester, Leeds and Viborg are presented in Table 6.13. There was no association demonstrated between rs4759044 and AAA in any cohort, all P = >0.05. There was no deviation from HWE in any cohort. Additionally the Imputed data from the discovery phase of the GWAS are presented; this SNP was not directly genotyped in the GWAS. Combination of the genotyping data from all 3 sites and the imputed GWAS discovery data yields a significant association between rs4759044 Odds Ratio = 1. 23 (95% confidence interval 1.14 to 1.31, P = $2.33x^{10-5}$).

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	TT	71	38		
	TC	90	66		
	CC	35	17	0.99 (0.88-1.12)	0.90
Leeds		n=216	n=254		
	TT	78	78		
	TC	87	119		
	CC	44	54	1.12 (0.89-1.41)	0.33
Viborg		n=503	n=196		
	TT	152	57		
	TC	247	89		
	CC	96	46	1.12(0.88-1.43)	0.35
Imputed				1.20 (1.11-1.29)	$5.37x^{10-8}$
Combined				1.23 (1.14-1.31)	$2.33x^{10-5}$

Table 6.13: Data combining laboratory genotypes for rs4759044 with Imputed results and meta-analysis of all data.

6.5.9 rs715498 genotyping results

rs715498 was selected for genotyping as it is in high LD (r2 = 0.26) with rs1466535 and is within a region of DNase hypersensitivity. The SNP genotyping results from Leicester, Leeds and Viborg are presented in Table 6.14. There was no association demonstrated between rs715498 and AAA in any cohort, all P = >0.05. There was no deviation from HWE in any cohort. Additionally the Imputed data from the discovery phase of the GWAS are presented; this SNP was not directly genotyped in the GWAS. Combination of the genotyping data from all 3 sites and the imputed GWAS discovery data yields an association between rs715498 Odds Ratio = 1.07 (95% confidence interval 0.99 to 1.15, P = 0.05).

Location	Allele	Cases	Controls	Odds Ratio (95% Confidence Interval)	P value
Leicester		n=203	n=128		
	GG	98	60		
	GA	86	55		
	AA	17	11	1.03 (0.75-1.42)	0.85
Leeds		n=216	n=254		
	GG	84	115		
	GA	83	109		
	AA	30	26	0.82 (0.61-1.12)	0.21
Viborg		n=503	n=196		
	GG	252	97		
	GA	214	82		
	AA	37	15	1.01(0.65-1.55)	0.97
Imputed				1.10 (1.02-1.19)	0.02
Combined				1.07 (0.99-1.15)	0.05

Table 6.14: Data combining laboratory genotypes for rs715948 with Imputed results and meta-analysis of all data.

6.5.10 SNPs associated with rs1466535 discussion

Laboratory genotyping did not demonstrate a significant association between AAA and any SNP genotyped. However combining data of the genotyping and imputation data did illustrate significant associations, most notably rs11172114 and rs4257011, Figure 6.5.

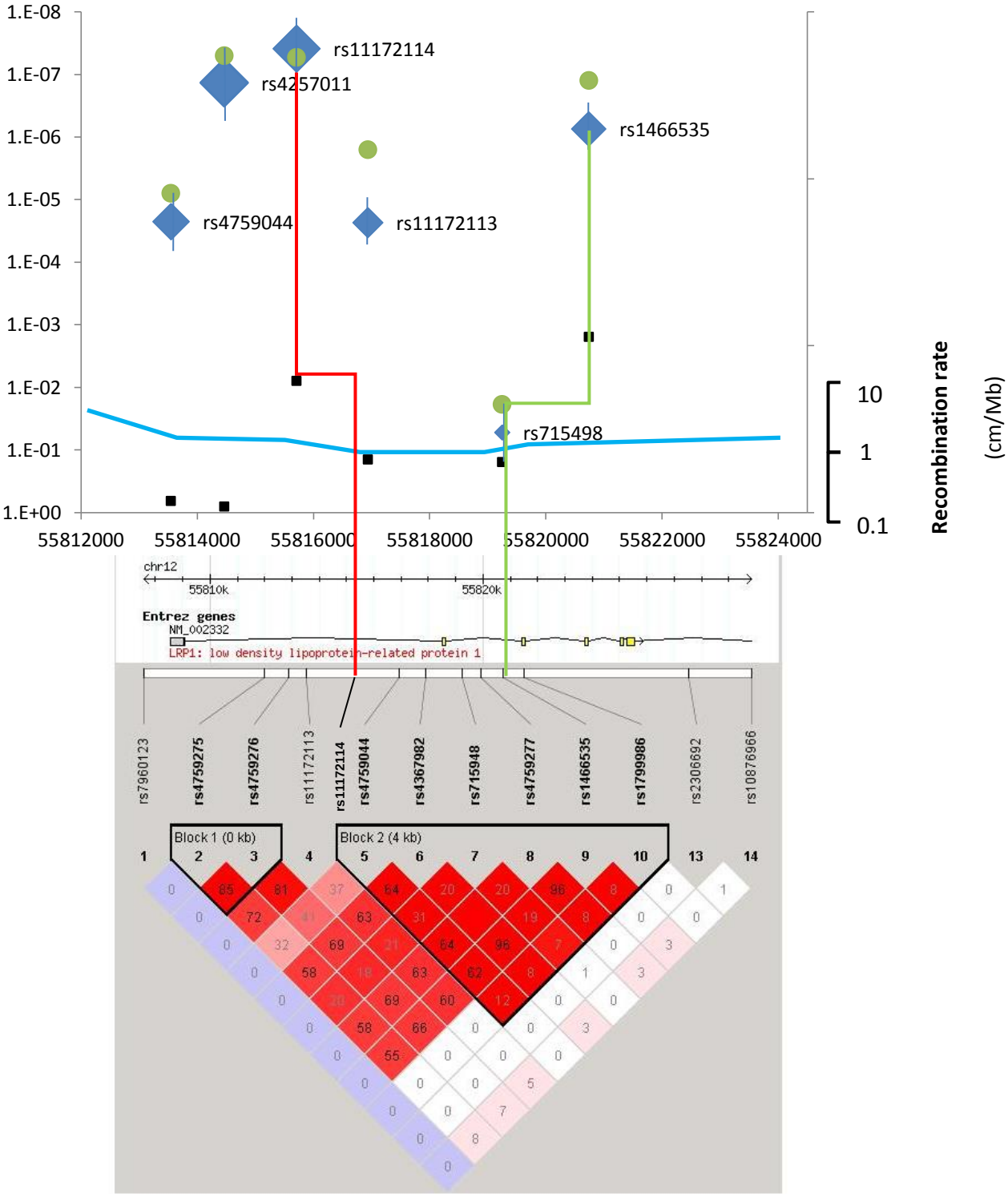

Figure 6.5**:** SNPs genotyped in this experiment. The small black squares = meta-analysis of the laboratory genotyping. The green circles = imputation data from the discovery phase of the GWAS. The blue diamonds = meta-analysis of the laboratory data and the imputation data. The bold blue line represents the regional recombination rate (right axis). The LD plot of the region is below this with the standard D^{1}/LOD Haploview colour scheme. rs1466535 is highlighted by the bold green line, rs11172114 is represented by the bold red line.

6.6 Haplotype Analysis

As several SNPs on the same chromosome have been genotyped it is possible to construct a haplotype from this data. A haplotype is defined as set of polymorphisms that are statistically associated. Haplotype analysis was performed using PHASE (v2.1, 2004) upon all cases that had undergone SNP genotyping [Stephens 2001 and Stephens 2005]. The haplotypes were estimated with PHASE in the AAA group and then the distribution between cases and controls was analysed. The predicted haplotypes of cases and controls was analysed with a permutation test. Pearson $\chi 2$ tests were used to compare the predicted haplotype frequencies within case and control groups.

The estimated haplotype frequencies resulted in 22 different haplotypes in cases and controls. Overall haplotype frequencies in cases and controls were significantly different ($p = 0.02$). The most commonly estimated haplotype was CTTCCA accounting for 32.4% of case and control haplotypes. Table 6.15 shows the most commonly estimated haplotypes with the overall frequency and the frequency within the case and control groups. Although some haplotypes were estimated to have a greater frequency in either group, none of these differences were statistically significant, and no single risk haplotype was identified. The TCCCCA haplotype was the closest to reaching statistical significance, the frequency in cases was 0.8% whereas in controls it was 0.4%, the resulting P value was 0.08 (Pearson $\chi 2$) .

Haplotype	Overall		Controls		Cases					
	Freq	SE	Freq	SE	Freq	SE	OR	Low OR	Hi OR	P – value*
CTTCCA	0.321	0.001	0.338	0.002	0.310	0.001	1.13	0.97	1.32	0.121
TCCTTG	0.302	0.002	0.300	0.002	0.303	0.002	0.98	0.84	1.15	0.841
TCCTCG	0.200	0.002	0.189	0.003	0.207	0.003	0.89	0.74	1.07	0.212
TCCCCG	0.087	0.002	0.088	0.002	0.087	0.002	1.01	0.78	1.31	0.920
CCCTCG	0.051	0.002	0.052	0.003	0.051	0.002	1.02	0.73	1.42	0.920
CCCCCG	0.007	0.001	0.006	0.002	0.008	0.001	0.70	0.30	1.65	0.410
TCCCCA	0.006	0.0004	0.008	0.001	0.004	0.0003	2.56	0.85	7.68	0.082
TTTCCA	0.005	0.001	0.006	0.001	0.005	0.001	1.17	0.43	3.17	0.764

Table 6.15: Estimated Haplotype frequencies, with standard error (S.E.) in controls and cases and odds ratio. Freq = frequency, SE = standard error, OR = Odds Ratio, * = P value from Pearson Chi Squared analysis.

6.7 SNP genotyping: discussion

The initial SNP of interest, rs217120, failed to maintain a statistically significant association when the data from 5 cohorts was combined with an inverse variant meta-analysis. As is the case with many genetic association studies, initial highly significant associations fail to maintain significance with replication studies. Interestingly the replication cohort from Belfast was actually of greater significant association than the initial discovery. However this cohort was both small and its validity must be called in to question; the control cohort deviated significantly from HWE, suggesting that the samples were not representative of their population. Subsequently the association of rs217120 and AAA was further weakened, the cohort from Leeds was not significant and the association between AAA and the *CTSC* SNP demonstrated a modestly significant association with AAA, P value = 0.047, but the effect was in the opposite direction to that from the initial experiment. In other words possession of the rare allele was protective in the Viborg cohort when it had been the risk allele in the initial Leicester and Belfast cohorts. Finally the cohort of samples from Leicester that had not been used in the initial analysis failed to demonstrate a significant association. Without the second Leicester cohorts results it may have been plausible to assume the initial finding was just a manifestation of a gene having a stronger effect within a subpopulation, however with these data it must be assumed the initial association between rs217120 was spurious and therefore did not warrant analysis beyond this stage.

In analysis of the rs1466535 polymorphism the origin of the SNP of interest is derived from a much more robust foundation. The Golden Gate array the lead to the discovery of the association between rs217120 and AAA included just under 900 individuals, the GWAS that lead to the discovery of the association of rs1466535 and AAA had in excess of 50 000. The difference in the statistical power to detect a true variation is vastly improved in the GWAS. Bown *et al.'s* GWAS was also multinational in nature, making problems such as over representation of a polymorphism within a small population highly unlikely. The SNP, rs1466535, was once again not statistically significant in the laboratory replication; however the direction of effect was consistent in each population and once combination via meta-analysis was performed the overall association was highly significant. This was also the case in the other SNPs genotyped, which is unsurprising as they were all known to be in high levels of linkage disequilibrium with the lead SNP from the GWAS. Most interestingly a polymorphism that had previously not been directly typed had a more significant association with AAA than rs1466535. This polymorphism was rs11172114, it is located 5027 bases upstream of SNP rs1466535, within the first intron of the LRP1 gene on chromosome 12. Additionally rs4257011 had an association with AAA that was more significant than rs1466535, this SNP is also located at the 5^1 end of LRP1 gene, 6273 bases up stream of rs1466535.

6.7.1 Chapter 6 Null Hypothesis

The Null Hypothesis was:

SNP genotyping

a. There is no significant association between *CTSC* and AAA
b. rs1466535 is the regional SNP in LRP1 with the most significant association to LRP1

Hypothesis “a” must be accepted as the meta-analysis of the association data between rs217120 and AAA failed to produce a significant P value.

Hypothesis “b” can be rejected as rs11172114 and rs4257011 had more significant levels of association to AAA than rs1466535.

Chapter 7: Re-sequencing of LRP1 exons

7.1 Re-sequencing

Analysis of the SNP genotyping data demonstrated consistently that the there is an association between LRP1 and AAA. The SNPs that significantly associate with AAA are all located to the 5^{l} end of the *LRP1* gene; therefore this region of the gene was the target for the re-sequencing.

Re-sequencing is the process of comparing the sequence of an individual with an accepted norm or reference sequence, to detect genetic variations. If polymorphisms are found within coding portions of genes a base change will be evident that could lead to an amino acid change or even introduce a premature stop codon, and this could obviously influence protein structure and or function. An example of a SNP that causes a disease is rs6025 with the *F5* gene on chromosome 1q23. This missense mutation causes a G to A substitution, resulting in an arginine to glutamine substitution at the 506 position in the resulting F5 protein. This results in the clotting disorder Factor V Leidin deficiency, associated with venous thrombosis, as a binding site on F5 is disrupted by the amino acid change [Bergrem *et al.* 2011].

There is now a publically available resource with over 95% of genomic variation available, The 1000 Genomes Project [The 1000 Genomes Project Consortium]. This catalogue of human variation provides information on vast numbers of polymorphism but cannot give information about the phenotype, i.e. if the patient has an aneurysm. In selecting the control group for this experiment USS confirms a normal aorta in these patients.

Re-sequencing was achieved in two stages. Firstly the first 3 exons at the 5' end of the *LRP1* gene, of cases and controls were amplified with a custom designed PCR (Polymerase Chain Reaction) and the resulting product were sequenced. Once sequence was obtained it was analysed for the presence of any novel polymorphisms. Exons were chosen as it was felt that a mutation within expressed DNA was more likely to influence phenotype than a non-coding polymorphism. Re-sequencing was focused upon these three exons as the strongest associations were seen in introns 1 and 2. The LRP1 gene contains 89 exons and the decision to resequence only 3 was in order to reduce time and cost (Figure 7.1)

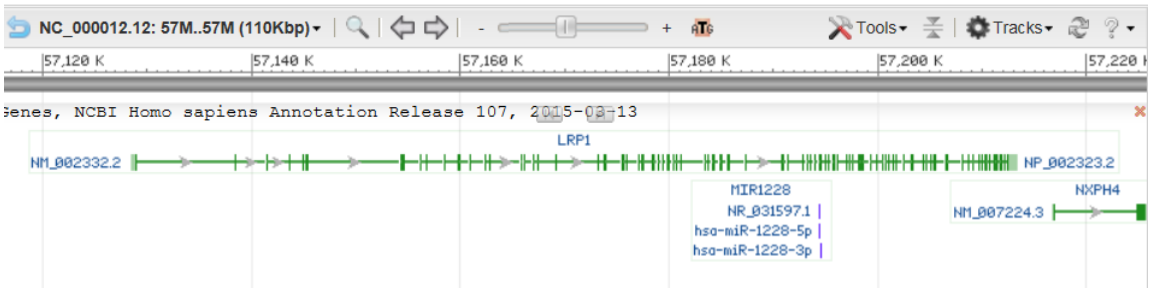

Figure 7.1: Image capture of a schematic of the human LRP1 gene taken from NCBI: Gene (ncbi.nlm.nih.gov/gene/), illustrating the 89 exons of LRP1.

The PCR technique was first reported in 1988 and utilises a thermo stable polymerase enzyme to cause extension of a DNA sequence (a primer) that is complimentary to a strand within the template DNA. Extension leads to duplication of the desired stand and will terminate upon reaching the second primer. In order to reproduce larger quantities of product a PCR reaction undergoes numerous cycles of heating and

cooling, allowing the Primer sequences to anneal, extend and then dissociate in order for unbound primers to anneal in the next cycle. The reaction requires a thermo stable enzyme as the temperature required to disrupt the DNA is around 95^0c and 35 cycles of heating and cooling are not uncommon. The essential components of a PCR reaction are therefore the template DNA, specific left and right sided primers (the beginning and end of the desired sequence to be replicated), a supply of dNTPs (deoxynucleoside triphosphates) the individual bases (A, T, C and G) that make up a strand of DNA, a thermo stable DNA polymerase enzyme (usually *Taq*) and a buffering solution (Figure 7.2).

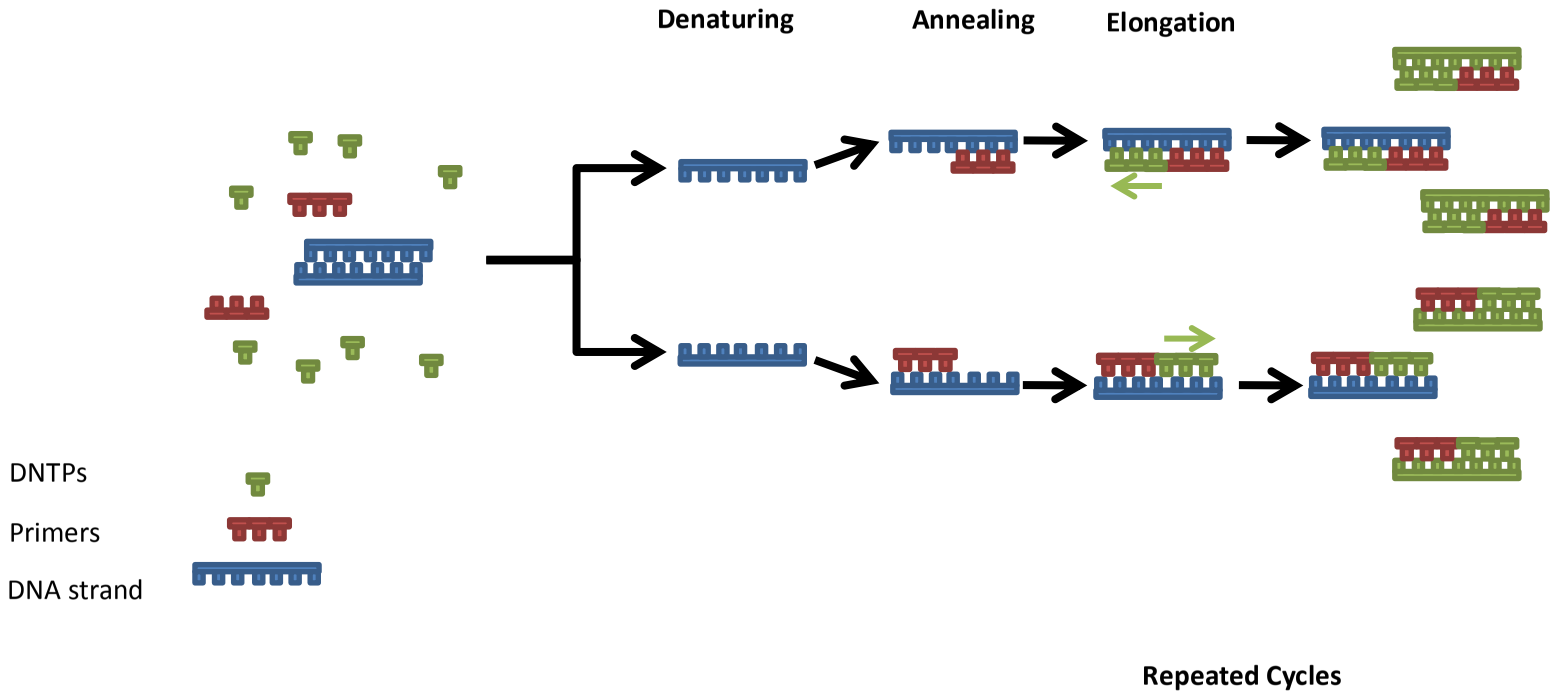

Figure 7.2: Schematic representation of PCR

7.2 Patients

PCR was performed on 192 patient samples (96 cases and 96 controls). The demographics are shown in table 7.1.

Demographic	Cases (96)	Controls (96)	P value
Age (range)	74 (54-90)	66 (63-85)	0.0001*
Males	87	91	0.41
Smoking	86	69	0.003
IHD	16	7	0.08
Hypertension	60	43	0.02
Diabetes	30	9	0.0003
Statin	68	46	0.005
Size (range)	5.3(3-10)	1.8(1.2-3.0)	--

Table 7.1: Demographics of the re-sequencing cohort. P values are calculated by Pearson Chi square analysis, unless * in marked indicating a t test.Key: Smoking = any history, past or current, of cigarette smoking, IHD = Ischaemic heart disease, Diabetes Type 1 or 2.

7.3 Primer Design

Custom primers were designed utilising Primer3, a free web based application [Untergrasser *et al.* 2012]. Primers were designed with at least a cuff of 90 bases either side of the exon in order to preserve the entire sequence of the exon. Primers were also designed so that each pair would have a complimentary melting temperature (tm), a GC clamp at the terminus of the right hand primer and the primers were screened against a mispriming library. The GC content would ideally be between 40 and 60% however this was not possible for Exon 1 as it lies within a GC rich region of the LRP1 gene. Primers for the first 3 exons are listed in table 7.2.

Exon	Primers (Left, right)	Product Size	Tm (^{0}c)
1	CTCCCCCGGGCAGCGCGTCA	810	59.70
	AGGCACCCCCTCCCGCGTCT		58.45
2	CCACCCCCTGACACCCCCAGGC	469	59.41
	AGCCCAACAGGCACCATGCCAAGCA		59.75
3	CCGAGGCCTCCTGAGATCTGAAACCCC	370	57.89
	GGCAGGGGACCAGGACAAGGCCTCA		60.26

Table 7.2: Primers used in the PCR reaction to amplify the first 3 exons of the LRP1 gene

7.4 Optimization

Having designed primers each set underwent several trial reactions where a variety of factors such as temperature and the addition of magnesium and DMSO [Roux *et al.* 2009]. The use of a temperature gradient allowed a range of temperatures to be tested in one experiment, the resulting PCR products were then placed in a 1% Acrilamide Agarose gel and electrophoresis was conducted. Despite *Taq* polymerase being commonly used in PCR, the decision was taken to use Phusion polymerase (New England Biolabs, Ipswich, Massachusetts, USA), which has been proven to have a low error rate ideal for a re-sequencing experiment such as this one [Frey *et al.* 1995].

All initial PCR reactions were made up to a standardised template as described in the Phusion Polymerase literature, reactions were conducted in 50µl volumes.

7.4.1 Temperature gradient

A gradient of temperature was trialed for each primer set, in order to determine the optimum annealing temperature (Figures 7.3 and 7.4).

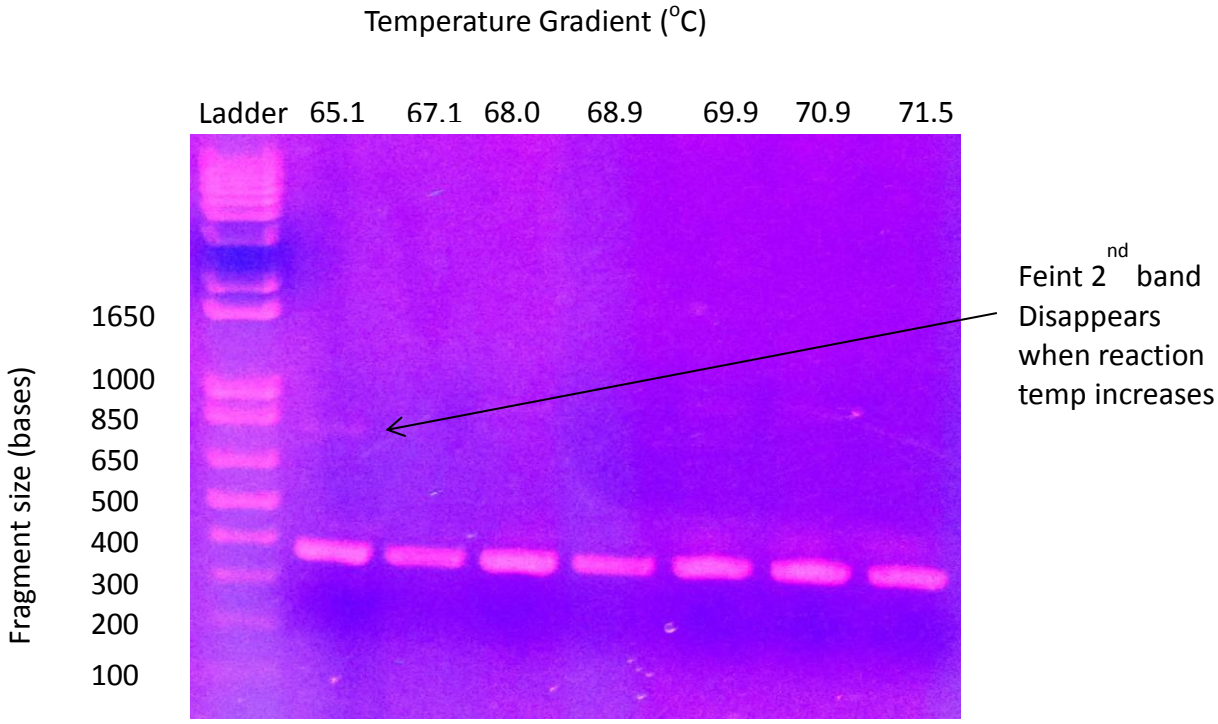

Figure 7.3: Example of temperature gradient PCR reaction. This gradient demonstrated that at a low temperature reaction there was a secondary product band at 65.1°C.

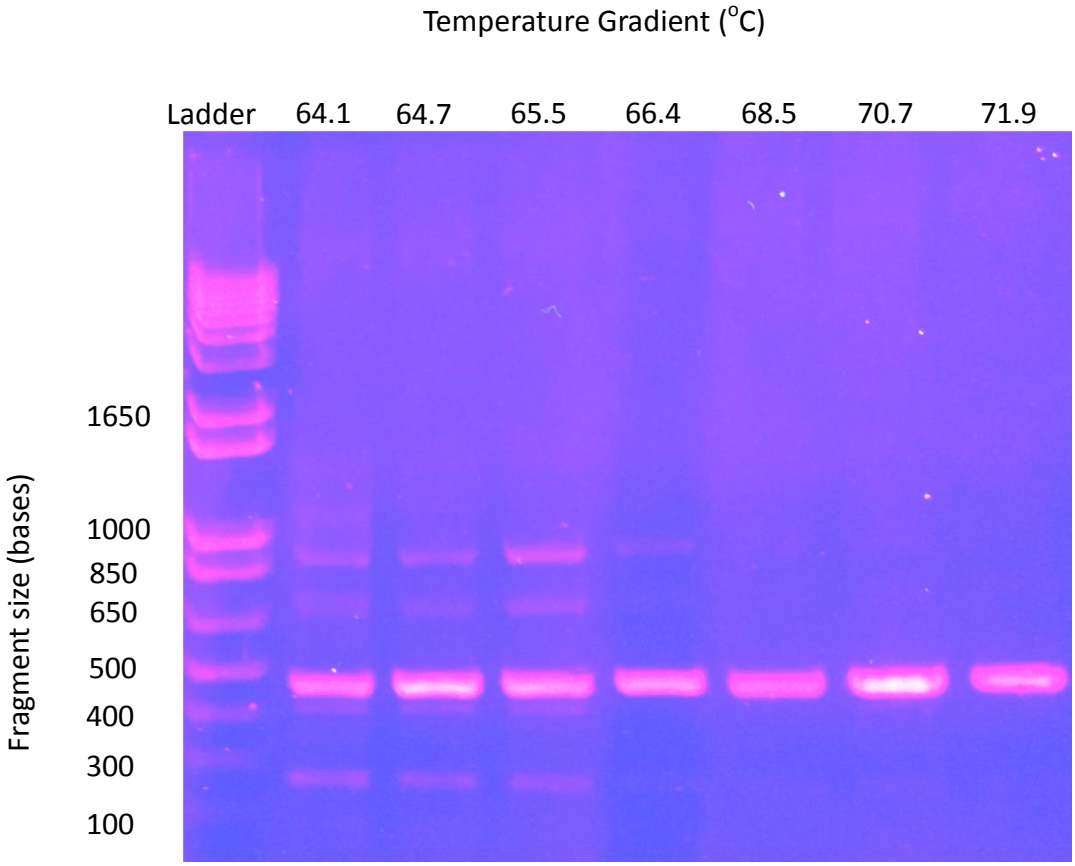

Figure 7.4: Example of temperature gradient PCR reaction. This gradient demonstrated that at a lower temperature reactions, below 64.4°C, there were multiple secondary products

7.4.2 Magnesium Concentration

Magnesium is an essential constituent of buffer solutions, Phusion polymerase is Magnesium dependent. Low magnesium concentrations are associated with low yields whereas excessive concentrations can lead to non-specific binding of the primer, leading to multiple different sized products (Figure 7.5).

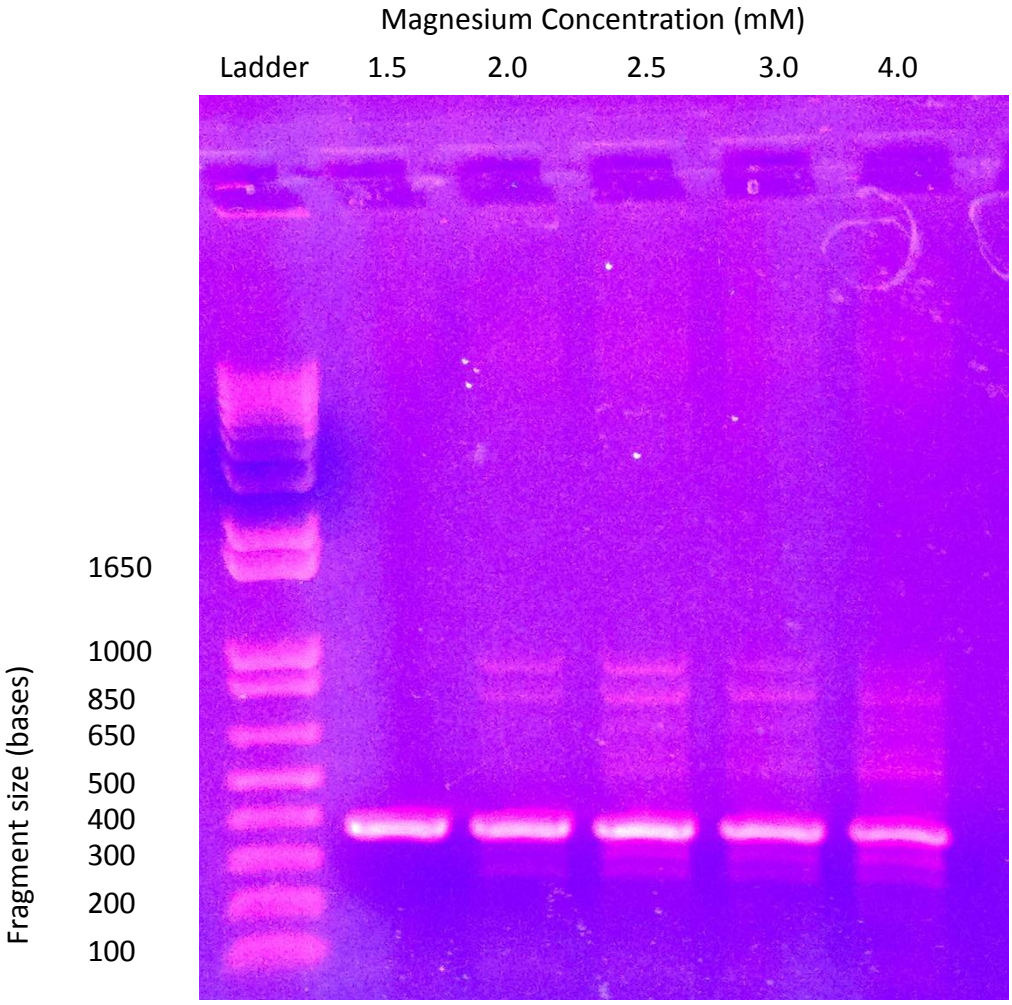

Figure 7.5: Example of Magnesium gradient PCR reaction. This MgCL gradient demonstrated that at a higher concentration of magnesium reaction there were multiple secondary products.

The addition of magnesium to the PCR reaction mixture lead to multiple product bands, indicating nonspecific binding, therefore concentration of Magnesium was maintained at 1.5mM (as supplied in the standard Phusion Polymerase Buffer).

7.4.3 DMSO Concentration

The addition of Dimethyl sulfoxide (DMSO) to a PCR reaction can inhibit DNA secondary structures from forming. Addition of DMSO was not required for the reactions to amplify Exons 2 and 3. Initially trial reactions with amplification of exon 1 were unsuccessful due to multiple product bands (Figure 7.6)

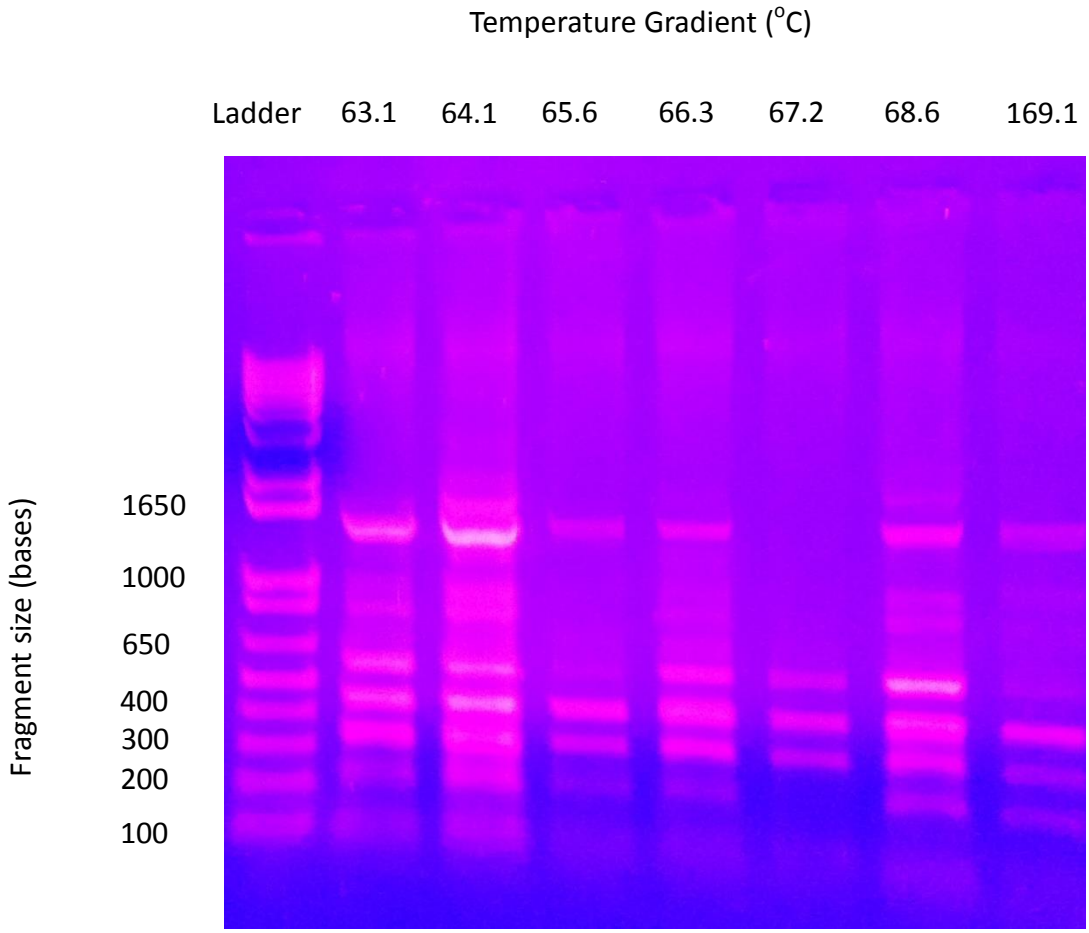

Figure 7.6: Example of temperature gradient in an attempt to amplify Exon 1 of LRP1. The use of the generic buffer lead to multiple bands, the products would be unsuitable for further analysis.

The “GC buffer” was utilised in the amplification of Exon 1 as it resides within a GC rich region. It was clear that the “GC buffer” lead to much less accessory product and therefore this buffer was used in all future reactions where Exon 1 of LRP1 was being amplified. The GC buffer contains DMSO at a final concentration of 3%. In order to determine if this was the sufficient volume of DMSO, a further 2% was added to make a final volume of 5% (Figure 7.7 and 7.8).

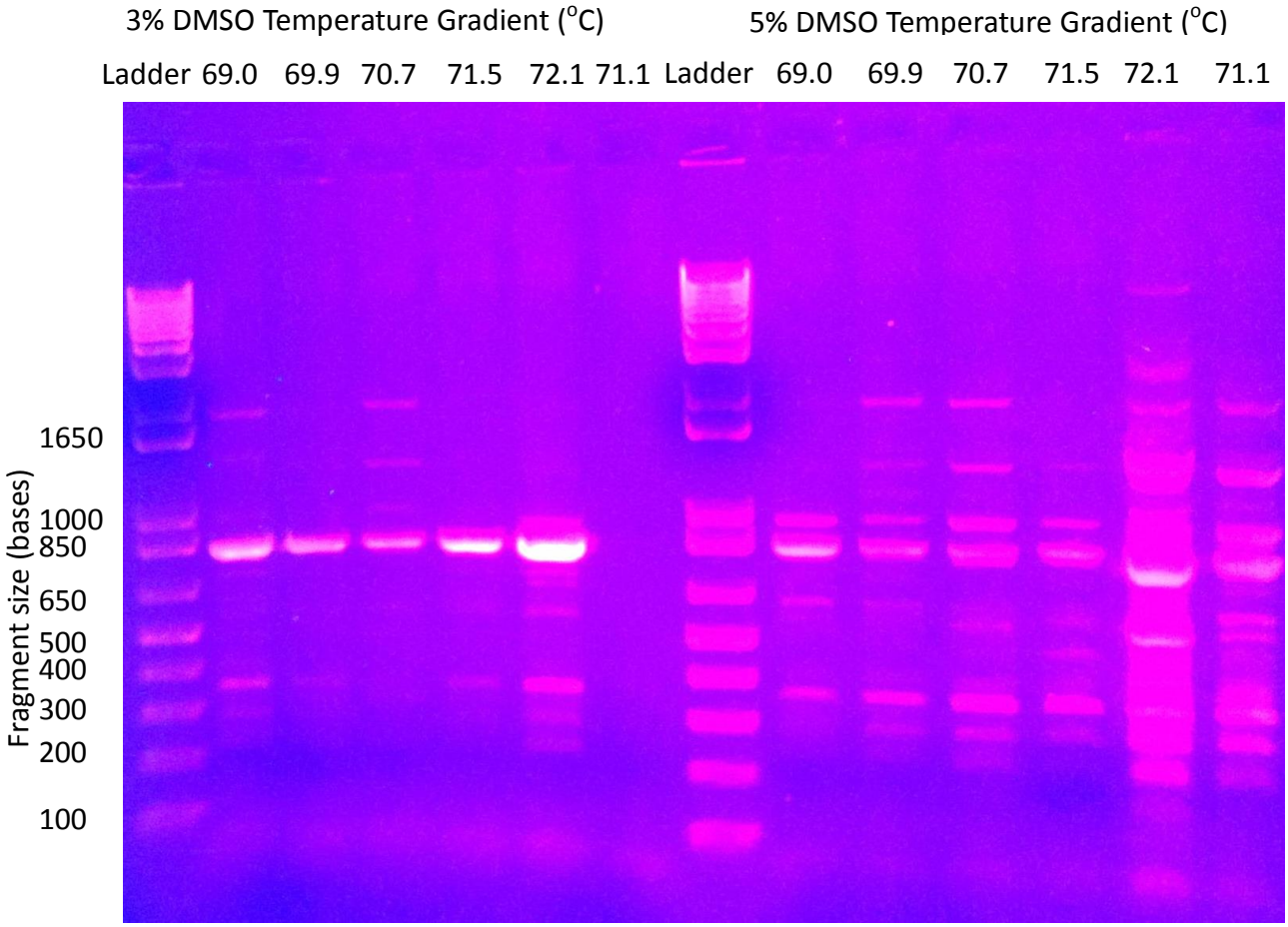

Figure 7.7: Temperature and DMSO gradient utilised in LRP1 Exon 1 amplification.

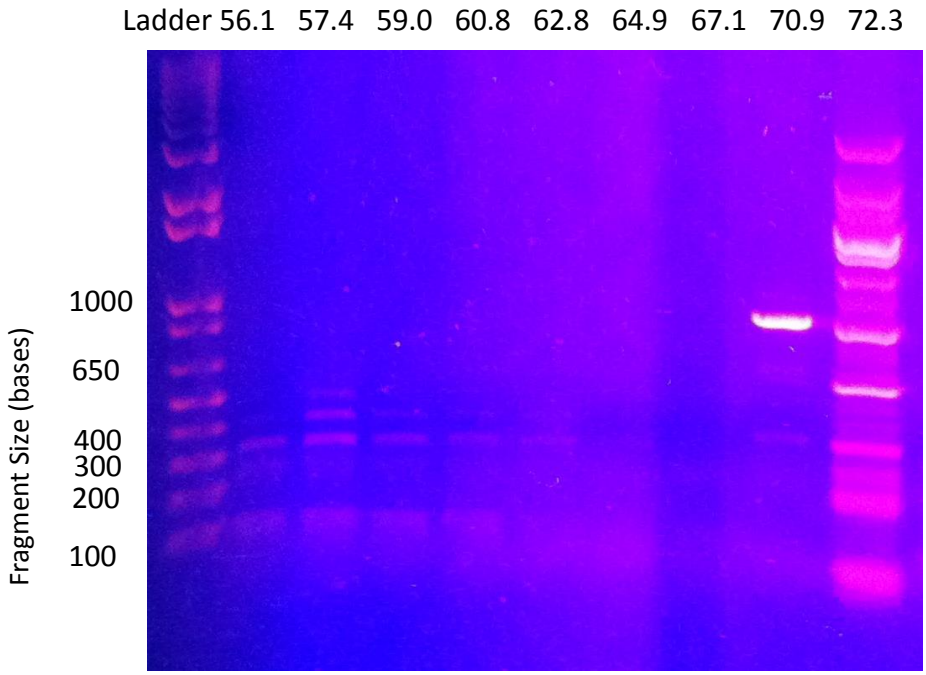

Figure 7.8: Lower Temperature Gradient and GC Buffer in LRP1 Exon 1 amplification

It was clear that additional DMSO was leading to non-specific binding; therefore no additional DMSO was required other than the content with the GC Buffer solution.

7.5 Reaction Reagents

The final reaction mixture for Exons 2 and 3 are displayed in Table 7.4, and Exon 1 in Table 7.5, temperature programs utilised were those in Table 7.6. PCR reactions were conducted in 96 well plates on a GS1 Thermal cycler (G Storm, Somerset, UK: Figure 7.9). Following completion of PCR reactions plates were stored within a -20^{o}C freezer.

Component	Volume	Final Concentration
5X Phusion HF Buffer	10 µl	1X
10 mM dNTPs	1 µl	200 µM each
Left Primer	1 µl	0.5 µM
Right Primer	1 µl	0.5 µM
DNA template	5 µl	
Phusion Polymerase	0.5 µl	0.02U/µl
H_2O	31.5 µl	

Table 7.4 Reagents utilised in Amplification of LRP1 Exons 2 and 3. Left and Right primers used were specific for the given Exon.

Component	Volume	Final Concentration
5X Phusion GC Buffer **Containing DMSO**	10 µl 1.5 µl	1X 3%
10 mM dNTPs	1 µl	200 µM each
Left Primer	1 µl	0.5 µM
Right Primer	1 µl	0.5 µM
DNA template	5 µl	
Phusion Polymerase	0.5 µl	0.02U/µl
H_2O	31.5 µl	

Table 7.5: Reagents utilised in Amplification of LRP1 Exon 1.

Step	Exon 1	Exon 2	Exon 3	Time
Initial Denaturation	98°C	98°C	98°C	10 minutes
35 Cycles	98°C	98°C	98°C	10 seconds
	71.5°C	72°C	71.5°C	30 seconds
	72°C	72°C	72°C	30 seconds
Final Extension	72°C	72°C	72°C	10 minutes
Hold	4°C	4°C	4°C	

Table 7.6: PCR machine programmes.

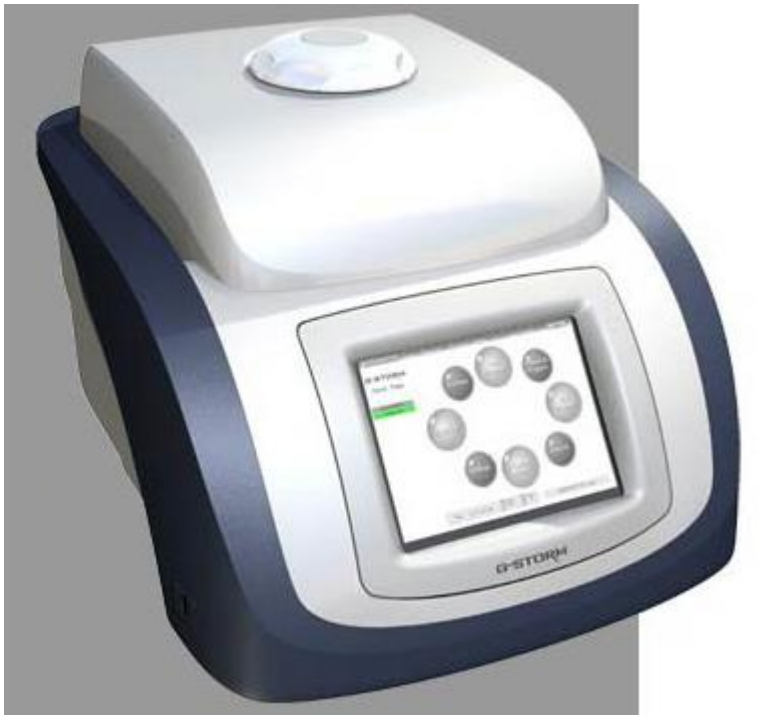

Figure 7.9: The GS1 Thermal Cycler: Utilised to Perform PCR reactions.

7.6 Sequencing

Sequencing of PCR products was undertaken by Source Bioscience (Nottingham, UK). Source utilise an Applied Biosystems 3730 xl DNA Analyzer and BigDye termination chemistry version 3.1 (Life Technologies, Carlsbad, California. USA.). This technology generates sequence via chain termination or Sanger Sequencing. Sanger sequencing is a popular method used to achieve sequencing of moderately sized products. Firstly DNA is purified with the 96 DNA Clean and Concentrator kit (Zymo, Irvine, California, USA). This removes contaminants such as DNA polymerases, nucleases and free dNTPs, that may affect the subsequent analysis of the PCR product. The 3730 xl DNA analyzer utilises a capillary array to aspirate the PCR product from the 96 well plate that the reaction was performed upon. Sanger Sequencing is achieved by using a form of PCR with the addition of fluorescently labelled dideoxynucleosidetriphosphates (ddNTP). There are four ddNTPs corresponding to the 4 DNA bases, ddATP, ddGTP, ddCTP, or ddTTP, each ddNTP being labelled with a different colored flurophor. The ddNTPs prevent the polymerase enzyme from extending that sequence any further. This leads to multiple products at varying lengths. These products can then be separated upon size by electrophoresis. An argon laser is subsequently used excite each strand in size order, causing the labelled ddNTP to fluoresce. Measurement of this fluorescence generates a trace of peaks and produces a chromatograph.

7.7 DNA analysis

Once chromatographs were generated for each sample they were analysed utilising the SeqManPro v11.2 programme from DNAStar (Madison, Wisconsin, USA). This software allowed analysis of individual samples by way of base calling (assignment of a base for a given chromatograph spike), alignment of the sequence and variant calls (identification of mutations within sample sequence). Visual inspection of chromatographs was performed in order to assess signal quality (see Figure 7.10 and 7.11). The Genbank file sequence (NG_016444.1) was imported into SeqMan Pro and used as the reference sequence for contig alignment [Benson *et al.* 2014]. Base position + 1 corresponded to A of the translation initiation codon ATG. Intronic sequence changes were identified based on the LRP1 genomic sequence and amino acid changes were identified based on the reference protein sequence (NP_002323.2). Allelic variations were assessed against the sequence data from 96 controls.

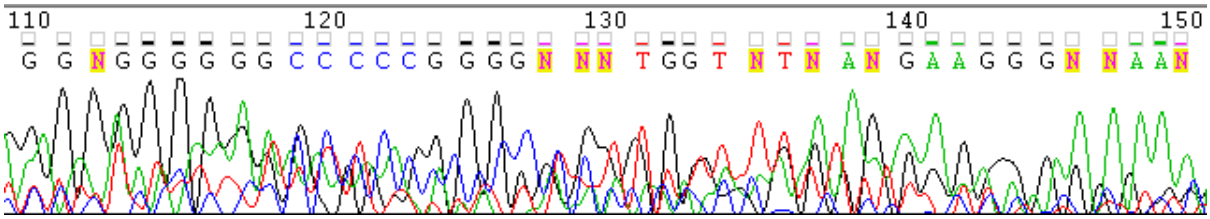

Figure 7.10: An example of very unclear signal, the results of which cannot be relied upon.

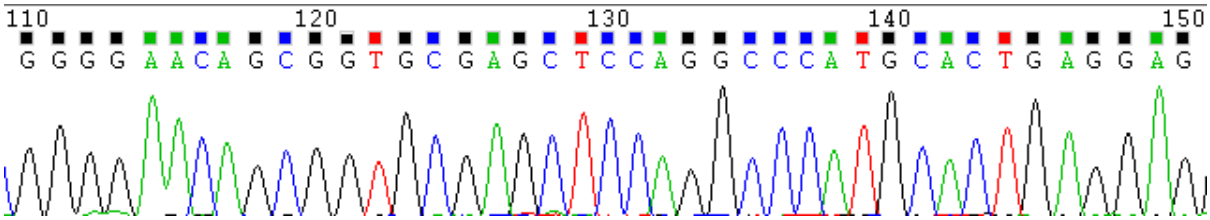

Figure 7.11: An example of clear signal, reliable results can be obtained when the sequence is clear.

7.8 Results of Mutation analysis

There was only one sequence variation identified from within the 3 exons that was consistently found within good quality signal tracing. This was a C>T polymorphism within exon 3, at position 12985 (See image 7.12).

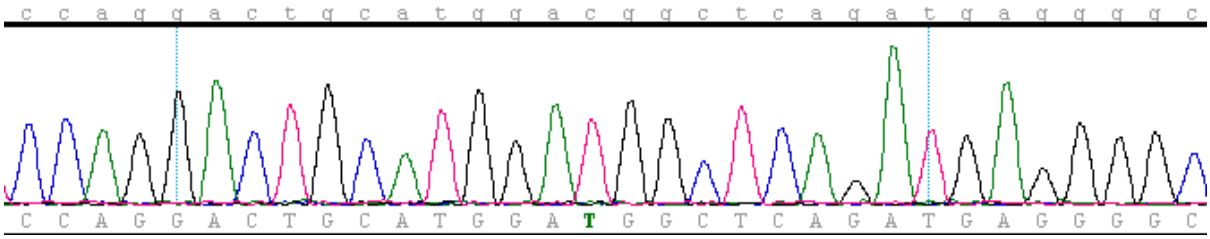

Image 7.12: Chromatograph displaying the variant, highlighted by the green T. The lower case sequence at the top of the image is the reference sequence

The variant has been reported previously, it is known as rs1799986 (HGVS c.[300C>T],p.D100D) (Image 7.13). This was not however a significant finding as it was a synonymous variant with high Allele frequency. This variant was detected in 19 cases and 18 control subjects.

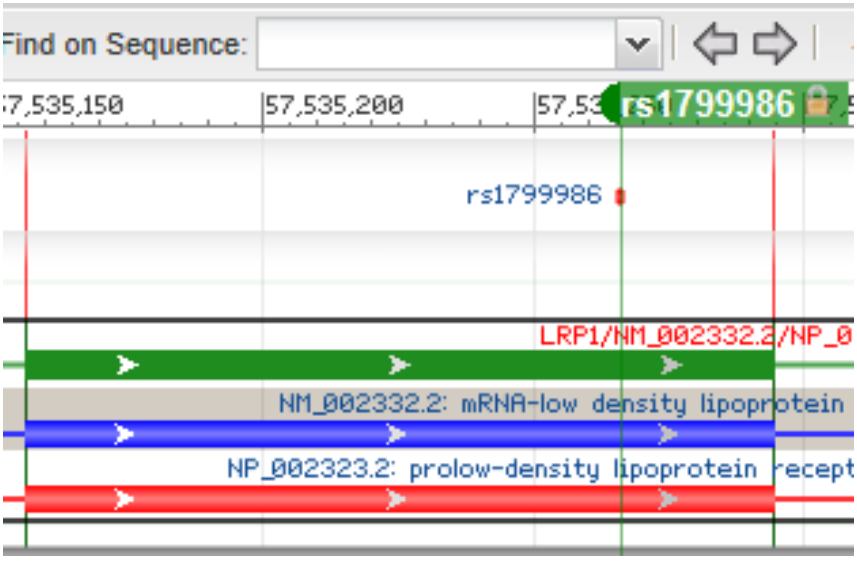

Image 7.13 : Image from Pubmed demonstrating the position of the rs1799986 SNP within the 3rd exon of LRP1.

7.9 Chapter 7 Conclusion

The first three exons of the*LRP1* gene were successfully amplified in a cohort of AAA patients and controls. Sequence analysis of these regions failed to identify any novel mutation.

7.9.1 Chapter 7 Null Hypothesis

The null hypothesis was:

“There is no variation within the exons of LRP1 associated the lead SNP from the re-sequencing experiment”.

The null hypothesis has to be accepted, as no variation was detected.

Chapter 8: Detection of Soluble LRP1 in plasma

8.1 Introduction

The association between AAA and LRP1 was demonstrated within analysis of single nucleotide polymorphisms, a genetic technique. As stated by Bown *et al.* the mechanism by which the rs1466535 polymorphism affects the aorta is un-clear [Bown *et al.* 2011]. Soluble LRP1 was not measured by Bown *et al..,* though LRP1 expression was determined via analysis of mRNA from descending aortic adventitial tissue, with a significant difference observed between the CC and TT homozygotes, (p =0.029) 1.19-fold (1.04–1.36). In order to determine if the systemic expression of LRP1 is affected by SNP genotype or disease status the freshly frozen plasma of both AAA cases and screened controls was analysed. A specific LRP1 detecting Enzyme-Linked Immunosorbent Assay (ELISA) was obtained and utilised to determine the concentration of soluble LRP1.

8.2 Enzyme-Linked Immunosorbent Assay (ELISA)

In order to quantify the levels of LRP1 protein within cases and controls an LRP1 specific ELISA was conducted. ELISA utilises specific antibodies to capture a protein of interest and the initiate a reaction that leads to a detectable colour change, the specific type of ELISA is known a sandwich ELISA (Figure 8.1).

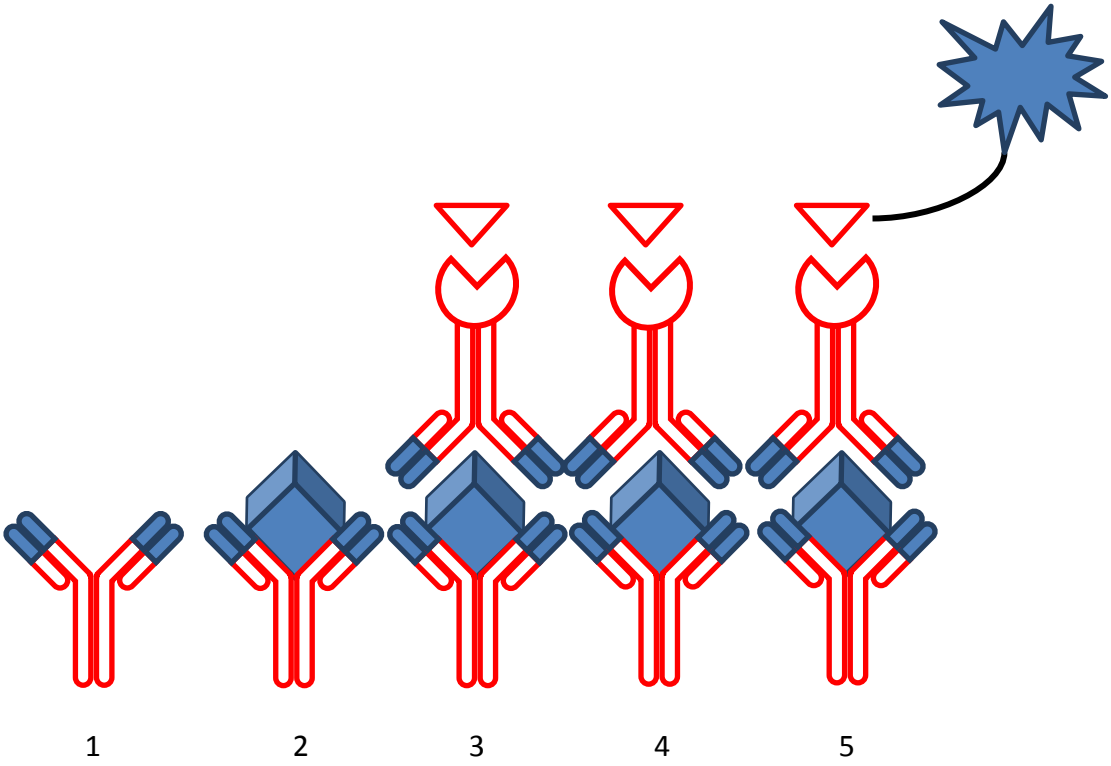

Figure 8.1: Schematic of the key stages of a Sandwich ELISA.

The stages of a Sandwich ELISA:

1. A specific antibody is pre-bound to a polyethylene microtitre plate
2. Plasma samples are incubated with the antibody, the protein of interest will bind to the bound antibody

3. A second specific antibody is exposed to the plate, this antibody is conjugated with an enzyme
4. The addition of a substrate for the conjugated enzyme
5. The action of the conjugated enzyme on the substrate leads to a detectable colour change.

In order to prevent non-specific activity there are multiple washes with a detergent solution between incubations.

Samples of patient plasma were obtained as in Chapter 5. Following thawing samples were diluted in order to perform an ELISA. Plasma sample were diluted to a 1 in 100 dilution as per the suggestion in the manufacturer Instruction Manual.

An ELISA specific for human LRP1 was obtained (USCN Life Sciences, Wuhan, Peoples Republic of China: Catalogue Number E91010Hu). The specific LRP1 antibody bound to the plate was a monoclonal antibody raised in mouse specific to the C terminus of the protein, the second antibody was a polyclonal antibody raised in rabbit. This kit utilises a second antibody that is conjugated to Biotin and a solution of avidin conjugated to horse radish peroxidase (HRP). Only wells on the test plate containing LRP1 would retain the biotin conjugated antibody and the avidin-HRP conjugate and therefore undergo a colour change, clear to yellow. The reaction was halted with the final addition of sulphuric acid, producing a blue coloration, this was then measured with a spectrophotometer (ELx808 Ultra Microplate Reader; Bio-Tek Instruments, Winooski, Vermont, USA) utilising a 450nm filter. Each individuals sample was analysed in 2 separate wells and a mean of these 2 results was taken. A linear trend was then obtained from known standard dilutions (ranging from 10000 to 13.7pg/nl) from the

same plate. The trend from the standard dilutions was then used to derive the concentration of sLRP1 in each sample from the mean optical density (Figure 8.3).

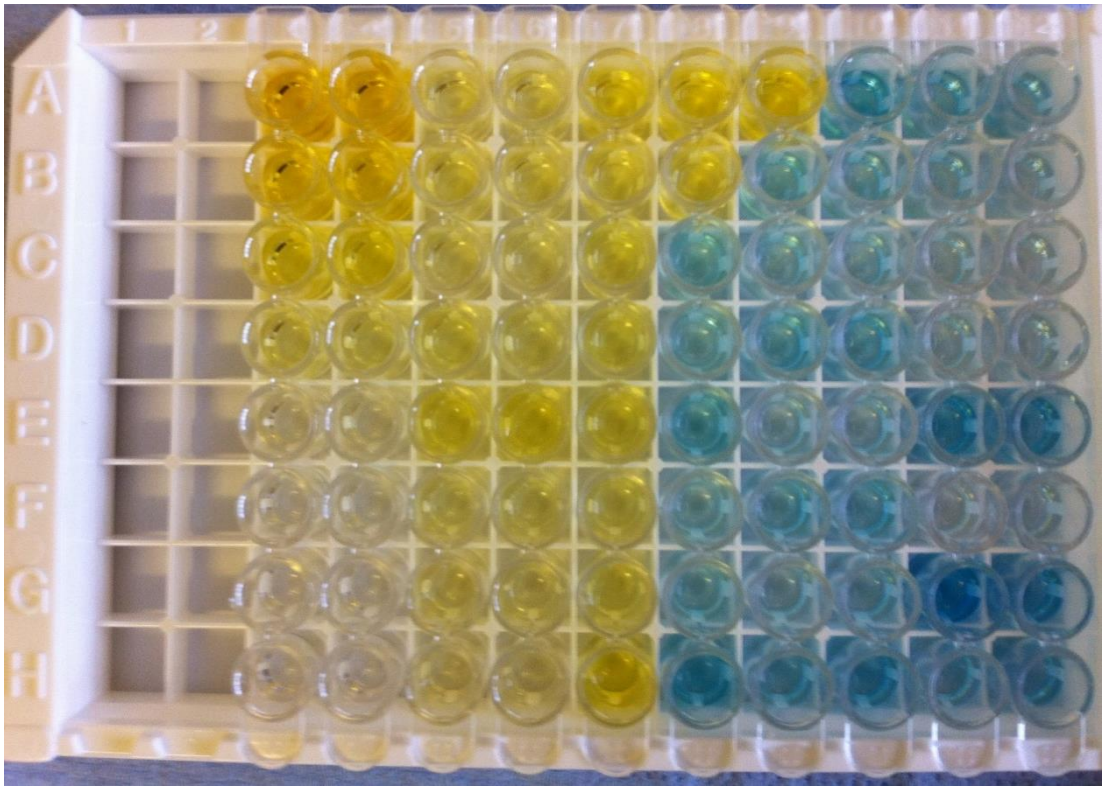

Figure 8.2:.An ELISA plate, in the process of reaction termination with the addition of sulphuric acid causing the yellow to blue colouration.

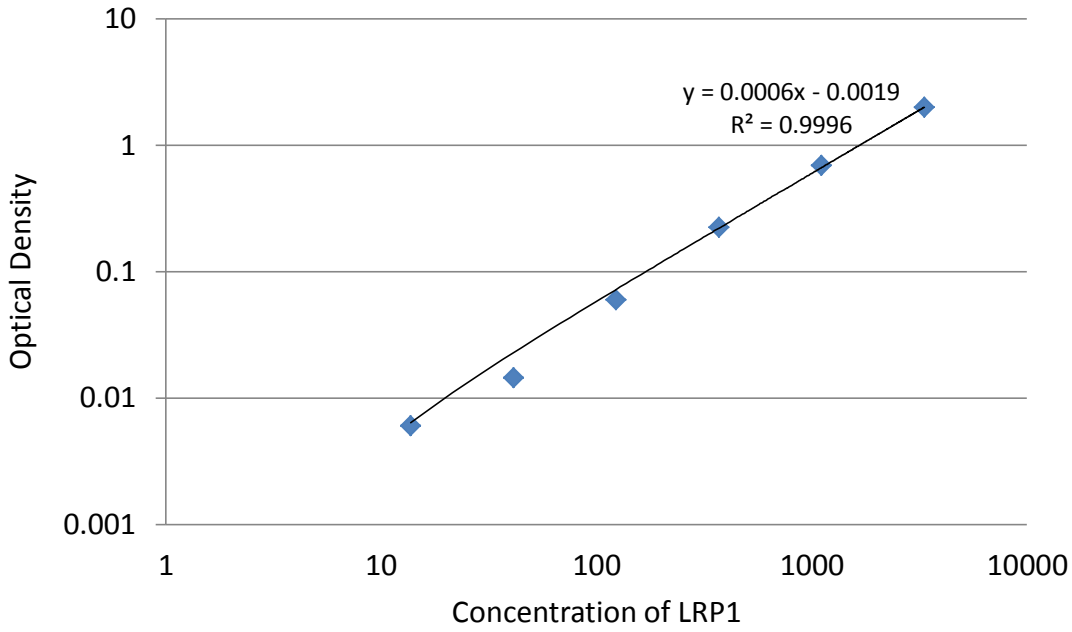

Figure 8.3: The Linear Trend Line created from the optical densities of the standard dilutions. The mean optical density from each sample can be referenced to this trend in order to determine the sLRP1 concentration.

8.3 Patient Demographics

The protein samples from 44 cases and 26 controls were collected and analysed in the ELISA. The mean age of cases was greater than that of controls, 74 years v 65 years (Table 8.1). The mean aneurysmal diameter of cases was 4.9cm, whereas the mean aortic diameter of controls was 1.7cm. A greater proportion of the control group had a previous history of smoking (38.5% compared to 27.3%). The percentage of patients suffering from diabetes mellitus type 1 was slightly greater in the case group (15.9% v 11.5%). As expected there were a greater percentage of patients in the AAA group with a positive family history for the disease.

	Cases	Controls	P value
Number	44	26	--
Mean Age	74	65	0.0001
Mean Size cm,(range)	4.9	1.7	--
Positive Smoking History (%)	12 (27.3)	10 (38.5)	0.48
Ischaemic Heart Disease (%)	21(47.7)	6(23.1)	0.05
Diabetes Mellitus (%)	7(15.9)	3(11.5)	0.82
Positive family history of AAA(%)	7(15.9)	1(3.8)	0.23

Table 8.1: Demographic data of the individuals in the ELISA experiment

8.4 Results

The aortic diameter and resulting concentration of each individual are contained within Figure 8.2. These samples were chosen for analysis as these were the most recently obtained plasma samples. USCN, the manufacturer of the ELISA kit, advised that samples be no older than 2 months in order to prevent contamination and loss of bioactivity.

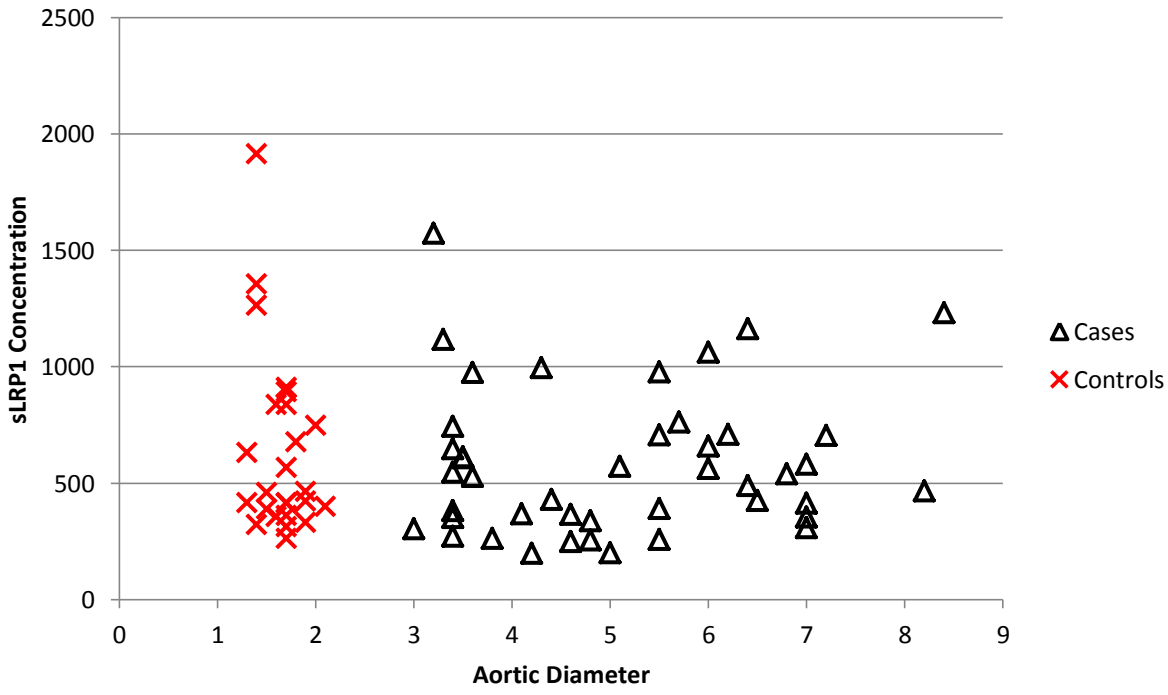

Figure 8.2. The graphical representation of the results of the ELISA experiment. The sLRP1 concentrations for each patient according to their aortic diameter.

8.4.1 Effect of Case/Control Status upon sLRP1 concentration

The difference in sLRP1 concentration of cases and controls was not significant, P value was 0.69 (Mann Whitney U test) Figure 8.4.

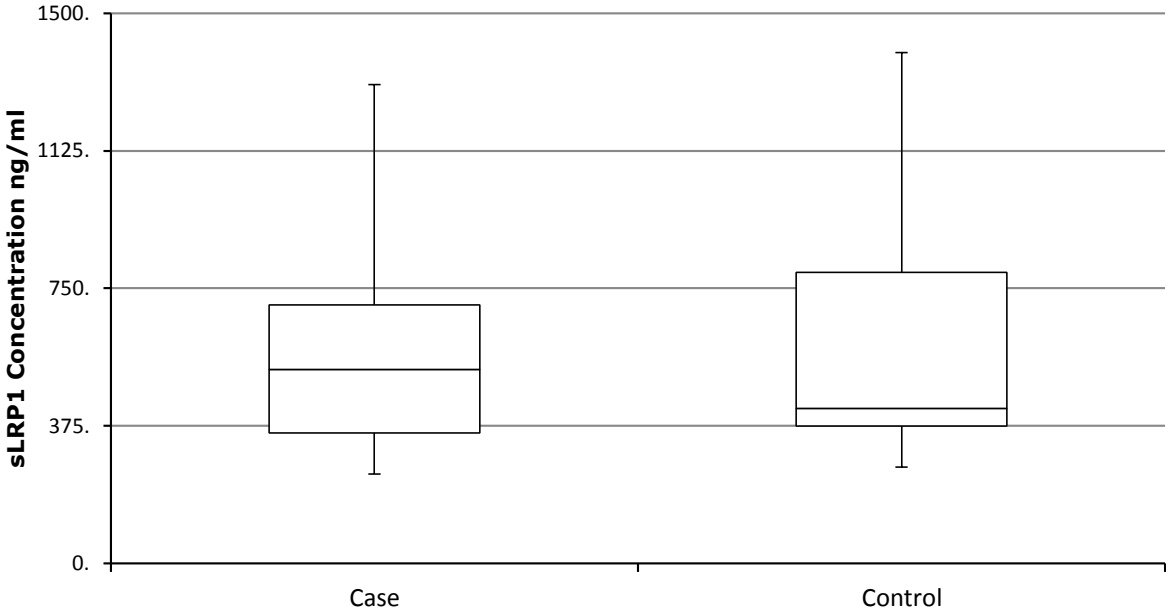

Figure 8.4:.The effect of case or control status upon sLRP1 concentration. The mean optical densities of the cases and controls are presented.

8.4.2 The Influence of genotype upon sLRP1 Concentration

There was no significant affect upon sLRP1 concentration when comparing the different rs1466535 genotypes, P=0.07 (Kruskal-Wallis) Figure 8.5.

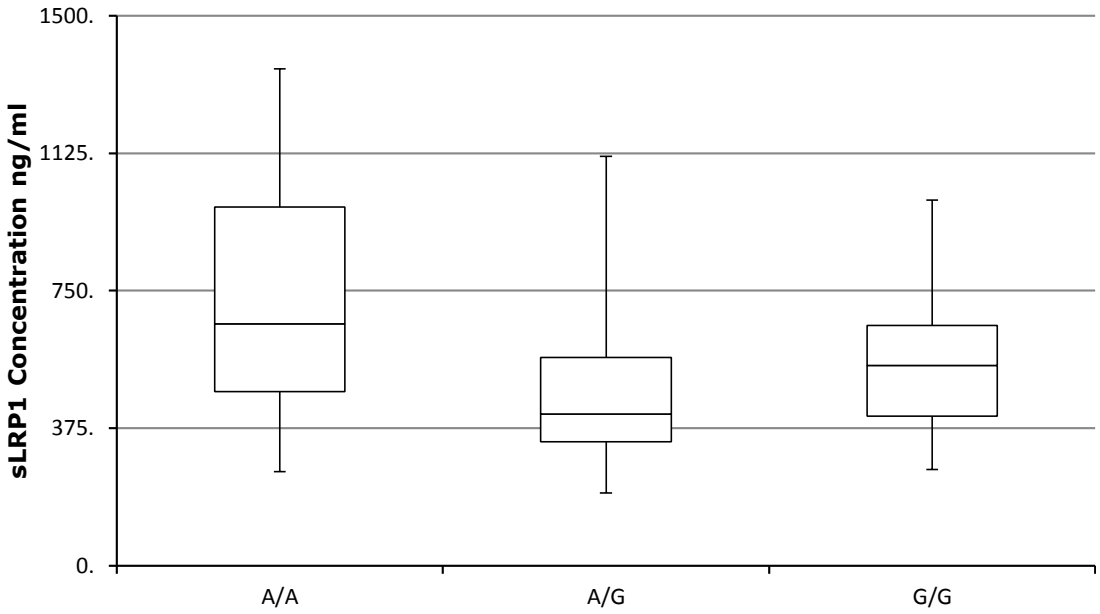

Figure 8.5.The effect of the rs1466535 genotype on sLRP1 concentration. The boxes represent the mean optical densities of samples comparing the 3 genotypes of rs1466535.

There was no significant affect upon sLRP1 concentration when comparing the different rs11172114 genotypes,P=0.02 (Kruskal-Wallis) Figure 8.6. This appears to demonstrate that there is a significance in the variation in the concentration of sLRP1 based upon rs11172114 status, however when comparing the 3 genotypes against one another the only significant variation is between the C/C and C/T genotypes.

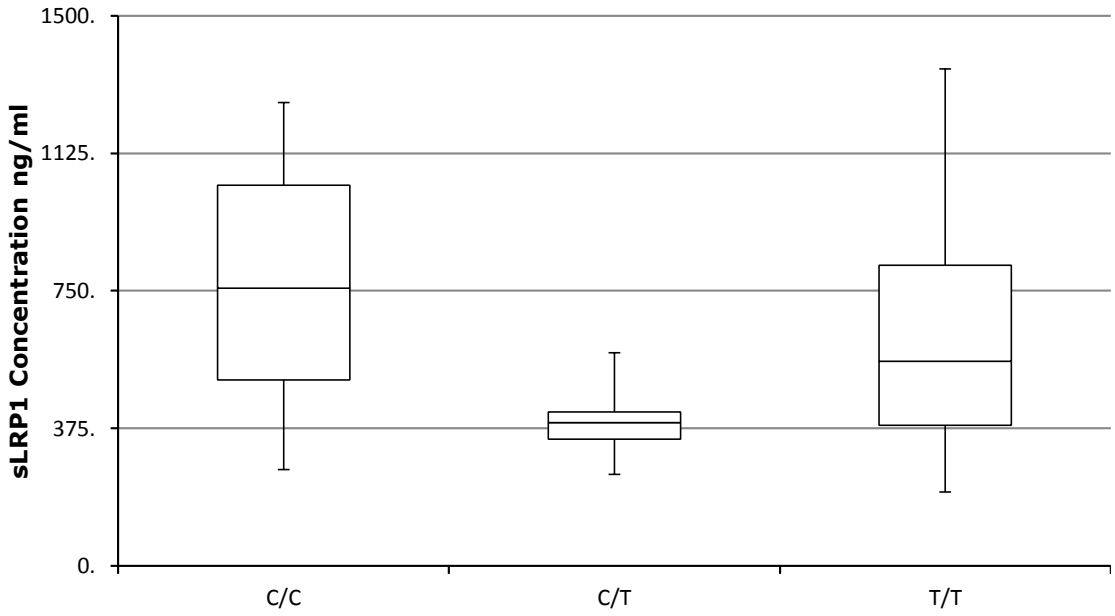

Figure 8.6: The effect of the rs11172114 genotype on sLRP1 concentration. The boxes represent the mean optical densities of samples comparing the 3 genotypes of rs11172114.

There was no significant affect upon sLRP1 concentration when comparing the different rs4257011 genotypes, P=0.05 (Kruskal-Wallis) Figure 8.7.

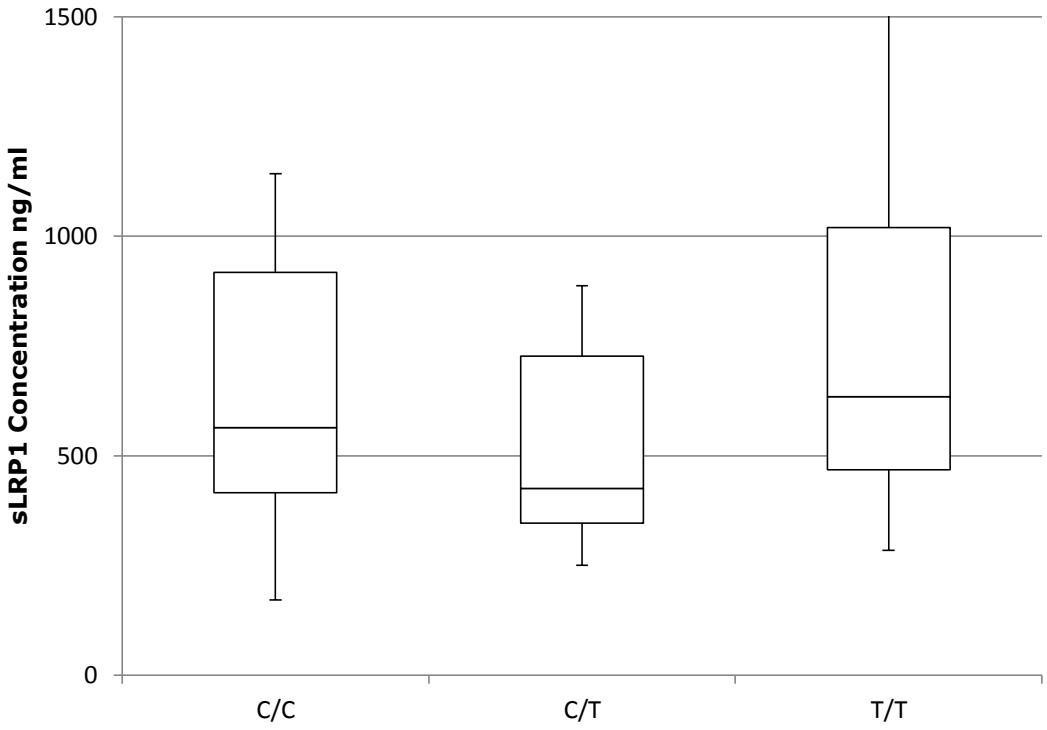

Figure 8.7: The effect of the rs4257011 genotype on sLRP1 concentration. The boxes represent the mean optical densities of samples comparing the 3 genotypes of rs4257011

8.5 Chapter 8 Conclusion

There was no significant variation seen in sLRP1 levels when cases are compared to controls. When genotypes were compared there was a significant difference in sLRP1 levels when rs11172114 C/C and C/T groups were compared. However the C/C group had very few members within it and this may lead to a falsely significant difference. It appears that the level of LRP1 is not influencing aneurysm status. There was not a significant difference seen between the rs1466535 and rs4257011. This ELISA does not indicate if case/control status or genotype has any influence on LRP1 activity or the morphology of the expressed protein as the antibody only binds to a portion of the LRP1 molecule, C terminus. This experiment does illustrate that the use of LRP1 as a biomarker for AAA progression is unsuitable.

8.5.1 Chapter 8 Null Hypothesis

The null hypothesis for this experiment was:

“the concentration of sLRP1 is unaffected by AAA status”.

The results demonstrate we cannot reject the null hypothesis.

Chapter 9; Immunohistochemical staining of Aortic biopsies

9.1 Chapter 9 Introduction

Although it has been proven that LRP1 is expressed in aortic adventitial tissue it has not been demonstrated whether the LRP1 protein is uniformly distributed across the arterial wall [Bown *et al.* 2011]. A histological feature of AAA is degenerative changes within the tunica media, it is therefore reasonable to suppose that there will be a significant proportion of LRP1 localising to this region of the arterial wall.

9.2 Immunohistochemistry of Aortic Biopsies

Aortic biopsies were obtained from patients undergoing open AAA repair who had been consented to allow for tissue retrieval and with the permission of the operating surgeon. Samples were retrieved directly from the operating theatre and immediately immersed in 10% Formalin solution. Subsequently samples were place in a 4^0c fridge for 24-48 hours.

9.2.1 Sample Preparation

Upon removal from formalin samples were immersed in liquid paraffin wax. Once set, usually after 24 hours, sections of tissue were cut from the wax tissue block at a width of 5µm on a microtome and placed on tissue slides that were pre-coated with Vectabond (Vector Laboratories, Inc., California USA). Before either Haematoxylin and Eosin (H&E) or EnVision G2 Doublestain protocols could be performed the slides required removal of any residual paraffin. Slides were incubated in a water bath at 65^0c for 10 minutes and then immersed in a series of alcoholic washes. The first stage was xylene (5 minutes, repeated once), followed by 100% IMS (industrial methylated spirit, 3 minutes, repeated once), 95% IMS (1 minute), 80% IMS (1 minute) and finally two washes in water (1 minute).

9.2.2 Haematoxylin and Eosin Staining

Each sample had one tissue slide stained with H&E in order to have a reference of normal microscopic anatomy. Initially slides were immersed in Mayer's Haematoxylin

for 5 minutes, then washed with water for 5 minutes. Slides were then counterstained with 1% eosin for 3 minutes and once again rinsed in water. In order to dehydrate the slides they were submerged in graded IMS. Finally slides were finished in xylene (two changes of 2 minutes each) and a cover slide was mounted with DPX (Sigma-Aldrich Ltd, Dorset, UK).

9.2.3 LRP1-Actin Double Stain

In order to determine the relation of LRP1 to actin in tissue samples a double stain kit was obtained from Dako (EnVision ™ Doublestain G2:Dako Denmark A/S, Glostrup, Denmark). Following the removal of paraffin slides were placed in a bath of 500ml 10mM Sodium Citrate (pH 6) and heated in a 700 watt microwave for 20 minutes in order to assist in epitope retrieval. This process was used to disrupt protein cross linkage that can occur during formalin fixation. The EnVision™ Doublestain procedure was followed as per the instructions from the manufacturer. In brief the process begins with blockade of endogenous enzymes, total incubation time used was extended to 10 minutes as primary staining demonstrate 5 minutes was insufficient to block the endogenous peroxidase in aortic tissue. The initial primary antibody was specific for αActin (Sigma-Aldrich Ltd, Dorset, UK) a major protein within muscle fibres, this was utilised at a concentration of 1:400. The anti αActin incubation was at room temperature (RT) (10 minutes), this was then cross linked to DAB via a dextran polymer conjugated with horseradish peroxidise. Addition of DAB+ Chromogen (5% 3,3'-diaminobenzidine tetrahydrochloride) (5 minutes) stained any reactive tissue brown. A second blocking solution was then applied (3 minutes) before a second primary antibody, a monoclonal reactive to LRP1 produced in mouse (Sigma-Aldrich).

This second primary was utilised at 1:100 concentration and incubated (20 minutes) before an antibody link solution (dextran polymer coupled to secondary antibodies against mouse and rabbit immunoglobulins in buffered solution) was added (20 minutes) to facilitate cross linkage with a secondary dextran polymer conjugated with alkaline phosphatase and affinity-isolated immunoglobulins was then added (20 minutes). The chromogen that reacted to the secondary antibody complex was Permanent Red (20 minutes). Finally the tissues were counter stained with Mayers Haemotoxylin solution (Sigma-Aldrich) (5 minutes). This resulted in actin within aortic biopsies being coloured brown, the LRP1 being coloured red and background tissues blue/purple. Cover slides were secured with Faramount Aqueous Mounting Medium (Dako).

9.3 Immunohistochemical Staining Results

In total 6 aortic biopsies were collected and processed, all from male subjects, mean age 67 years. The AAA were all infrarenal and biopsies were taken from the anterior aneurysmal wall near the site of maximal diameter. A significant cigarette smoking history, in terms of pack years, was observed in 5 subjects, and one patient denied any prior smoking history. None of the patients had a history of diabetes mellitus (DM). Only one patient had a positive family history of AAA, his mother. Ischaemic heart disease (IHD), including history of myocardial infarction, angina, coronary arterial angioplasty (with or without placement of coronary stent) or coronary artery bypass grafting (CABG), was seen in 3 of the patients.

ID	Age	Size (cm)	Pack years	IHD	Family History of AAA	rs1466535	rs1172114	rs4257011	LRP1 Localisation
B1	54	5.5	28	No	No	A/G	C/T	T/C	Medial Sparing
B2	77	6.3	79.5	Yes	No	G/G	--	C/C	All layers
B3	67	8.4	0	Yes	No	A/A	T/T	T/T	Intimal Sparing
B4	64	6.4	59	No	No	G/G	C/C	C/C	Medial Sparing
B5	70	6.0	82.5	No	Yes	A/G	C/T	T/C	Medial Sparing
B6	70	6.0	22	Yes	No	A/G	C/T	T/C	All layers

Table 9.1: Summary of the patient demographics whose aortic tissue biopsies were successfully obtained and underwent IHC to determine to location of LRP1. Pack Years relates to smoking history.

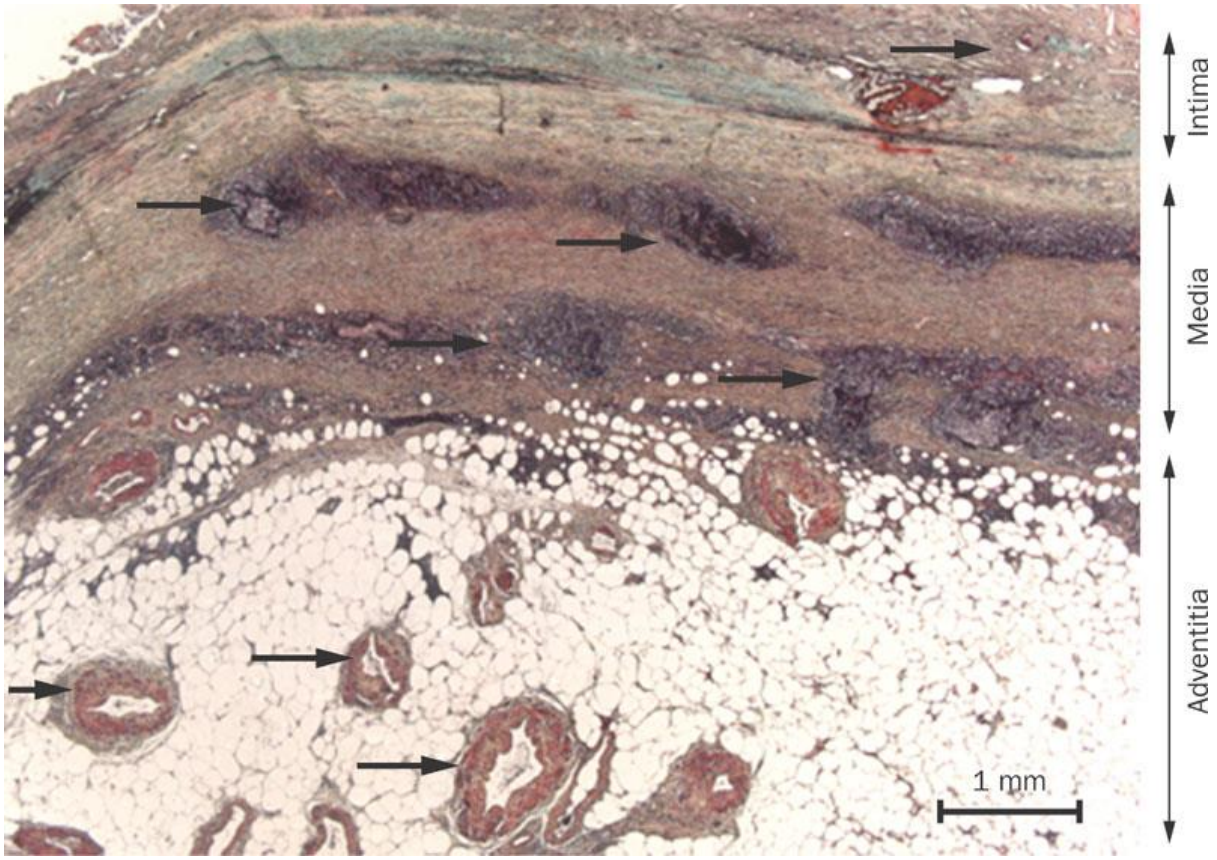

Figure 9.1: Biopsy of AAA stained with Movatt's Pentachrome staining, the arrows within the adventitia illustrate numerous arterioles whereas the arrows within the Media layer illustrate inflammatory cell infiltrates and the arrow within the intima highlights the presence of a cholesterol crystal. Reproduced from Hellenthal et al. 2009, with permission.

In order to determine the location of LRP1 within the arterial wall comparison was made to the figure taken from Hellenthal *et al.* [Hellenthal *et al.* 2009]. Analysis of the successfully processed samples demonstrated that in the majority of samples the intimal and adventitial layers of the biopsy demonstrated the most significant uptake of the anti-LRP1 stain (see results figures). The Dual stain demonstrated that there was little LRP1 present within the arterial wall, i.e. co-localised red and brown chromagen. Analysis of the slides from biopsy B3 were difficult as there did not appear to be clear intimal or adventitial layers, and the dual stained slide demonstrated greater uptake of red chromagen than the LRP1 only control slide. However in the other 5 samples it

was clear that the intimal layers demonstrated the greatest affinity for the LRP1-red chromagen. We can be confident that this is not just non-specific staining of the biopsy edge as in samples that were processed in the absence of LRP1 antibody (the Actin Only samples) there was no red chromagen binding evident in the intimal layer.

Aortic biopsy B1, uptake of red colouration, therefore LRP1, is seen in the adventitial and intimal layers with sparing of the medial layer (Figure 9.2).

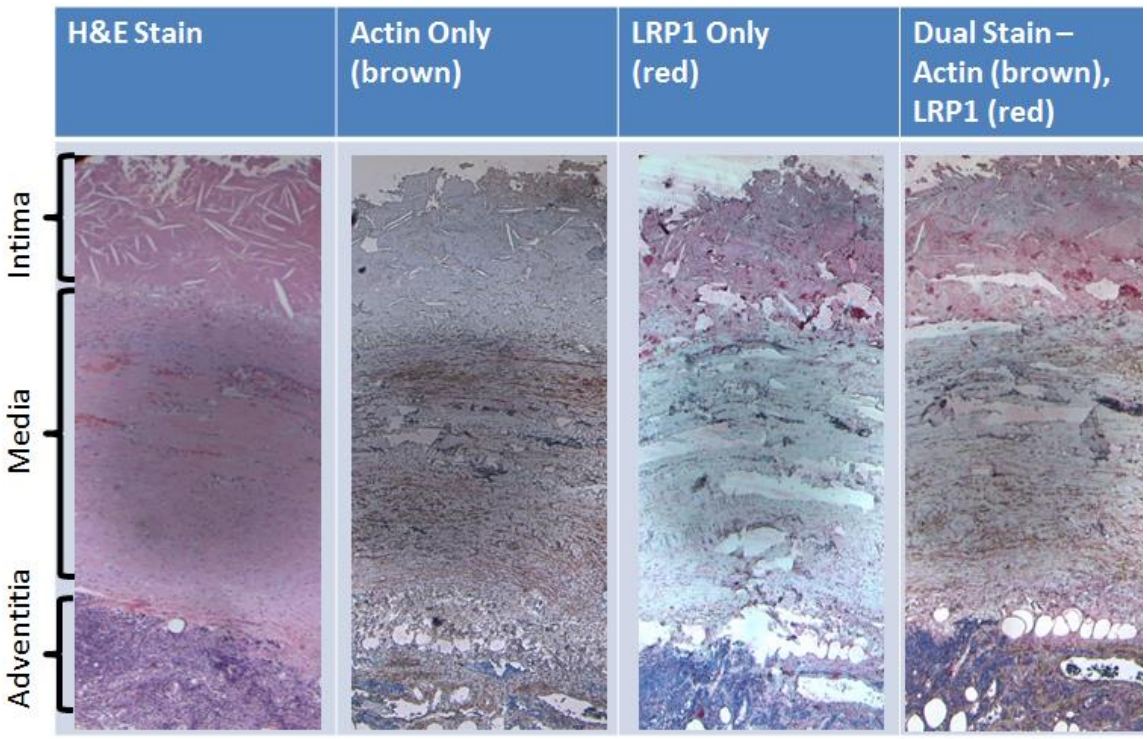

Figure 9.2: Images of the immuno histochemical staining of aortic biopsy B1, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

Aortic biopsy B2, uptake of red colouration, therefore LRP1, is seen within all layers of the aortic wall (Figure 9.3).

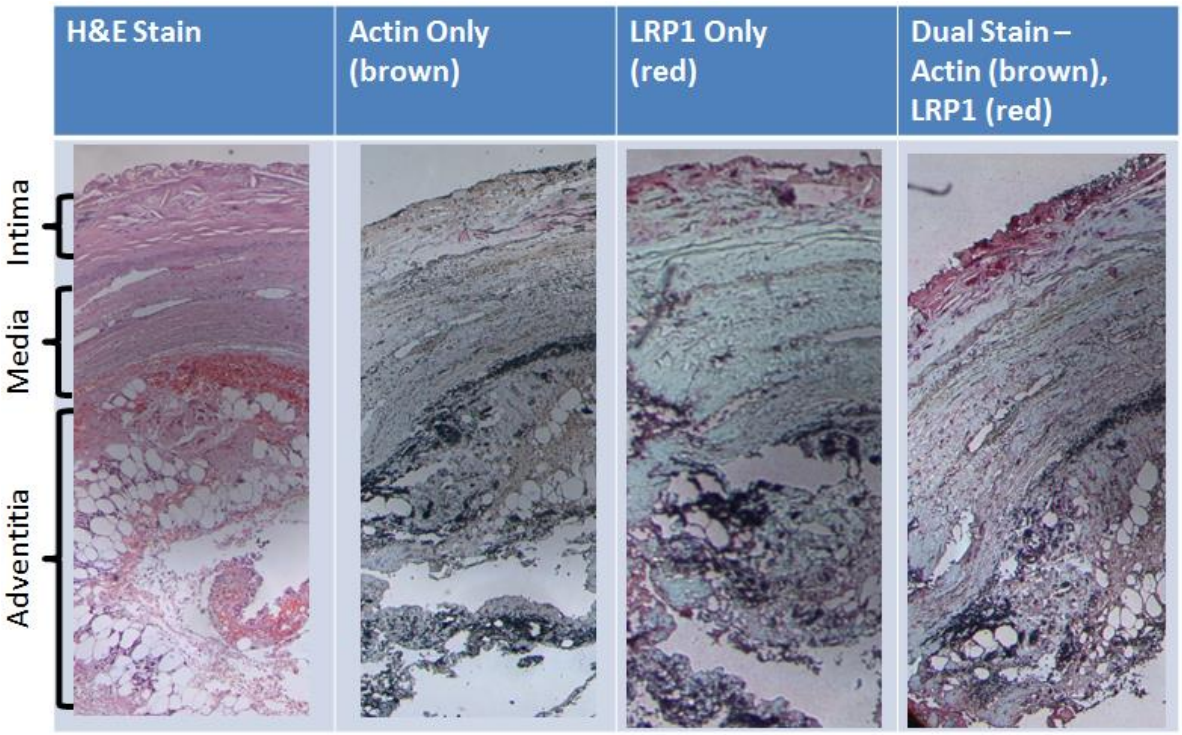

Figure 9.3: Images of the immuno histochemical staining of aortic biopsy B2, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

Aortic biopsy B3, uptake of red colouration, therefore LRP1, is seen within the medial and adventitial layers of the aortic wall, with Intimal sparing (Figure 9.4).

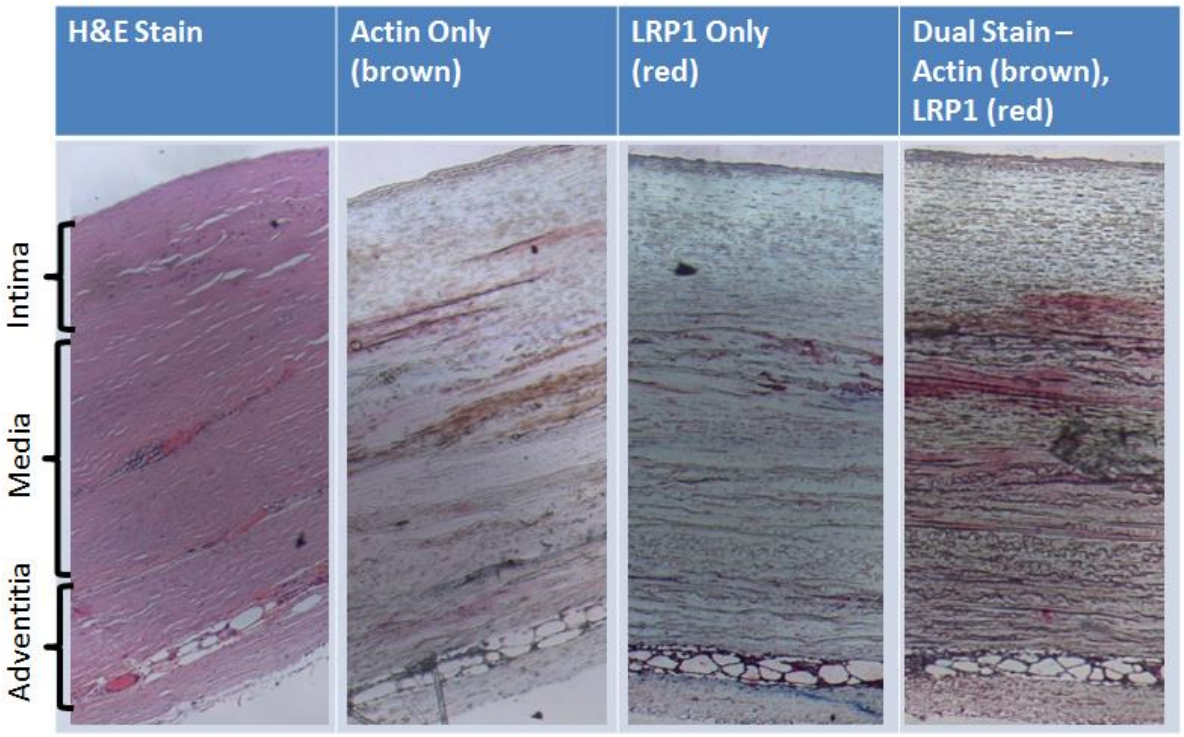

Figure 9.4: Images of the immuno histochemical staining of aortic biopsy B3, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

Aortic biopsy B4, uptake of red colouration, therefore LRP1, is seen in the adventitial and intimal layers with sparing of the medial layer (Figure 9.5).

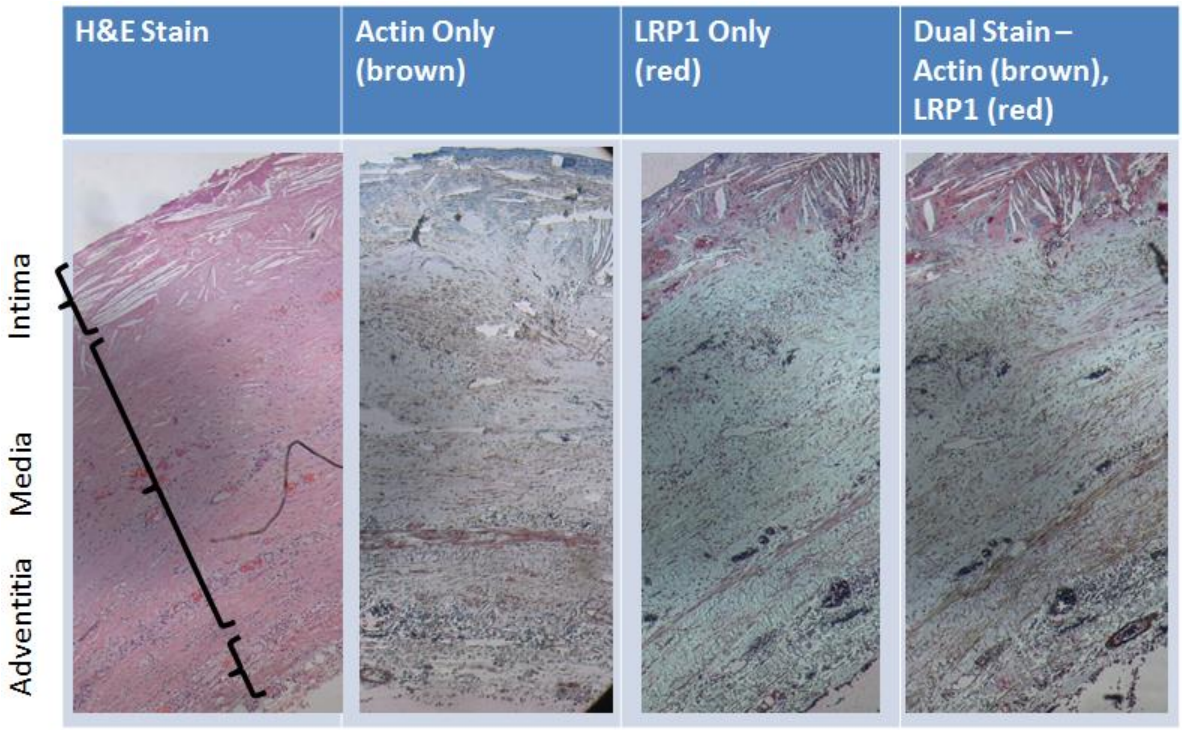

Figure 9.5: Images of the immuno histochemical staining of aortic biopsy B4, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

Aortic biopsy B5, uptake of red colouration, therefore LRP1, is seen in the adventitial and intimal layers with sparing of the medial layer (Figure 9.6).

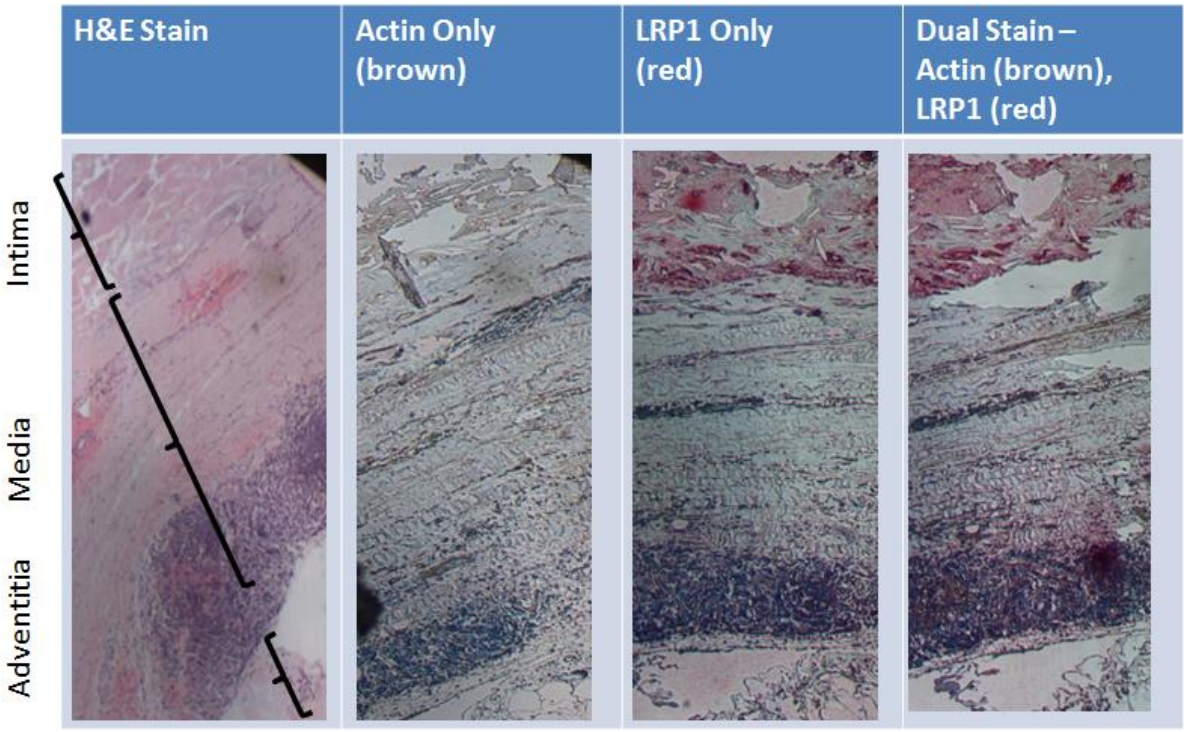

Figure 9.6: Images of the immuno histochemical staining of aortic biopsy B5, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

Aortic biopsy B6, uptake of red colouration, therefore LRP1, is seen in all the layers of the aortic wall (Figure 9.7).

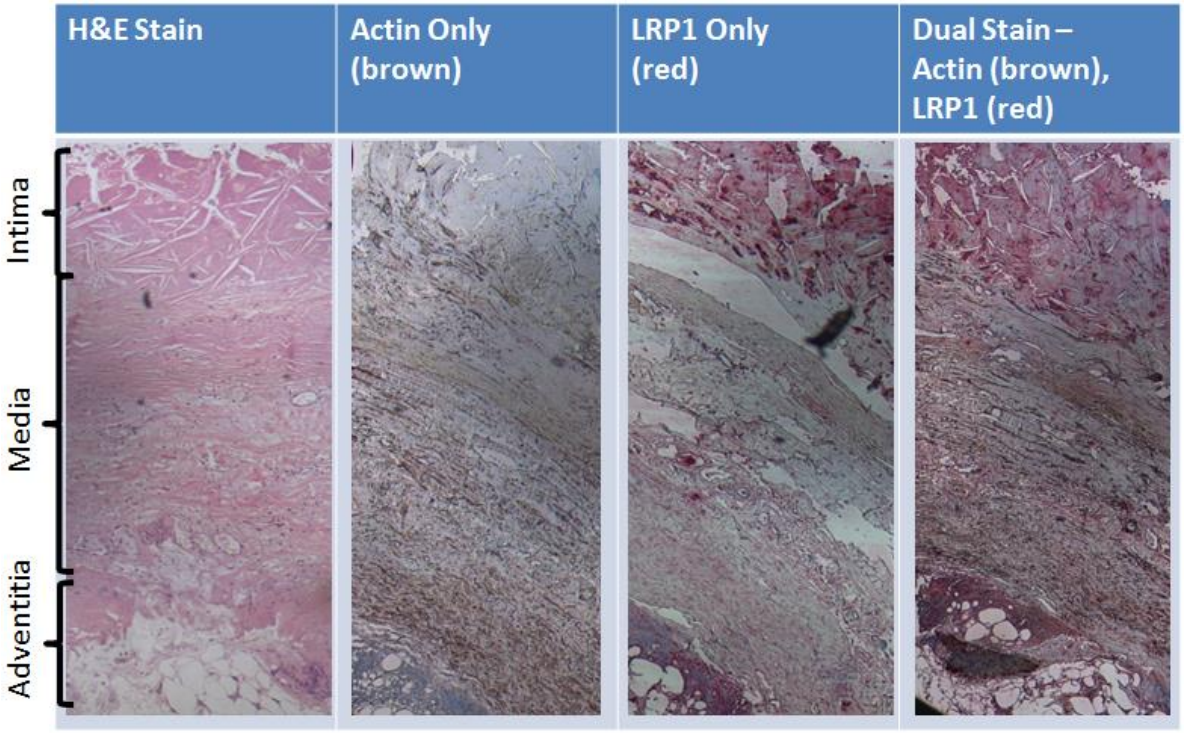

Figure 9.7: Images of the immuno histochemical staining of aortic biopsy B6, from left to right Haemotoxilin and Eosin stain, Dako Double Stain Actin only, Dako Double Stain LRP1 only, Dako Double Stain Dual stain with LRP1 and Actin, Magnification 200X.

9.4 Chapter 9 Conclusion

The location of LRP1 within the aortic wall has not previously been well characterised histologically. In the GWAS conducted by Bown *et al.* there was an analysis of LRP1 expression in the ascending aorta, however this was based upon mRNA extraction and not histology [Bown *et al*. 2011]. Subsequent to this experiment a group from Hong Kong has published an histological analysis of 10 aortic biopsies taken from patients with AAA and 12 healthy controls (from organ transplantation) [Chan *et al*. 2013]. This experiment also utilised reagents obtained from Dako, tough the LRP1 specific antibody utilised was obtained from Abcam (Cambridge, UK), whereas this experiment utilised an LRP1 antibody obtained from Sigma-Aldrich. Chan *et al.* reported that LRP1 was evident in the adventitial and medial layers of their samples, with relatively less LRP1 detected in AAA biopsies when compared to controls. This is partially in agreement with my study, LRP1 does localise to the adventitia, however I did not find uniform LRP1 positivity within the medial layers, and I did find intimal uptake in 5/6 samples. The analysis I have conducted supports LRP1 being found in the intimal layer as well as the adventitial layer. The potential reasons for this disparity are numerous. The 2 populations that the AAA patients arise from are not uniform, one is Asian the other European, and there may be different tissue specific LRP1 expression patterns in the two populations. Different antibodies were used to detect the LRP1, the Sigma-Aldrich antibody is raised against an immunogen that corresponds to amino acids 188-201 of the LRP1 protein the immunogen in the Abcam antibody corresponds to amino acids 1-172 of LRP1. Therefore the 2 antibodies are reactive to different regions of the LRP1 protein, conformational changes in the protein or ligand binding may affect

reactivity of the two antibodies. Both my experiment and Chan *et al.'s* report had small numbers of patients recruited and this may introduce sampling error. This analysis is also hampered by the lack of control tissue. Every effort was made to obtain informed consent for tissue collection however with a move to minimally invasive techniques there are less open AAA repairs conducted and biopsy retrieval is not feasible from EVAR. Amendments to the ethical approval for the study were made in order to obtain tissue from both hospital post-mortems and from unused aortic tissue from donor kidneys used in cadaveric transplant, however no additional samples were collected during the research period. The advantage is that over time a bank of aortic specimens will accumulate and the technique demonstrated here can be utilised once the sample size is large enough.

9.4.1 Null Hypothesis

The null hypothesis for this experiment was:

"LRP1 is widespread in aortic tissue".

The result of the immunohistochemical analysis of 6 aneurysmal aortic tissue biopsies is that the null hypothesis is accepted. It was expected that LRP1 would localise to a single arterial wall layer, this was not observed in these samples.

Chapter 10: Discussion

10.1 Introduction to Discussion

Abdominal aortic aneurysms are a life threatening condition that predominantly affects males in the later years of life. AAA are a disease with a genetic component. Several genes have significant association with AAA based upon meta-analysis of multiple association studies. Utilizing a genome wide association study approach the LRP1 gene significantly associated with AAA, the SNP rs1466535 reaching a genome wide significance [Bown *et al.* 2011]. This thesis described the efforts to determine if another SNP within LRP1 had a more significant association with AAA. It also described the analysis of the first 3 exons of the LRP1 gene, to determine if a novel mutation could be found within the coding regions of the gene to explain how LRP1 is affecting the aortic wall. Subsequently the levels of soluble LRP1 in plasma were assayed in order to determine if case/control status or genotype influenced LRP1 concentration. Finally aortic biopsies were stained in order to determine which layer of the aortic wall LRP1 localises within.

10.2 Summary of Findings

There were 4 experiments undertaken in order to investigate genetic polymorphisms and AAA, looking at not only individual polymorphisms but functional implications of different genotypes.

10.2.1 SNP genotyping

Specific TaqMan probes were used to perform SNP genotyping on AAA cases and controls. The rs217120 polymorphism failed to significantly associate with AAA when data from 4 geographical cohorts was meta-analysed. As this SNP failed to associate it was not selected for further analysis. Had only one (small) replication cohort been used then it is likely that further analysis of the SNP would have taken place, despite the association not being significant. This highlights the importance of appropriately sized replication cohorts as advocated by Ioannidis *et al.* [Ioannidis *et al.* 2001].

The analysis of SNPs associated with rs1466535 revealed several polymorphisms that positively associated with AAA however rs11172114 and rs4257011 had a more significant association than rs1466535.

10.2.2 Re-sequencing

PCR amplification of exons 1, 2 and 3 of LRP1 was followed by sequencing of the PCR products. Having successfully sequenced the 3 exons closest to the highly significant

polymorphisms there was no novel mutation discovered in the analysis of the resulting sequence.

10.2.3 Detection of Soluble LRP1 in plasma

Utilising a sandwich ELISA the concentration of sLRP1 was assayed. Neither case/control status nor SNP genotype (rs1466535, rs4257011 and rs11172114) led to significant variation in sLRP1 concentration.

10.2.4 Immunohistochemical staining of Aortic biopsies

Six aortic biopsies were obtained and successfully immunohistochemically stained for LRP1 protein. The distribution of LRP1 within tissue was not uniform between samples but appeared to spare the intimal layers in some cases.

10.3 General Limitations

There are several limitations of this thesis. In terms of the cohorts utilised for SNP genotyping and ELISA, the demographics were poorly matched between the case and control cohorts, such as age, smoking and medical history. This was addressed where possible however ideally there would be no significant variation in the demographics of the case and control groups. There were also relatively few samples included in the ELISA and immunohistochemistry experiments. The small numbers used in the ELISA experiment was primarily due to the avoidance of using plasma samples that had been in storage for greater than 3 months. This was a recommendation of the manufacturer in order to avoid the loss of bioactivity and contamination. The small number of samples used in the tissue staining experiment was due to the small number of patients undergoing open aneurysm repair currently, reducing the number of potential biopsy donors. As the technique of endovascular repair expends the harvesting of aortic biopsies is certain to be a recurring issue.

10.4 Future directions

This thesis has not determined if the LRP1 found in AAA cases has the same function as the LRP1 found in healthy controls. There are a variety of potential ligands for LRP1 (as discussed in Chapter 3). The ability of LRP1 in cases and controls could be determined using any of a variety of techniques [de Jong *et al.* 2005]. A known ligand for LRP1, such a RAP (Receptor associated Protein) would be labelled with a fluorescent or radioactive marker, could be used to determine LRP1 binding affinity.

10.5 Practical uses for SNPs associated with AAA

There are implications for the use of SNP genotypes in the rationalisation of screening for AAA. In 2010 Pashayan *et al.* published a model for personalised screening of both breast and prostate cancers [Pashayan *et al.* 2010]. Utilising multiple SNPs identified from GWAS (31 prostate cancer and 18 breast cancer susceptibility loci) the model reduced the number of men eligible for screening, improving the efficiency. It is plausible that the rs11172114 SNP along with other significantly associated polymorphisms (such as SORT1 SNP, rs599839 and LDLR SNP, rs6511720) could be used to stratify risk in screening patients and determine the interval between an initial screening scan at age 65 and recall for follow up scans.

10.6 Conclusion

AAAs continue to be a significant cause of mortality and the only treatment remains to be surgical repair. Significant risk factors for AAA include a history of cigarette smoking, ischaemic heart disease and positive family history. As heritability is a risk factor it follows that there is a genetic component to AAA. There have been multiple studies that have attempted to identify genetic polymorphisms that significantly associate with AAA. Several genes have been implicated by these case control studies. However a large number of the genes implicated have not been replicated in other studies therefore genome wide association studies have recently been utilised in order to avoid the errors that were encountered in case control studies. This thesis has determined that the LRP1 SNPs, rs11172114 and rs4257011, significantly associate with AAA. Despite this association I have not found there to be any sequence variant in the exons surrounding rs11172114 and rs4257011. Additionally serum levels of LRP1 were not found to be significantly different between cases and controls and between different SNP genotypes. Finally staining of aortic tissue biopsies failed to elucidate which layer of the aneurysm LRP1 localises within. LRP1 continues to play an important role in AAA pathogenesis however currently the role it plays has not been defined.

10.7 Corrections

Following my *viva voce* September 2015 at Leicester University the following corrections were suggested by my examiners Prof. Alun Davies and Prof. Shu Ye, my responses are italicised:

1. Check and follow guidelines regarding the referencing system used in theses of the University of Leicester. *This has been addressed and is consistent throughout.*

2. Some information about and a diagram of the LRP1 gene. *This has been added to chapter 7.*

3. Amend and clarify hypotheses 3 and 4, as discussed during the viva. *This has been amended.*

4. Measurements of DNA concentration and purity (260/280 ratio), as discussed during the viva. *This has been amended.*

5. A bit more of the appreciation on the possible difference between the 4 groups. *Ideally I would have liked to analyse to data from the sLRP1 ELISA based upon groups of control, small AAA, medium AAA and large AAA, as there may be genetic differences as the pathological processes of AAA progress, however as I had so few samples I felt this would have reduced the impact of my findings.*

6. An acknowledgement that some of the graphs would benefit from appropriate axis marking with respect to units. *This has been amended.*

7. Comment on the lack of statistical input from a statistician, especially with respect to the meta-analyses. *I learned the process of meta-analysis from my supervisor, however I did not have instruction from a statistician, and this would have likely improved the presentation of my data analysis*

8. Comment why he used Box and whisker plots rather than scatter plots in 8.4,8.5,8.6,8.7. *This form of presentation was utilised as I felt the output of the scatter plots was not of sufficient image quality.*

9. The limitations of chapter 9, especially as no negative or positive controls used and no comment on reproducibility of these results. *The analysis of the aortic tissue biopsies is limited by the absence of normal aortic biopsies and the absence of control tissues that are known to express LRP1. My results were in partial agreement with Chan et al. illustrating the potentially for reproducibility.*

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