AN INVESTIGATION INTO
CANDIDATE GENES FOR
ABDOMINAL AORTIC ANEURYSMS

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Abstract

Objectives
There is growing evidence to suggest that abdominal aortic aneurysm (AAA) is a complex disease with multiple environmental and genetic risk factors. Susceptibility genes have been investigated through multiple underpowered candidate gene studies, which has led to the association of numerous genes with conflicting evidence. This study aimed to investigate the commonly cited associations between polymorphisms of the matrix metalloproteinase-9; MMP9(-1562C>T), tissue inhibitor of metalloproteinase-1; TIMP1(+434C>T) and TIMP1(rs2070584), platelet activating factor acetylhydrolase; PLA2G7(-994G>T), estrogen receptor beta; ESR2(+1730 A-G) and Heme oxygenase 1; HMOX1(GT)n genes and AAA in a large powered study to provide definitive evidence of any association.

Materials & Methods
A case-control study was performed of 1,202 patients with AAA and 1,059 screened control subjects. DNA was extracted from whole blood and genotyping was performed using polymerase chain reaction based restriction fragment length polymorphisms (PCR-RFLP).

Results
Two polymorphisms (ESR2 (OR 1.42, P<0.0001) and HMOX1 (OR 1.99, P<0.0001)) showed a potential association with AAA. One polymorphism (TIMP1 rs2070584) could not be genotyped despite using 2 different methods. Polymorphisms of the MMP9 (OR 0.99, P=0.82), PLA2G7 (OR 0.76, P=0.29) and TIMP1 (+434C>T) (OR 0.94, P=0.46) genes did not show an association with AAA.

Conclusion
We have demonstrated an association between polymorphisms of the ESR2 and HMOX1 genes and AAA, although further work is essential to confirm this association. Contrary to other published data, no such association was seen in common polymorphisms of the MMP9, TIMP1, and PLA2G7 genes and AAA.
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Publications & presentations arising from this thesis

Publications


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Presentations


Abbreviations

AAA  Abdominal Aortic Aneurysm
AAAQIP Abdominal Aortic Aneurysm Quality Improvement Programme
ACE  Angiotensin Converting Enzyme
ACEI Angiotensin Converting Enzyme Inhibitors
CI   Confidence Interval
CFA  Common Femoral Artery
CO   Carbon monoxide
COPD Chronic obstructive pulmonary disease
CRP C-Reactive Protein
CT   Computed Tomography
CVA  Cerebrovascular Accident
DM   Diabetes Mellitus
DNA  Deoxyribonucleic acid
dNTP Deoxynucleoside triphosphates
DUSS Duplex Ultrasound Scan
ECM  Extracellular matrix
EDTA ethylenediaminetetraacetic acid
EVAR Endovascular Aneurysm Repair
FEV1 Forced Expiratory Volume in 1 second
GP   General Practitioner
HDL  High density lipoprotein
HO1  Heme oxygenase 1
HWE  Hardy-Weinberg Equilibrium
IHD  Ischaemic Heart Disease
ITU/ICU Intensive Therapy/Care Unit
IVC  Inferior Vena Cava
LDL  Low density lipoprotein
MI   Myocardial Infarction
MMP  Matrix Metalloproteinase
MRC  Medical Research Council
OR   Odds Ratio
PAF  Platelet activating factor
PAFAH Platelet activating factor acetyl hydrolase
PAP  Plasmin Anti-plasmin
PCR  Polymerase chain reaction
PVD  Peripheral Vascular Disease
QALY Quality Adjusted Life Year
RAAA Ruptured Abdominal Aortic Aneurysm
RCT  Randomised Control Trial
RE   Restriction Enzyme
RFLP Restriction fragment length polymorphism
SEP  Serum Elastin Peptides
SNP  Single nucleotide polymorphism
TIMP Tissue Inhibitor of metalloproteinase
UKSAT United Kingdom Small Aneurysm Trial
INTRODUCTION

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Anatomy of the Abdominal Aorta

The aorta is the largest artery in the human body and traverses the thorax and abdomen. It originates from the left ventricle of the heart and terminates at its bifurcation in the abdomen into the common iliac arteries. It is subdivided into several parts. The abdominal aorta begins behind the diaphragm at the lower border of the 12th thoracic vertebra. The oesophagus, cisterna chyli and the inferior vena cava are some of the important visceral structures surrounding the proximal part of the abdominal aorta. The abdominal aorta then descends in the retroperitoneum giving off many vital branches, which supply the abdominal structures. The coeliac trunk, supplying the liver, spleen and parts of the stomach arises at the level of the 12th thoracic vertebra. The superior and inferior mesenteric arteries are the other two unpaired branches to arise from the aorta at 1st and 3rd lumbar vertebra respectively, and supply a large majority of the bowel. The paired renal arteries arise between the superior and inferior mesenteric arteries at the 2nd lumbar vertebra. Other branches arising from the aorta include the paired suprarenal, gonadal and inferior phrenic arteries as well as four lumbar arteries and median sacral artery (Figure 1).
The infrarenal abdominal aorta lies to the left of the midline anterior to the 1\textsuperscript{st} to the 4\textsuperscript{th} lumbar vertebrae and is covered superiorly by the 3\textsuperscript{rd} and 4\textsuperscript{th} parts of the duodenum. Distally, the aorta bifurcates at the level of the 4\textsuperscript{th} lumbar vertebra into right and left common iliac arteries.
Definition of abdominal aortic aneurysm (AAA)

An arterial aneurysm is defined as ‘a permanent localised (focal) dilation of an artery having at least a 50% increase in diameter compared to the expected normal diameter of the artery in question’ (Johnston KW 1991) (Figure 2). In the above-mentioned article, Johnston et al produced expected values for arterial diameters at different levels along the aorta, in both sexes using ultrasound and CT measurements. However, the range of normal values quoted was large and therefore led to inaccuracies in reporting true aneurysms. Lederle et al (Lederle FA Oct 1997) have also shown that age, gender, race, and body size have a small but statistically significant effect on infrarenal aortic diameter. The authors recommended a simpler radiological definition of aortic diameter of \( \geq 3 \text{cm} \) to be used to define AAAs.

Figure 2: Abdominal aortic aneurysm (Reproduced from http://www.circulationfoundation.org.uk)
Incidence

The actual incidence of AAA has been shown to vary with age, peaking at 65 years with an incidence of 0.67% among the UK male population over one year. In the same study, incidence was found to be lowest at age 50 (0.08%) and fell after the age of 65 to reach an incidence of 0.285% at age 85. (Figure 3) (Vardulaki KA 1999).

Figure 3: Estimated incidence of abdominal aortic aneurysms (Reproduced from Vardaluki KA 1999)

Much of the incidence data is based on mortality data or data derived from hospital admissions. This largely represents the incidence of the acute presentation of AAA and due to the asymptomatic nature of the disease, the actual incidence may be much higher.
An early epidemiological study published in 1984 raised the suspicion of an upward trend and suggested a three-fold increase in AAA between 1951 and 1980 (Figure 4) (Melton LJ 3rd 1984).

**Figure 4**: Age- and sex-adjusted incidence of abdominal, thoracic, and total aortic aneurysms among Rochester, Minnesota, residents by five-year time periods, 1951-1980. (Reproduced from Melton LJ 3rd 1984)

A study published 4 years later also confirmed an increase in the incidence of ruptured AAA from 9.2 to 17.5/100,000 patient years during an 8-year period (Mealy K 1988). In 1989, Fowkes et al reported a rise in age standardised mortality by 20 fold in men to 47.1 per 100,000 population and 11 fold in women to 22.2 per 100,000 population, which was attributable to deaths from AAA (Fowkes FG 1989). They also proved a rise in hospital admissions of men with AAA from 1968 to 1983. These large increases have been attributed to an ever-aging population; increases in the number of smokers;
the onset of screening programmes and improved diagnostic tools. (Sakalihasan N 2005).

Whilst the above results are useful, they do not comment on the trend over the past decade and can therefore be argued as historical data. Best et al looked at the changing incidence of AAA in Scotland between 1981 and 2000 and confirmed the continuing increase in the incidence of AAA, with age-adjusted mortality rates for AAA increasing 2.6-fold and rise in hospital admissions by 3-fold during this time period. (Figure 5 & 6) (Best VA 2003).

**Figure 5:** Age-adjusted rates of elective and emergency hospital admission for abdominal aortic aneurysm in Scotland between 1981 and 2000 (Reproduced from Best VA 2003)
Figure 6: Age-adjusted mortality rates for abdominal aortic aneurysm in men and women in Scotland between 1981 and 2000 (Reproduced from Best VA 2003)

However, more recent data has not confirmed this trend and has indicated that it may be reversing. Two recent articles from Australasia have reported a declining rate of hospitalisation and mortality from AAA between 1991 and 2007 (Norman PE 2011); (Sandiford P 2011). A similar study from Choke et al examined mortality data along with hospital admission and procedure data in England and Wales between 2001 and 2009. This study reported a declining rate of AAA mortality (35.7%), admission for ruptured AAA (29.3%) and emergency AAA repair (35.5%). Admission for elective AAA remained stable but was seen in an older population, with an increase (17.2%) in the numbers of elective repairs (Choke E 2012).
**Prevalence**

Much of the data regarding prevalence of AAA has been gathered using population screening and autopsy studies. The prevalence of screened AAA in men in England varies between 1.3% and 12.7%, but is largely dependent on the age of the screened group and the given definition of AAA (Wilmink AB 1998). Similar prevalence rates (0.6% to 10.7%) are found when analysing studies outside the UK. Autopsy studies from the UK, Sweden and USA show a prevalence of between 2.3% and 4.3% in men of age 50 and above with this figure falling to between 1.2% and 2.1% in women of the same age. (Turk K 1965); (McFarlane MJ 1991); (Bengtsson H Jan 1992); (Bengtsson H Nov 1992). Interestingly, in a Japanese screening study of 348 people, no AAA was detected and a mean infrarenal diameter of 18.7mm was found (Takei H 1995).

**Risk Factors**

Potential causes for the development of AAA can be considered as non-modifiable or modifiable risk factors.
**Non-Modifiable risk factors**

**Gender**

Gender was first speculated as a risk factor after a case report demonstrating an increased prevalence amongst brothers was published (Clifton MA 1977). Since then, epidemiological studies have shown the prevalence of AAA to be six times greater in men than women (Plumeekers HJ 1995); (Scott RA 2002). A recent meta-analysis across 6 studies showed that male sex had the strongest association with AAA with an odds ratio of 5.7 (Cornuz J 2004).

**Age**

Age is a strong risk factor for AAA, with older patients more likely to have an AAA. AAA that are seen in younger patients are usually due to the presence of a connective tissue disorder. The prevalence of AAA with increasing age was examined by Vardulaki et al who found this to be 2.7% in those aged between 65 and 69; 3.9% aged between 70 and 74 and 4.4% in those aged between 75 and 79 years old (Vardulaki KA 2000). One of the largest population based studies on AAA (19,478 subjects) was published in 2003 by Rodin MB et al (Rodin MB 2003). The results showed that men of ages 60-64 years were nearly five times more likely to have an aneurysm than men of 40-44 years old. This trend was the same for women, whereby those aged between 60-64 years old were more than seven times more likely to have an aneurysm than those aged 40-44 years old.
Family History

Along with age, family history of AAA also appears to be a strong risk factor and will be mainly addressed in ‘Chapter 4. Genetic basis of AAA’. Family history of an AAA in a first-degree relative has been shown to increase the risk of an AAA by 4.33 fold (95% Confidence Interval (CI) 1.32 to 14.23) (Salo JA 1999). In this study, male sex and increasing age were also shown to increase risk, and led the authors to conclude that the highest risk for developing AAA is seen in aging brothers of patients with known AAA. More recently, a Swedish study has been conducted to investigate the risk of developing an AAA for first-degree relatives of patients with AAA. The overall relative risk of family history compared to no family history was 1.9 (95% CI 1.6-2.2) (Larsson E 2009).

Race

It is a well-established fact that AAA is predominately a disease of white Europeans. A study comparing the incidence of AAA in ‘whites’ and ‘blacks’ in North Carolina in 1985 concluded that there is an increased incidence of AAA in the white male when compared to white females and in both sexes from the black community (Johnson G Jr. 1985). This trend has also been documented in other studies (Lederle FA Oct 1997) with one particular study suggesting an odds ratio of 0.29 between black and white males (LaMorte WW 1995).

Whilst there has been an increase in the incidence of AAA seen in China, when considering the population of the country, the incidence is much less
than that seen in Europe and North America (Cheng SW 1998). In Japan, there were no AAA detected through a screening study of 348 Japanese residents of a rural community (Takei H 1995) and a larger study that recruited 1,591 men of ages greater then 60 years detected only 0.3% of patients with an AAA (Adachi K 2000).

Several studies have also been conducted on the UK Indo-Asian population. A study conducted in Bradford, UK, where there is a high cluster of the Indo-Asian community suggested that hypothetically, there should be 28 new AAA seen per year in this population. Over the 7 years of the study, 233 patients with AAA were identified from the local population, none of which were Indo-Asian (Spark JI 2001). Hobbs et al found that despite having high risk factors for vascular disease, the prevalence of AAA and peripheral vascular disease in Asian patients is much lower than would be expected in an age and sex matched Caucasian population (Hobbs SD 2006). Furthermore, a large study (18,431 Caucasians, 446 Asians and 137 other ethnicities) published from Leicester has shown a prevalence of 0.45% in the Asian population at age 65 when compared to 4.69% of the Caucasian population (Salem MK 2009).

**Modifiable risk factors**

**Smoking**

Smoking has been identified by several studies as the most important modifiable risk factor for the development of AAA (Lederle FA Mar 1997); (LaMorte WW 1995); (Blanchard JF 2000). A large multicentre study showed
a relative risk of an AAA (4cm or larger) was 5.6 times higher in smokers than non-smokers (Lederle FA Mar 1997). However, while there is overwhelming evidence regarding a positive relationship, it is still unclear whether this is a dose-dependent relationship (Kahn HA); (Doll R 1976); (Hammond EC 1969) or dependent on duration of smoking (Wilmink TBM 1999). MacSweeney et al compared growth of AAA among smokers and non-smokers over 3 years and found a statistically significant rate of growth among those that smoked (0.9mm/year in non-smokers and 1.6mm/year in smokers; P = 0.038) (MacSweeney ST 1994).

Hypertension

Results from studies assessing the link between hypertension and AAA have been varied. Whereas some show positive associations (Strachan DP 1991); (Reed D 1992), this is not replicated in other studies (Plumeekers HJ 1995) (Collin J 1989). A recent meta-analysis that included 9 studies and over 28,000 screened patients has revealed that hypertension only has a weak positive association with AAA (OR 1.33, 95% CI 1.14-1.55) (Cornuz J 2004).

Hyperlipidaemia

The Tromsø study from Norway found that low serum high density lipoprotein cholesterol was associated with an increased risk for abdominal aortic aneurysm (Singh K 2001). However, other studies since then have not come to the same conclusions. Blanchard et al did not find clinical
hypercholesterolaemia, serum levels of total cholesterol, low or high density lipoproteins to be associated with AAA formation (Blanchard JF 2000).

_Diabetes Mellitus_

There is no definitive evidence that associates diabetes mellitus with AAA. Data from several studies have shown either no relationship or even an inverse relationship with diabetes (Golledge J 2006); (Lederle FA Mar 1997); (Pleumeekers HJ 1995); (Blanchard JF 2000). A meta-analysis of 6 studies across 28,462 patients with screen-detected AAA showed no relationship with an odds ratio of 1 (95% CI 0.80-1.26) (Cornuz J 2004).

_Other Cardiovascular diseases_

Peripheral vascular disease (PVD) and myocardial infarction (MI) have been shown to have moderate associations with AAA (OR 2.50 and 2.28 respectively) (Cornuz J 2004). It is unlikely that PVD and MI are independent risk factors for AAA, rather that all 3 diseases are caused by commonly associated causative factors.

_Atherosclerosis_

Historically, atherosclerosis was believed to cause aneurysm formation because of the similarities in risk factors between the two diseases (Golledge J 2010). However, more recently, this theoretical link has been questioned and it has been concluded that both diseases may in their own right be unique due to the differences in underlying pathophysiological mechanisms (Diehm N
2007). Histologically, there appears to be a localisation of macrophages in the media of the aneurysmal aortic wall, whereas this process appears to occur in the subintima in atherosclerotic disease.

**Clinical Manifestations of AAA**

Whilst a large majority of AAA remain symptom free, the few that are symptomatic can be devastating. Asymptomatic AAA are generally diagnosed incidentally through inadvertent findings on radiological imaging performed for other purposes, usually for assessment of the renal or gastrointestinal tracts. Some patients actually notice a pulsation in their abdomen and self-present to their General Practitioner (GP). More recently, with the onset of screening programmes, asymptomatic AAA have been detected through routine scans.

Complications from AAA can be both life and limb threatening. Thrombus in the wall of the aneurysm can lead to thromboembolic disease leading to ischaemia, usually seen in the legs and feet. Left untreated, this may lead to tissue necrosis (Figure 7) and sepsis, requiring amputation and possibly resulting in death.
Rare complications include fistulisation of the AAA into surrounding structures such as the duodenum, causing severe gastrointestinal haemorrhage or into the inferior vena cava (IVC) leading to sudden heart failure (Figure 8). The commonest and most dangerous complication of AAA is that of rupture.

Figure 7: Gangrene of the toe

Figure 8: Non-enhanced abdominal CT showing an 11 cm infra-renal inflammatory AAA with the inferior vena cava adherent to its medial wall (arrow) (Bhogal R 2007)
Treatment

Operative management is currently the only method used to prevent rupture of AAA. Medical treatment is used to control the risk factors mentioned above such as hypertension and hyperlipidaemia. Although these do not directly treat the AAA, the presumption is that controlling the risk factors may limit the extent of growth of the aneurysm and reduce the overall cardiovascular risk of death.

Medical Treatment

Statins

Statins are generally used to halt the progression of atherosclerosis via plaque stabilisation, but have also shown other beneficial effects, such as reducing levels of C-Reactive Protein (CRP). However, this may or may not have any effect on AAA growth (Gotto AM Jr 2007). Whilst the link between hyperlipidaemia and AAA growth is weak, there have been studies showing the benefits of statin therapy on AAA. Treatment with simvastatin has been shown to suppress the development of AAA in both normal and hypercholesterolemic mice, but the overall mechanisms were felt to be more than just due to the lipid-lowering effects (Steinmetz EF 2005). Another study comparing growth rates of AAA in patients with and without statin usage (Figure 9), showed a statistically significant (P=0.001) decrease in mean AAA size in those patients on statins (2mm per year, n=59) compared to those without (3.6mm per year, n=91) (Schouten O 2006).
However, many of the studies assessing the benefit of statin therapy on AAA size are observational and level I evidence is limited. This is largely due the difficulty in isolating statin use for AAA alone. As discussed above, many of the risk factors for AAA, PVD, ischaemic heart disease are similar, and therefore most patients will already be on a statin for one of these 3 diseases. As an observation, we continue to see increase in aortic sizes despite patients complying with statin therapy. A recent meta-analysis examined 12 studies involving 11,933 individuals and reported no significant reduction of AAA size with statin therapy (Twine CP 2011). This study also suggested that the positive results obtained are all due to low quality evidence, as suggested above. However, since then 2 further studies have been published reiterating...
a reduction in aortic size with statin therapy (Karrowni W 2011); (Periard D 2012).

**β-Blockers**

Historically, β-blockers had been thought to decrease AAA growth rates (Gadowski GR 1994); (Leach SD 1988). However, 2 multi-centre randomised controlled trials have suggested otherwise. Both studies were conducted using propranolol and did not show this to inhibit aneurysm expansion. However poor compliance rates amongst both studies have led them to be widely criticised (Lindholt JS 1999); (Propranolol Trial Investigators 2002).

**Angiotensin Converting Enzyme Inhibitors (ACEI)**

Experimental studies in rats have shown that treatment with ACEI suppresses the development of elastase-induced AAA (Liao S 2001). Hackam et al recently published a large study (15,326 patients) comparing the effects of ACEI on patients admitted with AAA and found that ACEI use within 3 to 12 months of admission was less frequent among those admitted for aneurysm rupture (OR 0.82, CI 0.74 to 0.9). These effects were specific to ACEI and were not seen with other classes of anti-hypertensive agent (Hackam DG 2006). In direct contrast, Sweeting et al conducted a prospective trial of 1,701 patients from 93 hospitals between 1991 and 1995 (United Kingdom Small Aneurysm Trial data), and found the complete opposite. There was a statistically significant (P=0.009) difference in growth rates between patients taking ACEI (3.33mm per year in 169 patients) compared to the control
population (2.77mm per year in 1532 patients) (Sweeting MJ 2010). To clarify the literature and investigate whether ACEI reduce AAA growth rate, a pilot RCT has been developed and is currently recruiting (AARDVARK trial investigators 2011).

Antibiotics

Tetracyclines
Animal studies have demonstrated that doxycycline could prevent aneurysm development in the elastase-induced rat model (Petrinec D 1996). A small patient based prospective double-blind randomized placebo-controlled study showed that aneurysm expansion rates in the doxycycline group were much lower than the placebo group, although this did not reach statistical significance. The study also found that whilst doxycycline treatment had no clear effect on antibody titres at 6-month follow-up, CRP levels in the doxycycline group were significantly lower than the baseline levels (Mosorin M 2001). The trial was however criticised due to the assumptions made on a total of 32 patients, which led to an invited commentary by Baxter to conclude that doxycycline has no effect on aneurysm expansion (Baxter BT 2001). Dodd et al have recently conducted a systematic review into the benefit of doxycycline in reducing AAA growth rates (Dodd BR 2011). This study only identified 6 RCT and 2 cohort studies (total of 255 patients examined) in humans that provided conflicting evidence. They concluded that the evidence for any beneficial effects of doxycycline on AAA growth rates was weak.
**Macrolides**

Antibiotic therapy has largely been suggested as a treatment for AAA due to the finding of Chlamydia pneumoniae in atherosclerotic plaques and the aortic wall in AAA (Nieto FJ 2002); (Lindholt JS Aug 2001). This hypothesis was initially examined in a human randomized double-blind controlled trial using roxithromycin and found that in comparison to placebo, roxithromycin (300mg daily for 4 weeks) reduced the expansion rate of AAA by 44% (P=0.02) in the first year but only 5% (P=0.64) in the second year (Vammen S 2001). However, subsequent prospective randomised controlled trials have not proven that AAA growth could be slowed by this treatment. The use of macrolides have also been investigated in coronary artery disease and have not shown to be beneficial (Grayson JT 2005); (Burkhardt U 2004).

**Surgical Treatment**

The UK Small Aneurysm Trial (UKSAT) assessed the preferred method of management of small AAA (4cm to 5.9cm), either early surgery or ultrasound surveillance. The trial found that elective surgical repair was not associated with long-term survival advantage for patients with small AAA (The UKSAT Participants 1998). An American study published 4 years later found that survival was not improved by elective repair of AAA smaller than 5.5cm, even when operative mortality is low (Lederle FA 2002). Therefore, due to this overwhelming evidence, surgery is generally not performed for elective AAA until an aortic diameter of 5.5cm is reached. Surgery can then be performed in one of 2 ways; open or endovascular repair.
Open Repair

The first successful open repair of an AAA was performed by Charles Dubost in 1951, whereby the AAA was resected and replaced by a cadaveric homograft (Cervantes J 2003). The next breakthrough in aortic surgery came when a synthetic graft was used to repair an AAA and was published in the Annals of Surgery in 1966 by Oscar Creech Jr entitled ‘Endo-aneurysmorrhaphy and treatment of aortic aneurysm’ (Creech O Jr 1966) (Figure 10). At the time, this technique was seen as a breakthrough in vascular surgery due to the many failed operations on the aneurysmal aorta including several ‘non-resective’ operations such as aneurysm ligation, wrapping and differing forms of aneurysm thrombectomy combined with bypass procedures. To this day, very little has changed with this technique of open repair of AAA.
Figure 10: Diagrammatic procedure for open AAA repair (Reproduced from Creech O Jr 1966)

The technique requires a laparotomy to enter the abdomen (Figure 11).
The various structures overlying the abdomen are then retracted before the retroperitoneum is entered. The vasculature is assessed and dissected to allow clamping of the vessels (aortic neck and iliacs) before repair. The aneurysm is opened longitudinally and any back-bleeding into the sac from the inferior mesenteric or lumbar arteries is controlled with sutures. A prosthetic graft (usually dacron) is sutured to the proximal and distal ends of the aneurysm, acting as a tube repair. The blood flow is then restored and the aneurysm sac is used to cover the new graft before the abdomen is closed.
Chapter 1. Abdominal Aortic Aneurysms

Endovascular repair

First performed by Volodos in 1987 and published by Parodi in 1991 (Parodi JC 1991), endovascular repair (EVAR) quickly gained popularity due to encouraging results of several large randomised controlled trials. Results from the EVAR1 (United Kingdom EVAR Trial Investigators 2010) and DREAM (Prinssen N 2004) trials, together with the EUROSTAR registry (Harris PL 2000) all showed benefits of EVAR as compared to open repair in terms of peri-operative mortality. The EVAR1 trial showed a three-fold reduction in mortality of EVAR as compared to open repair (4.7% and 1.7% respectively, P=0.009). Due to the minimally invasive nature of the technique, the EVAR1 trial also showed reduced ITU stay, hospital stay and lower operative times. However, there are problems with this technique. Only certain patients with favourable anatomy are able to undergo EVAR repair and a recent paper found that only 49% are suitable (Slater BJ 2008). The EVAR1 trial also highlighted expense as a major block to the success of EVAR. There are also new complications, such as endoleaks, stent migration and fracture associated with EVAR which not only increase costs due to the need for continued monitoring but may also be problematic for the patients if secondary interventions are required. The EVAR1 trial showed that the proportion of secondary interventions required after EVAR was more than that for open repair (9.8% vs. 5.8%; P= 0.025). Long term follow-up data (median 6 years) did not suggest any significant difference in death rates from any cause (adjusted hazard ratio 1.03; 95% CI 0.86 – 1.23; P=0.72) (United Kingdom EVAR Trial Investigators 2010).
EVAR involves intraluminal stenting of the aneurysm. The operation can be performed without the need for general anaesthetic and without performing a laparotomy. Both groins are exposed and the arteries are identified. A guidewire is then passed up the common femoral artery (CFA) to the level of the renal arteries, where an aortogram is performed using a C-arm image intensifier. The device containing the collapsed stent-graft is then passed retrogradely up the CFA, iliacs and aorta to the correct position before being deployed. The stent-graft then expands in position and forms a channel for the blood to flow through which excludes the aneurysm sac (Figure 12). A completion angiogram is performed to check the final position of the graft before closing the groins. Patients can be sent straight back to the ward and do not require intensive care monitoring.
Figure 12: Endovascular AAA repair
(Reproduced from http://www.cardiosmart.org)
Summary

Aneurysmal disease of the abdominal aorta is essentially a disease commonly seen in elderly Caucasian males, mainly caused by smoking, and to a much lesser degree hypertension, and has associations with other cardiovascular diseases such as PVD and MI. Whilst most AAA are detected by chance, or more recently from screening, identification is important as rupture can be life-threatening. Clinical research has focussed on medical management of AAA to slow the progression of the disease and surgical management to determine the best way of repair.
Chapter 2. Pathogenesis

Structure of the normal aortic wall

The structure of the aorta is the same as any other artery. There are three distinct layers arranged in concentric circles (Figure 13).

Figure 13: Cross section of the artery
(Reproduced from Encyclopedia Britannica online)

The intima is the innermost layer and is made up of an endothelium that rests on a layer of connective tissue. The middle layer, named the media, consists of a large amount of elastin, and gives the aorta its ability to expand and
accept larger volumes of blood. The outermost layer is thin and is called the adventitia. It is made up of strong connective tissue and collagen.

**Intima**

The intima is the innermost layer of the artery wall and extends from the luminal surface, which is lined by endothelium to the internal elastic lamina. This layer is generally very thin and only contains a few scattered leukocytes, smooth muscle cells and connective tissue fibres.

**Media**

The media extends from the internal elastic lamina to the adventitia and consists of tightly packed smooth muscle cells along with elastin and collagen fibres. The smooth muscle cell layer is surrounded by a common basal lamina, closely associated to an interlacing network of type 1 collagen fibres. Surrounding these is a system of elastic fibres forming in essence a musculoelastic fascicle. These are generally responsible for the tensile strength, resilience and structural integrity of the aortic wall.

**Adventitia**

The adventitia is composed of fibrocellular connective tissue and contains a network of vasa vasorum composed of small arteries, arterioles, capillaries, venous channels and nerves that control smooth muscle tone. The innermost portion of the media is nourished by circulating intraluminal blood and the
outermost by these small vessels that penetrate the outer wall (vasa vasorum) (Figure 14).

**Figure 14:** Layers of the arterial wall (Reproduced from Kangasniemi K 1997)

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**Structural pathology of the aneurysmal wall**

The gross abnormality within the wall of an aneurysm is the loss of medial elastin and smooth muscle cells. This leads to a reduction in the tensile strength of the artery and compensatory excessive deposition of adventitial collagen and subsequently thickening of the arterial wall. The disruption of the extracellular matrix causes a widespread inflammatory infiltrate and decreased proteoglycan synthesis.

**Elastin**

Elastin is a connective tissue protein arranged into alternating hydrophobic and basic domains. As its name suggests, its elastic properties allow tissues
to stretch rapidly and to resume their normal shape. Deterioration of aortic elastin is believed to play an important role in the development of AAA (Halloran BG Jun 1995). It is not known why the infrarenal aorta is particularly susceptible but some have suggested it is due to a reduction in the number of elastic lamellae along the length of the aorta (Halloran BG Jul 1995).

Dobrin et al investigated the effects of elastases and collagenases on arteries. This study concluded that a degradation in arterial elastin leads to ‘vessel dilatation, decreased vessel distensibility, and vessel elongation which can cause tortuosity’, and that a reduction in collagen produced ‘increased vessel distensibility and rupture’ (Dobrin PB 1994). The experiments were interpreted to show that degradation in elastin leads to expansion whilst degradation of collagen leads to rupture. The authors however have since accepted that ‘neither degradation of elastin nor of collagen produced the true gross enlargement characteristic of human aneurysms’ (Dobrin PB 1994).

Further studies have investigated the potential enzymes that could lead to the degradation of these key structural components. One such class of proteinases that causes degradation of both collagen and elastin is the matrix metalloproteinases (MMP), and will be discussed later in the chapter. Breakdown of elastin leads to the accumulation of elastin degradation products, which are chemoattractants for many cell types including inflammatory cells and fibroblasts. The influx of these inflammatory cells leads to an enhanced protease activity (Bhatti 2003). Several animal models have shown that elastase infusion leads to an inflammatory reaction that
causes AAA formation. This can be prevented by inhibiting recruitment of inflammatory cells, or by blocking MMP activity by using an inhibitor such as doxycycline (Anidjar S 1992); (Boyle JR 1998); (Petrinec D 1996); (Ricci MA 1996)

The effects of decreased elastin in the vessel wall can be demonstrated in patients with Marfan’s disease. This inherited genetic disorder is caused by a mutation in the \textit{FBN1} gene, which encodes for fibrillin-1. This protein is responsible for the correct formation of the extracellular matrix including the biogenesis and maintenance of elastic fibres. The clinical manifestations of Marfan’s disease are extensive but also include aortic aneurysms.

\section*{Collagen}

Whilst the compliance of the aorta is due to elastin, the strength is attributed to collagen. Collagen is the most abundant protein in mammals and has a huge role in tissue strength. It is present in ligaments, tendons, cartilage, bones, teeth and skin. Although sixteen different types of collagen exist, approximately 90\% of all collagen is either type 1, 2, or 3. The majority of the aortic wall collagen is type 1 (75\%) and the remainder is type 2 (25\%), both of which contain fibrillar domains characterised by a triple helix structure capable of extensive cross-linking.

The effects of deranged collagen synthesis can be seen in patients who suffer with Ehlers Danlos type 4. This is a genetic disorder that causes a defect in the type-III collagen synthesis and leads to a tendency to form aneurysms.
Although the ratio of type 1 to type 3 collagen remains unaltered in AAA there is an increase in the overall level of collagen when compared to the other aortic wall proteins (Rizzo RJ 1989).

**Inflammation and the Immune Response**

Characteristic histological features of the aneurysmal aortic wall include chronic adventitial and medial inflammatory cell infiltration, elastin fragmentation and degeneration, medial attenuation along with medial neovascularisation and decreased vascular smooth muscle cells (Shimizu K 2006). Chronic inflammation of the aortic wall causes the lymphomonocytic infiltrate and the vascular smooth muscle to secrete ECM degrading enzymes and pro-inflammatory cytokines and chemokines (Shah PK 1997).

Inflammation plays a key part in the formation of AAA and there is some evidence to suggest that the intensity of the inflammatory reaction correlates well with the size of the aneurysm (Anidjar S 1992). Freestone et al also demonstrated a higher density of inflammatory cells in the walls of larger AAA as opposed to small (4-5.5 cm) aneurysms (Freestone T 1995).

The aortic thrombus has also been suggested to play an important role in the inflammatory process but the mechanism of action remains unclear. Inflammatory cells such as polymorphonuclear neutrophils, T cells, B cells, macrophages, and mast cells filter from the bloodstream via the luminal thrombus directly to the wall of the aneurysm. These cells secrete various
inflammatory factors such as cytokines and leukotrienes that have a direct effect on the aortic wall. There is also evidence to suggest that these inflammatory cells access the vessel wall via the vasa vasorum (Herron GS 1991). It has also been suggested that inflammatory cells in the thrombus release active proteases such as matrix metalloproteinases (MMP9), which lead to degradation of the aortic wall (Fontaine V 2002). The intraluminal thrombus has also been blamed for causing functional hypoxia at the intimal and inner medial layers, which subsequently leads to neovascularization, inflammation, and potentially aneurysmal disease (Vorp DA 2001). A recent review by Michel et al has demonstrated that protease activity in AAA originates from the thrombus, rather than directly from the aortic wall. This may be due to the association of a thinner arterial wall, extensive elastolysis, lower density of smooth muscle cells in the media or a higher immuno-inflammation in the adventitia with an intraluminal thrombus (Michel J-B 2011).

**Matrix Metalloproteinases (MMPs)**

The MMPs are a family of enzymes whose main function is degradation of the ECM. In healthy individuals, this function is useful in processes such as wound healing, pregnancy and parturition, bone resorption and mammary involution (Parsons SL 1997); (Visse R 2003). MMPs can be classified in a variety of different ways but they are all proteases that degrade at least one component of the ECM. Other similarities between them are seen through common amino acid sequences, the presence of a zinc ion and inhibition by chelating agents and tissue inhibitors of metalloproteinases (TIMPs). All
MMPs are synthesized in the latent form (zymogen) and are secreted as proenzymes that require extracellular activation.

The common structure of MMPs consist of 3 domains; the pro-peptide, the catalytic domain and the haemopexin-like C-terminal domain which is linked to the catalytic domain by a flexible hinge region. Activation of the MMPs occurs via removal of the propeptide segment, which forms part of the ‘cysteine switch’. This contains a conserved cysteine residue which interacts with the zinc in the active site and prevents binding and cleavage of the substrate keeping the enzyme in an inactive form (Figure 15). Activation results in exposure of the active site zinc-binding domain.

**Figure 15:** Common structure of the MMP (Reproduced from Yong VW 2011)

Aneurysm formation has been suggested to be as a result of overactivity of MMP which would theoretically in turn cause exaggerated degradation of the aortic wall and subsequently lead to aneurysm formation.
**Tissue Inhibitor of Metalloproteinases (TIMP)**

As the name suggests, the TIMPs are inhibitors of MMPs. Therefore, an imbalance of MMP/TIMP ratio resulting in overexpression of MMP could also be achieved by downregulation of TIMP. This may potentially lead to aneurysm formation.

**Heme oxygenase 1 (HO1)**

Heme oxygenase is an essential enzyme in heme catabolism, cleaving heme to form biliverdin. Biliverdin is then converted to bilirubin by biliverdin reductase (Figure 16).

![Figure 16: Enzymatic reaction of heme oxygenase](Reproduced from Otterbein LE 2000)

Heme oxygenase occurs as 2 isoenzymes; heme oxygenase -1 and -2. Heme oxygenase 1 (HO1) is expressed by vascular smooth muscle cells and has potent anti-inflammatory and antioxidant capacity (Otterbein LE 2000). It has therefore been suggested that downregulation of this enzyme would lead to a
lower anti-inflammatory effect and therefore an increased risk of AAA formation.

**Platelet Activating Factor Acetylhydrolase (PAFAH)**

Platelet Activating Factor (PAF) is a potent phospholipid activator and mediator of many leukocyte functions, as well as platelet aggregation and inflammation. It is produced by neutrophils, basophils, platelets and endothelial cells. PAF is inactivated by the enzyme Platelet Activating Factor Acetylhydrolase (PAFAH). Platelet-activating factor acetylhydrolase protects low density lipoprotein (LDL) against oxidative modification, which is thought to be important in preventing atherosclerosis (Watson AD 1995). A single point mutation has been identified in a PAFAH gene (PLA2G7) that causes reduced (heterozygote) or complete abolition (homozygous) of the enzyme activity, and has therefore been suggested in association with AAA (Unno N Feb 2002).

**Sex hormones**

AAA’s are more commonly seen in men than women, with a lag of approximately 10 years in the incidence between the two sexes. Although no direct reason is attributed to this, some have suggested that it could be due to the protective effects of female sex hormones (Alcorn HG 1996). Although not tested in human subjects, estrogen in particular has also been shown to affect connective tissue metabolism in female monkeys (Register TC 1998). Several studies have been performed on animal models (mainly mice) that have
shown a protective effect of estrogen on AAA formation (Ailawadi G 2004); (Martin-McNulty B 2003).

Further evidence of the association of estrogen and AAA is supported by a study by Grigoryants et al 2005 who analysed the effect of tamoxifen (a selective estrogen receptor modulator (SERM)). This study found that tamoxifen inhibits the development of AAA in male rats, possibly due to an inhibition of neutrophil infiltration of the aortic wall (Grigoryants V 2005).

**Summary**

AAA are characterised by a change in the structure of the aortic wall, namely loss of medial elastin and smooth muscle cells and a compensatory increase in collagen. Although the cause behind this remains unknown, inflammation, the immune response, infection and genetics have all been postulated. These factors may work in isolation or synergistically to produce the features seen in aneurysmal disease.
Rupture is the most devastating outcome of an AAA. The classical triad of symptoms required to make a diagnosis of ruptured AAA are said to be sudden-onset severe abdominal or back pain, hypovolaemic shock and a pulsatile abdominal mass. However, in clinical practice, a high index of suspicion is required as many factors such as pain prevent a full assessment.

According to recent data, approximately 7,000 men die from a ruptured AAA in England and Wales each year (AAAQIP 2011). The frequency of rupture varies from region to region but appears to be particularly high in the UK, 13 cases per 100,000 persons, when compared to other countries: 4.4 in the USA, 4.9 in Finland and 6.9 in Sweden (all per 100,000 persons) (Pearce WH 2011). Whilst there is no known cause for this difference, the possibilities range from complex environmental and genetic to simply a difference in data coding between countries.

Predictors of Rupture

Although the acute pathophysiological events leading to rupture are not known, there is a relationship between size of the aneurysm and rupture. The
United Kingdom Small Aneurysm Trial (UKSAT) demonstrated that aneurysms less than 4cm have a 0.3% annual risk of rupture, those between 4cm and 4.9cm have a 1.5% annual risk of rupture and those between 5cm and 5.9cm have a 6.5% annual risk of rupture. (Brown LC 1999); (Figure 17).

**Figure 17**: Survival without abdominal aortic aneurysm (AAA) rupture by size category of last measured aortic diameter (Reproduced from Brown LC 1999)

Other risk factors associated with rupture from this study included female sex, reduced FEV1 (forced expiratory volume in 1 second), current smoking and a higher mean blood pressure. The authors found ruptured AAA occurred with a smaller diameter in women than in men, although no real explanation was found for this. Some studies have also demonstrated that continued smoking could cause the aneurysm to grow faster (MacSweeney ST 1994) and increased blood pressure was postulated to cause rupture from an increased pressure on the aortic wall.
Potential biomarkers have been studied as predictors of growth rate and subsequent rupture. Biomarkers may also change conservative management of AAA by suggesting optimal surveillance intervals, and may even help to explain the mechanisms behind AAA rupture. However, as yet, no definitive biomarkers have been identified.

Only serum elastin peptides (SEP) and plasmin-antiplasmin (PAP) complexes have shown strong correlation with AAA growth and rupture. Several authors have shown a positive correlation between SEP and AAA growth and rupture. (Lindholt JS 1997); (Lindholt JS Dec 2001); (Petersen E 2001); (Lindholt JS Aug 2001). One group has shown that SEP can predict AAA rupture with a sensitivity and specificity of 67% and 60%, increasing to 83% and 66% when the SEP is combined with the last aortic diameter measurement (Lindholt JS Dec 2001). PAP was initially investigated by Lindholt et al in 2003 in a group of 70 men with small AAAs. They found a significant positive correlation with growth rate, and when combining this with the aneurysm size they found a sensitivity and specificity of 83% (Lindholt JS Oct 2001). The same group have also examined the effect of insulin-like growth factor (IGF) as a potential biomarker for AAA and found serum IGF-I to positively correlate with AAA size and growth rate (P=0.016 and P=0.004 respectively) (Lindholt JS 2011).

Insulin-like growth factor binding protein 1 (IGFBP-1) (Ramos-Mozo P 2012) and thioredoxin (Martinez-Pinna R 2010) have also been suggested as novel biomarkers for AAA.
The matrix metalloproteinases (MMP) and their inhibitors (TIMP) have also been assessed as potential biomarkers in 2000 by Lindholt et al. MMP2, MMP9, TIMP1 and TIMP2 were studied in a small group of 36 patients which found that only MMP9 correlated significantly with expansion rate (Lindholt JS 2000). In another small study, blood plasma levels of MMP9 were found to be significantly higher in patients with AAA (n=25) as opposed to patients with aorto-occlusive disease (n=15) or healthy controls (n=5), although there did not seem to be any correlation between actual levels of MMP9 and AAA size (Hovsepian DM 2000).

A larger study was conducted by Eugster et al in 2005 using a screened population of 987 men of which 76 had an ‘aortic dilatation’. This study did not find a correlation between MMP9 and AAA. However, the ‘aortic dilatation’ was classed as equal to, or greater than 25mm and the screened population was set at a young age for AAA (55 to 70 years old), which may introduce inaccuracies in the dataset (Eugster T 2005).

**Screening**

In the absence of other methods of detecting AAA, imaging has become the mainstay of diagnosis in the general population. Duplex ultrasound scanning (DUSS) is the investigation of choice as it is a valid, safe, fast and acceptable method of screening with an estimated sensitivity and specificity of 98% and 99% respectively (Allen PI 1987). The main problem with DUSS is that it remains heavily operator dependant.
A recent Cochrane review has assessed screening for AAA in 4 large worldwide randomised controlled trials comprising of 137,233 patients (Cosford PA 2007). The trials were from Chichester, UK (Scott RA 1995); Viborg, Denmark (Lindholt JS 2008); Perth, Western Australia (Norman PE 2004) and an MRC trial in the UK (Multi-centre Aneurysm Screening Study) (MASS) (Ashton HA 2002). This review concluded that there was ‘evidence of a significant reduction in mortality from AAA in men aged 65 to 79 years who undergo ultrasound screening’ (OR 0.6). No benefit was found in women, although this was only examined in one study (n=9,342, Scott RA 1995); (Figure 18); (Figure 19). In the analysis, mortality included death from rupture and from surgery for aneurysm repair (elective or emergency). The Chichester trial also reported fewer ruptured AAA in the screened group (12 from 7,887) than the control group (22 from 7,888). All 4 papers show a significant increase in rates of surgery in the screened group (OR 2.03). The MASS trial took this further and attempted to quantify cost-effectiveness of screening. They reported 47 fewer deaths from AAA through screening at 4 year follow-up which equated to £36,000 per Quality Adjusted Life Year (QALY).
### Figure 18: Screening versus no screening for AAA. Death from AAA.  
(Reproduced from Cosford PA 2007)

<table>
<thead>
<tr>
<th>Study or subgroup</th>
<th>Screened</th>
<th>Unscreened</th>
<th>Odds Ratio M-H,Random,95% CI</th>
<th>Weight</th>
<th>Odds Ratio M-H,Random,95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chichester</td>
<td>10/3205</td>
<td>17/3228</td>
<td>1.08 % 0.93, 1.21</td>
<td>100.0</td>
<td>0.59 [0.27, 1.29]</td>
</tr>
<tr>
<td>MASS</td>
<td>65/33839</td>
<td>113/33961</td>
<td>71.1 % 0.42, 0.78</td>
<td></td>
<td>0.58 [0.42, 0.78]</td>
</tr>
<tr>
<td>Western Australia</td>
<td>18/19352</td>
<td>25/19352</td>
<td>18.1 % 0.39, 1.32</td>
<td></td>
<td>0.72 [0.39, 1.32]</td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>56396</td>
<td>56541</td>
<td>100.0 % 0.47, 0.78</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total events: 503 (Screened), 476 (Unscreened)
Heterogeneity: not applicable
Test for overall effect: Z = 0.83 (P = 0.40)

### Figure 19: Aneurysm-related mortality over 4 years of follow-up by randomisation group (Reproduced from Ashton HA 2002)

![Aneurysm-related mortality over 4 years of follow-up by randomisation group](image)

<table>
<thead>
<tr>
<th>Time since randomisation (years)</th>
<th>Cumulative mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Number at risk**

- **Control**: 33961 33162 32231 31196 17860
- **Invited**: 33839 33035 32117 31173 17920
More recently, 10-year follow up data has been published from the MASS trial. The authors concluded that the mortality benefit of screening men aged 65-74 years of age for AAA was maintained up to 10 years, with cost effectiveness improving over time. The study identified an absolute risk of 0.46% from AAA related mortality in the screened group compared to 0.87% in the control group, giving a relative risk reduction of 48% (95% CI 37%-57%). Using 10-year trial data, the cost per man invited for screening was £100 (Thompson SG 2009).

_Treatment & Outcomes_

The only treatment for rupture is to repair the aneurysm through either open or endovascular techniques. Historically, open repair was the only known technique but mortality varied widely between 15% (Stokes J 1973) to 90% (Ascer E 1996), with an accepted rate of approximately 45% (Barry MC 1997; Shackleton CR 1987). Despite advances in intensive care and emergency medicine, anaesthetic and radiological techniques and improvements in surgical methodology, there has only been a small decline in mortality rates from open surgical repair of ruptured AAA, as shown by a 50 year meta-analysis by Bown et al in 2002 (1954 to 1997, n= 21,523 patients) (Bown MJ 2002). The authors also suggested that this improvement in mortality would level off to an optimum point with time (Figure 20). Another meta-analysis conducted by Hoornweg LL et al in 2008 only included studies published since 1991 and showed no improvement in mortality rates (n= 60,822) (Figure 21) (Hoornweg LL 2008).
**Figure 20**: Meta-regression plot of mid-date of study against operative mortality (Reproduced from Bown MJ 2002)

**Figure 21**: Meta-regression of 115 studies reporting on % overall mortality against mid-point of the study (Reproduced from Hoornweg LL 2008)
One suggestion to reduce mortality is to perform endovascular repair for ruptured AAA. In the elective setting, this has seen a three-fold reduction in mortality and reduction in other parameters such as hospital and intensive care stay, intraoperative blood loss and morbidity (United Kingdom EVAR Trial Investigators 2010).

EVAR, however, does have novel complications such as endoleaks and an increase in re-intervention rates. Whilst cost and limitations of resources continue to be hurdles for emergency EVAR, there is no guarantee that anatomical suitability will be met. Several papers have shown that anatomical suitability for EVAR in ruptured AAA ranges between 20% to 49% (Slater BJ 2008); (Rose DF 2003). In theory, EVAR should be beneficial to a patient with a ruptured AAA as it reduces the biological stress required by an open repair. For a long time, it was considered that due to ethical, financial and resource issues, performing a randomised controlled trial would be difficult. However, a small pilot RCT was performed by Hinchliffe et al and found identical rates of mortality in the EVAR and open repair group. A similar rate of morbidity and hospital stay were also detected (Hinchliffe RJ 2006). In 2009, a large multicentre trial known as the Immediate Management of the Patient with Rupture: Open Versus Endovascular repair (IMPROVE) aneurysm trial was setup to determine ‘whether a policy of endovascular repair improves the survival of all patients with ruptured AAA’ (Powell JT 2009). The remainder of the evidence regarding mortality and morbidity rates after EVAR for ruptured AAA arises from small observational or retrospective studies.
**Meta-analysis and Systematic Review of EVAR for ruptured AAA**

To determine the extent of mortality and morbidity across the worldwide published literature for EVAR in ruptured AAA, I conducted a meta-analysis and systematic review (Rayt HS 2008). Medline (1950 to 2007) and Embase (1980 to 2007) databases were searched and limited to articles in English and regarding human studies. Abstracts of all identified articles were either reviewed online or obtained from local and national libraries and reviewed before being enrolled into the study. Manual searches of reference lists of relevant articles and book chapters were also conducted.

The main criterion that was sought for inclusion into the study was the availability of data on mortality rates after emergency EVAR for ruptured AAA. Articles were not restricted due to design of study (retrospective, prospective, observational etc), operative techniques, or stent-graft design. Articles that only gave data on acute symptomatic, as opposed to ruptured AAA were excluded. Case reports, review articles, letters, editorials, series of less than 5 patients and articles that focused on one group of patients (eg octogenarians) were also excluded.

In addition to study demographics (dates of study, length, type and location etc), mortality and morbidity outcomes for patients undergoing emergency EVAR for ruptured AAA were recorded for each study. Mortality rates were taken as in-hospital or 30-day mortalities. Difficulties arose when recording
morbidity data as studies used individual classification criteria. Due to this, all morbidity ranging from mild (wound infections) to severe (organ failure) was grouped together. Separate meta-analyses were conducted for mortality and morbidity. Publication bias was assessed used funnel plots and Egger’s test.

From a total of 813 articles that were identified, only 31 articles giving data on 982 patients met the study inclusion criteria. Only 21 of these studies gave data on morbidity. Of the patients undergoing emergency EVAR, 86% were men (based on data reported in 25 studies). The average age in the open repair group for RAAA from the 14 studies that reported it was 72.4 years compared to 74.1 years in EVAR group (5 studies). Twenty studies reported the average size of the AAA preoperatively using either mean or median statistics. The arithmetic mean of these statistics gave an average preoperative AAA size of 7.28cm.

The overall pooled estimate for mortality rate of ruptured AAA after treatment with EVAR from all 31 studies was 24% (95% CI 20–28%) (Figure 22). There was statistically significant heterogeneity between study results on the log odds scale ($\chi^2 = 58.73; \text{d.f.} = 30; P = 0.001$) resulting in an $I^2$ value of 49% indicating moderate heterogeneity between studies.
The overall pooled estimate from 21 studies for morbidity post EVAR (in those who survive) for RAAA was 44% (95% CI 33%-55%) (Figure 23). Again, heterogeneity between studies ($\chi^2 = 81.23$; d.f. = 20; $P < 0.001$) was statistically significant resulting in an $I^2$ value of 75% indicating a high degree of heterogeneity. Un-weighted average procedure time from the 20 studies that reported this outcome was 155.1 minutes. Intraoperative blood loss was reported in 14 studies with an un-weighted average at 523 ml. Although 21 studies reported the total hospital length of stay at 10.1 days, only six studies recorded Intensive Care Unit (ICU) stay (un-weighted average 113 hours).
To investigate whether mortality and morbidity systematically varied over time, mid-point of each individual study was included as a covariate. There was no suggestion of such a relationship with the analyses producing p-values of 0.67 and 0.93 for mortality and morbidity respectively. This implies that there is little evidence to suggest a significant change in morbidity or mortality over time in this dataset.

The duration of the procedure compared to mid-point of study does seem to have a negative relationship with a lower intra-operative time for the more recent studies (20 studies; P=0.024), implying that the intra-operative time for
ruptured EVAR reduces with experience. There was no significant association when comparing average blood loss (13 studies; P=0.132), hospital stay (21 studies; P=0.235) or ICU stay (6 studies; P=0.202) with the mid-point of study. In summary, although average procedure time seems to be less since 1994, there is no significant improvement in average blood loss, hospital and ICU stay.

In the funnel plot for mortality (Figure 24), there was a large degree of asymmetry especially in the lower right hand corner of the plot suggesting possible publication bias. A formal regression test for publication bias (which tests how a best line fit through the funnel deviates from a vertical line) supports this visual interpretation (p<0.001). The funnel plot for morbidity (Figure 25) is also irregular but it is less easy to discern where exactly studies are missing and thus speculate on the cause for this distribution of outcome estimates. The test for asymmetry results in a P value of 0.006.
An overall mortality rate of 24% for EVAR after ruptured AAA compares favourably to the average value of 45% that is quoted for open repairs of ruptured AAA. However, there are several factors that must be taken into
account when analysing this dataset. Firstly, there are only a small number of studies that have been considered, some with only a small number of patients. Whilst this may give inaccurate results, we must also remember that this is the full extent of the published literature on this topic. Secondly, the majority of the studies included are retrospective or observational studies. Very few RCT’s have been performed on this subject. Finally, although the actual pooled estimate of mortality is low, we must also factor in the high level of publication bias, which may make the actual figure for mortality higher.

**Summary**

The biggest risk factor for rupture is the size of the AAA. Other weaker risk factors for rupture include female sex, a decreased FEV1, current smokers or higher mean blood pressure. Aims to identify small asymptomatic AAA have been pursued. Whilst screening programmes have been successful, other measures such as determining biomarkers have only had limited usage. Although treatment for rupture remains largely unchanged, the onset of EVAR may lead to improved survival.
Chapter 4. Genetic basis of AAA

**Genetic association studies**

Detection of genetic association can be identified thorough several different methods. Segregation analysis assesses the way a disorder is transmitted in families to establish the mode of inheritance. Like familial studies, segregation analysis requires data to be collected on probands and their families to avoid bias. The probands themselves need to be unbiased samples from a population of known size to allow frequency estimates to be correct. An advantage of segregation analyses is that it provides a ‘best-fit’ model of inheritance, but can also provide estimates of disease allele frequency and penetrance. However, in complex disease such as AAA, a single pattern of inheritance is unlikely.

Candidate gene analyses focus on identifying the causative gene using knowledge of disease pathophysiology. They are generally simple to perform, easy to design and use established genotyping techniques. Analysis is conducted on genomic DNA; which is readily available and allows multiple genes to be studied using the same methodology. However, this type of analysis requires prior knowledge of the polymorphisms and pathology of the disease before it can be utilised. Meaningful results also require large sample sizes of both cases and controls. Collection of control samples may be problematic and ethically challenging.
Many people consider genome wide association studies (GWAS) the ‘gold-standard’ in genomic analysis of association. All the benefits of candidate gene studies apply without the need for prior knowledge of disease pathology. It is completely unbiased and assesses multiple markers. The potential problems however are that large sample sizes are needed, which is particularly difficult in recruitment of control groups. This is usually overcome by recruiting members of large registries, for example the blood donor service. As these patients cannot usually be excluded for the disease in question without undergoing further tests, which may not have ethical approval, an assessment of the prevalence of the disease in this group is estimated. This can obviously lead to inaccurate results. GWAS are generally expensive to run and may have logistical problems with recruitment. Genotyping is usually run on high throughput machines such as the GoldenGate assay, and require specialist equipment and knowledge for interpretation of results. As a large number of markers are usually run simultaneously, multiple hypothesis testing can be a problem and finding significance can be difficult. Markers generally focus on established polymorphisms meaning that novel or rare polymorphisms may be missed. If significant markers are found, they may not correlate with functionality so further experiments to required to delineate this.

**Familial studies**

The first suggestion that AAA may represent part of a complex genetic disease occurred in 1977 when Clifton reported a case of three brothers who
underwent surgery for AAA. This report fuelled debate regarding a genetic link (Clifton MA 1977).

Since then many studies have been published to assess the prevalence of AAA in families. Initially these studies used questionnaire based methods and later moved to identify disease within patients. The earliest of these studies was published by Norrgard et al in 1984 and used a questionnaire to assess the occurrence of clinically diagnosed and ruptured AAA among families of 200 patients with AAA. This study found 9 cases of AAA in siblings of 468 patients who were known to have AAA (1.9%). More so, 7 of the 204 deceased siblings had also died of ruptured AAA (3.4%) (Norrgard O 1984).

A further 11 studies have been conducted to assess the familial prevalence of AAA based on interview or questionnaire techniques. The largest was from Lederle et al in 1997 who assessed 985 patients and found a familial prevalence of 9.2% (Lederle FA 1997). The highest prevalence was seen in a study by Salkowski et al in 1999, who examined a much lower number of patients and identified a 26.4% prevalence (Salkowski 1999). All studies were investigated by Kuivaniemi et al who combined the results to give a combined prevalence from the 11 studies of 12.7% (Table 1) (Kuivaniemi H 2008).
Chapter 4. Genetic basis of AAA

Table 1: Familial prevalence of AAA based on interviews & questionnaires
(Reproduced from Kuivaniemi H 2008)

<table>
<thead>
<tr>
<th>Author</th>
<th>Study date</th>
<th>Country</th>
<th>Patients surveyed (n)</th>
<th>Patients with positive history (n)</th>
<th>Familial prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norrgard</td>
<td>1984</td>
<td>Sweden</td>
<td>87</td>
<td>16</td>
<td>18.4</td>
</tr>
<tr>
<td>Johansen</td>
<td>1986</td>
<td>USA</td>
<td>250</td>
<td>48</td>
<td>19.2</td>
</tr>
<tr>
<td>Powell</td>
<td>1987</td>
<td>UK</td>
<td>56</td>
<td>20</td>
<td>35.7</td>
</tr>
<tr>
<td>Johnston</td>
<td>1988</td>
<td>USA</td>
<td>666</td>
<td>41</td>
<td>6.1</td>
</tr>
<tr>
<td>Cole</td>
<td>1989</td>
<td>Canada</td>
<td>305</td>
<td>34</td>
<td>11.1</td>
</tr>
<tr>
<td>Darling</td>
<td>1989</td>
<td>USA</td>
<td>542</td>
<td>82</td>
<td>15.1</td>
</tr>
<tr>
<td>Majumder</td>
<td>1991</td>
<td>USA</td>
<td>91</td>
<td>13</td>
<td>14.3</td>
</tr>
<tr>
<td>Verloes</td>
<td>1995</td>
<td>Belgium</td>
<td>313</td>
<td>39</td>
<td>12.5</td>
</tr>
<tr>
<td>Lederle</td>
<td>1997</td>
<td>USA</td>
<td>985</td>
<td>91</td>
<td>9.2</td>
</tr>
<tr>
<td>Lawrence</td>
<td>1998</td>
<td>USA</td>
<td>86</td>
<td>19</td>
<td>22.1</td>
</tr>
<tr>
<td>Salkowski</td>
<td>1999</td>
<td>USA</td>
<td>72</td>
<td>19</td>
<td>26.4</td>
</tr>
<tr>
<td>Rossaak</td>
<td>2001</td>
<td>New Zealand</td>
<td>248</td>
<td>48</td>
<td>19.4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>3701</td>
<td>470</td>
<td>12.7</td>
</tr>
</tbody>
</table>

The first study to assess the prevalence of AAA among first-degree family members that used sonographic methods was published in 1989 by Bengtsson et al and found a 28.6% prevalence among brothers and 5.8% among sisters of patients with AAA (Bengtsson H 1989). A total of 18 studies used this method, and again were studied by Kuivaniemi et al (Kuivaniemi H 2008) who found a combined prevalence of 19.5% in brothers and 5.7% in sisters of patients with AAA (Table 2).
Table 2: Prevalence of AAA among first-degree family members based on ultrasonography (Reproduced from Kuivaniemi H 2008)

<table>
<thead>
<tr>
<th>Study</th>
<th>Date</th>
<th>Country</th>
<th>Brothers* (%)</th>
<th>Sisters* (%)</th>
<th>Other* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bengtsson</td>
<td>1989</td>
<td>Sweden</td>
<td>10/35 (28.6)</td>
<td>3/52 (5.8)</td>
<td></td>
</tr>
<tr>
<td>Collin</td>
<td>1989</td>
<td>UK</td>
<td>4/16 (25)</td>
<td>0/15 (0)</td>
<td></td>
</tr>
<tr>
<td>Webster</td>
<td>1991</td>
<td>USA</td>
<td>5/24 (20.8)</td>
<td>2/30 (6.7)</td>
<td>7/103 (6.8)</td>
</tr>
<tr>
<td>Adamson</td>
<td>1992</td>
<td>UK</td>
<td>5/25 (20)</td>
<td>3/28 (10.7)</td>
<td></td>
</tr>
<tr>
<td>Bengtsson</td>
<td>1992</td>
<td>Sweden</td>
<td>9/62 (14.5)</td>
<td></td>
<td>7/103 (6.8)</td>
</tr>
<tr>
<td>van der Lugt</td>
<td>1992</td>
<td>Netherlands</td>
<td>16/56 (28.6)</td>
<td>3/52 (5.8)</td>
<td></td>
</tr>
<tr>
<td>Adams</td>
<td>1993</td>
<td>UK</td>
<td>4/23 (17.4)</td>
<td>1/28 (3.6)</td>
<td>6/23 (26.1)</td>
</tr>
<tr>
<td>Moher</td>
<td>1994</td>
<td>Canada</td>
<td>9/48 (18.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitzgerald</td>
<td>1995</td>
<td>Ireland</td>
<td>13/60 (21.7)</td>
<td>2/65 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Larcos</td>
<td>1995</td>
<td>Australia</td>
<td></td>
<td>0/52 (0)</td>
<td></td>
</tr>
<tr>
<td>Baird</td>
<td>1995</td>
<td>Canada</td>
<td>7/26 (26.9)</td>
<td>3/28 (10.7)</td>
<td></td>
</tr>
<tr>
<td>Jaakkola</td>
<td>1996</td>
<td>Finland</td>
<td>4/45 (8.9)</td>
<td>1/78 (1.3)</td>
<td></td>
</tr>
<tr>
<td>van der Graaf</td>
<td>1998</td>
<td>Netherlands</td>
<td>26/210 (12.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salo</td>
<td>1999</td>
<td>Finland</td>
<td></td>
<td>11/238 (4.6)</td>
<td></td>
</tr>
<tr>
<td>Rossaak</td>
<td>2001</td>
<td>New Zealand</td>
<td></td>
<td>4/49 (8.2)</td>
<td></td>
</tr>
<tr>
<td>Frydman</td>
<td>2003</td>
<td>Australia</td>
<td>64/150 (42.7)</td>
<td>20/126 (15.9)</td>
<td></td>
</tr>
<tr>
<td>Ogata</td>
<td>2005</td>
<td>Canada</td>
<td>11/98 (11.2)</td>
<td>4/147 (2.7)</td>
<td>0/31 (0)</td>
</tr>
<tr>
<td>Badger</td>
<td>2007</td>
<td>N. Ireland</td>
<td>8/136 (5.8)</td>
<td>2/122 (1.6)</td>
<td>0/42 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>186/952 (19.5)</td>
<td>44/771 (5.7)</td>
<td>37/638 (5.7)</td>
</tr>
</tbody>
</table>

*number of individuals identified with AAA/number of individuals examined by USS. Others refers to other relatives other than brothers or sisters of the AAA individual.

By this point, questions were being raised regarding screening possibilities for first-degree relatives. Salo et al used USS to screen a sufficient number of family members and controls to define a high-risk subgroup that would benefit most from screening. Eleven percent of AAA siblings (all brothers) had ultrasonographic evidence of an AAA, whereas only 1.4% of controls...
(both men and women) had an AAA. Family history was found to increase the risk for AAA by 4.3-fold (95% CI, 1.3-fold to 14.2-fold), male sex by 12.2-fold (95% CI, 2.6-fold to 56.6-fold), and age (by decade) increased the risk by 1.9-fold (95% CI, 1.2-fold to 3.3-fold). The authors attributed the highest risk of developing AAA to aging brothers of probands and quoted a prevalence of AAA of 18% in the population of siblings over 60 years old (Salo JA 1999)

**Segregation Studies**

This was first studied by Tilson et al in 1984 who examined fifty families with clustering of AAA in at least two first degree relatives. They concluded that the inheritance pattern was likely to be autosomal if only one gene is responsible, but refused to exclude multigene mechanisms (Tilson MD Apr 1984). In another study by the same authors, inheritance patterns in 16 families with a total of 41 affected individuals were studied. The results pointed towards both X-linked and autosomal dominant forms of the disease, with the X-linked variant as the more common type. Again a multifactorial mechanism of inheritance could not be excluded (Tilson MD Feb 1984).

Powell and Greenhalgh followed up this work by evaluating genetic predisposition to AAA from 60 consecutive patients presenting for AAA repair. One third of patients had one first degree relative with AAA. Eight percent of 320 first degree relatives (9% of 128 parents or 7% of 192 siblings) had an AAA. The study also reported that familial AAA tend to occur at a younger age and that families with affected women are at a higher risk of developing
AAA. Genetic analysis revealed an estimated heritability (the proportion of phenotypic variation in a population that is attributable to true genetic variation among individuals as opposed to environmental factors) of 70% (Powell JT 1987).

Segregation analyses conducted by Majumder et al also assessed different modes of inheritance for AAA in first-degree relatives of 91 probands. The hypothesis that AAA are not a genetic disease was strongly rejected, with statistically significant evidence in favour of a genetic model. Controversially, they suggested that susceptibility to AAA should not be viewed as a multifactorial disease and is instead due to a single autosomal recessive gene (Majumder PP 1991). These results were not replicated by a study conducted by Verloes in 1995, who whilst agreeing with Majumder’s evidence for a single gene effect, found this to show dominant inheritance, but with a 1:250 frequency of the morbid allele in which the ex-dependent penetrance increases with age to reach 0.4 in men over the age of 80 years (Verloes A 1995).

A larger multicentre (USA and European) study identifying 233 families with at least 2 individuals with AAA giving a total of 653 AAA patients was conducted in 2003. The study identified 3 modes of inheritance. Almost three-quarters (72%) of families showed an autosomal recessive inheritance (as suggested by Majumder). The inheritance pattern in the remainder was split; with a quarter showing autosomal dominance and only 3% of families showing
autosomal dominance but with incomplete penetrance (Kuivaniemi H Feb 2003).

In 2010, the Swedish Twin Registry was analysed by Wahlgren et al to analyse the role of heredity and environmental factors in the development of AAA. The registry contains data on twins born in Sweden since 1886 and identified 265 twins with AAA. The authors found that there was a 24% probability that the monozygotic twin of a person with an AAA would also have the disease, whereas this was only 4.8% in dizygotic twins. A heritability of 70% of the total trait variance was estimated with the remaining variance explained by environmental factors. The authors concluded that their findings provided ‘epidemiologic evidence that heritability contributes to aneurysm formation’ (Wahlgren CM 2010).

**Candidate Gene Studies**

Fuelled by the above evidence of a genetic background to AAA, a search began for the causative genes. The first chosen candidate genes to be examined were those that encoded constituents of the arterial wall with biologically plausible mechanisms thought to lead to AAA formation.

**Collagen**

Although many different types of collagen exist, 90% of all human collagen is made up from types I-IV.
Type 3 collagen

Coded by the gene COL3A1, Type 3 collagen is also found in arterial walls and is responsible for tensile strength (Vandenberg P 1993). A defect or deficiency in the COL3A1 gene leads to the rare genetic disorder known as Ehlers-Danlos IV which is characterised by fragility of blood vessel walls and organ membranes, leading to rupture or aneurysm development.

Individual reports have shown there to be a relationship between a deficiency in type 3 collagen, possibly caused by a mutation the COL3A1 gene and the presence of AAA (Loosemore TM 1988); (Kontusaari S 1990). However, larger series have shown mixed results. Powell et al studied three polymorphisms of the type 3 collagen gene; 2 within the gene and one just outside but lying ‘sufficiently close to the 3’ region of the gene to act as a marker for variation in this region’. Surprisingly, a positive association was only found between the outlying polymorphism, rather than with the other 2 polymorphisms. Whilst they concluded that there was no evidence for a single common mutation in type 3 collagen predisposing to AAA, they did suggest that changes in the collagen gene may cause weakness in the aortic wall and make patients susceptible to AAA formation (Powell JT 1993).

Type 1 collagen

Type 1 collagen is the most abundant type of collagen found in the human body being present in scar tissue, tendons, skin and artery walls. It is produced by the COL1A1 and COL1A2 genes.
Although Minion et al showed an increase in type 1, alpha 1 procollagen expression in patients with AAA as opposed to patients with atherosclerosis disease or those with normal aorta, there is currently no association with the \textit{COL1A1} or \textit{COL1A2} gene and AAA (Minion DJ 1993).

\textbf{Matrix Metalloproteinases (MMP)}

The MMPs are a large family of matrix degrading enzymes that are required in normal circumstances for tissue remodelling and repair. However, abnormal expression of the MMP series can lead to excessive inflammation and tumour invasion. It has been suggested that derangements in the MMP mechanism could lead to the formation of AAA (Table 3, page 68). Activated MMPs weaken the aortic media by causing destruction of elastic fibres and smooth muscle cells.

A whole series of MMPs exist and although some have tried to classify them, this has proved more difficult than originally thought due to the mixed activity of each protein. The most common classification is made in terms of MMP function; the collagenases, gelatinases, stromelysins, and the membrane-type MMPs. Busuttil was one of the first to suggest that that AAA formation may be due to collagenolytic activity localised to the aneurysm wall (Busuttil RW 1980).

Increased expression of \textit{MMP1} was first shown to be associated with the formation of AAA by Irizarry in 1993 (Irizarry E 1993), although a weak expression was also seen in patients with normal aortas. These findings have
also been confirmed in other studies (Annabi B 2002); (Tamarina NA 1997). Newman et al published two separate reports in 1994 which implicated not only $MMP1$ with the formation of AAA but also $MMP3$ and $MMP9$ (Newman KM 1994).

Whilst studies relating $MMP3$ overexpression with the presence of AAA are limited (Carrell TW 2002); (Higashikata T 2004), $MMP9$ has since been widely implicated. $MMP9$ was first shown to be present in the aneurysmal aorta by Newman in 1994 (Newman KM 1994). Studies since then have looked at actual smooth muscle cells from aortic specimens (Patel MI 1996), PCR based analysis of tissue (Tamarina NA 1997); (Elmore JR 1998); (Yamashita A 2001) and plasma (McMillan WD 1999) levels of MMP. All have shown a positive association with higher levels of $MMP9$ seen in AAA patients. One of these studies (Tamarina NA 1997) showed $MMP9$ to be the predominant metalloproteinase expressed in AAA as its mRNA levels were more than 20 times higher than $MMP1$ and twice as high as $MMP2$. Jones et al (Jones GT 2003) subsequently identified a functional C-1562T polymorphism that was commonly seen in patients with AAA than in normal controls or in patients with peripheral vascular disease (adjusted odds ratio of 2.41 and 2.94 respectively). Despite this overwhelming evidence, others studies have failed to find such positive results. A study by Yoon et al did not identify a link between the $MMP9$ gene and AAA in a section of the Finnish population (Yoon S 1999).
The role of \textit{MMP2} in aneurysmal disease is unclear. Whereas some studies state that there is a positive association with increased levels of \textit{MMP2} and AAA formation (McMillan WD 1995); (Patel MI 1996); (Davis V 1998); (Crowther M 2000), others have shown non-significant differences in expression of \textit{MMP2} in AAA and aorto-occlusive disease (Yamashita A 2001) or even no elevation in levels of \textit{MMP2} (Elmore JR 1998).

More recently, \textit{MMP8} has been implicated as high concentrations of this protein have been detected in the aneurysmal aortic wall (Wilson WR 2005); (Wilson WR 2006).
Table 3: Summary of MMP SNPs investigated for association with AAA (Reproduced from Thompson AR 2008)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Author (et al)</th>
<th>Year</th>
<th>Cases/controls</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>-1607G&gt;GG</td>
<td>Ogata</td>
<td>2005</td>
<td>387/425</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP2</td>
<td>-1306C&gt;T</td>
<td>Eriksson</td>
<td>2005</td>
<td>455</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP2</td>
<td>-955A&gt;C</td>
<td>Ogata</td>
<td>2005</td>
<td>387/425</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP3</td>
<td>-1612 5A/6A</td>
<td>Yoon</td>
<td>1999</td>
<td>47/174</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP3</td>
<td>-1171 5A/6A</td>
<td>Ogreita</td>
<td>2005</td>
<td>387/425</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP9</td>
<td>-1562C&gt;T</td>
<td>Jones</td>
<td>2003</td>
<td>414/203</td>
<td>(P=0.03) Not significant</td>
</tr>
<tr>
<td>MMP9</td>
<td>(CA)(_h)</td>
<td>Yoon</td>
<td>1999</td>
<td>47/174</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP10</td>
<td>+180A&gt;G</td>
<td>Ogata</td>
<td>2005</td>
<td>387/425</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP12</td>
<td>-82A&gt;G</td>
<td>Eriksson</td>
<td>2005</td>
<td>455</td>
<td>Not significant</td>
</tr>
<tr>
<td>MMP13</td>
<td>-77A&gt;G</td>
<td>Ogata</td>
<td>2005</td>
<td>387/425</td>
<td>Not significant</td>
</tr>
</tbody>
</table>
**Tissue Inhibitors of Metalloproteinase (TIMP)**

The TIMPs are naturally occurring inhibitors of MMPs. There are 4 main types (*TIMP1-4*).

Diminished levels of TIMP in the aortic matrix were initially implicated in the formation of AAA in 1991 (Brophy CM 1991). However, two years later, a study published data pointing towards a deficiency of TIMP from local tissue conditions rather than from mutations in the primary structure of the gene (Tilson MD 1993). In 1999, Wang et al identified one nucleotide variant in each of the *TIMP1* and the *TIMP2* coding sequences. The *TIMP2* nt 573 was the only polymorphism that showed a significant difference between AAA patients and controls (Wang X 1999). A follow-up study by Ogata et al isolated genetic variations in *TIMP1* and *TIMP3* that were associated with AAA (Ogata T 2005). In contrast, a recent article by Hinterseher et al analysed the entire coding region of the *TIMP1* gene but failed to show any association between genetic variations and AAA (Hinterseher I 2007).

**Platelet Activating Factor Acetyl Hydrolase (PAFAH)**

Deficiency of *PAFAH* gene (*PLA2G7*) was initially studied by Miwa et al and found to cause severe respiratory symptoms in asthmatic children and was thought to have an autosomal recessive heredity (Miwa M 1988). The molecular basis for this defect was later described as a single point mutation (a guanine to thymidine substitution at position 994) in exon 9, leading to a reduced enzymatic activity with heterozygote and no activity with
homozygotes. Interestingly, no polymorphism was identified in a random sample of 108 Caucasian North Americans (Stafforini DM 1996). Unno et al were the first to consider a link between this mutation and the formation of AAA, in the Japanese population. They found that the frequency of the mutant ‘T’ allele was significantly higher in AAA patients than in control subjects in the plasma PLA2G7 gene and quoted an odds ration of 2.48 for development of an AAA if the ‘T’ allele is present (Unno N 2002).

**Heme Oxygenase 1 (HMOX1)**

Heme oxygenase (HO) is an enzyme that catalyses the degradation of heme into biliverdin, iron, and carbon monoxide. Although there are 3 known isoforms of the enzyme, of particular interest is HMOX1, which is expressed by vascular smooth muscle cells in response to stress, and therefore has anti-inflammatory and anti-oxidant properties.

A polymorphism in the 5’ flanking region of the human HMOX1 gene was described and tested for Parkinson’s disease and Alzheimer’s disease (Kimpara T 1997). Shortly after this, a study by Wang et al found HMOX1 to be highly induced in atherosclerotic lesions of human aortas (Wang LJ 1998). To date, Schillinger et al are the only group to have studied the association of this polymorphism with AAA. Seventy AAA patients were compared to 61 unmatched healthy atherosclerotic-free controls. They found that AAA patients were less frequent carriers of the short (<25 GT) repeats than healthy subjects. They concluded that short alleles, which caused upregulation of
HMox1, were protective anti-inflammatory agents in the formation of AAA (Schillinger M 2002).

**Estrogen Receptor Beta (ESR2)**

Two main observations led Massart et al to hypothesise that AAA onset is possibly due to protective effects of female sex steroids. Firstly, AAA occurs with an increased male preponderance. Secondly, the incidence in the female population over 65 years is about 6% (versus 14% for male), showing that AAA onset in women appears to lag 10 years behind that of men. Whilst several complex pathogenetic mechanisms have been proposed for AAA development, Massart hypothesised that this was due to the protective effect of female sex steroids. In a study looking at the estrogen receptor (alpha and beta) and the progesterone receptor (PR), across 225 healthy controls and 99 AAA patients, they concluded that a common ESR2 gene polymorphism may contribute to a risk of AAA susceptibility. No association was found between the other female sex steroids and development of AAA (Massart F 2004).

**Angiotensin Converting Enzyme (ACE)**

The ACE gene was suggested for investigation in the association of AAA as it was deemed biologically plausible, mainly due to prior research in other cardiovascular diseases.

Hamano et al looked at one specific polymorphism of the ACE gene which involves either an insertion ('I' allele) or deletion ('D' allele) of a 287-base-pair
(bp) sequence within intron 16 in 125 AAA patients and 153 randomly selected matched controls. They came to the conclusion that the distribution of ACE genotypes and allele frequencies in the control and AAA groups was not significantly different (Hamano K 1999).

Pola et al compared the distribution of ACE genotypes between 56 normotensive patients with AAA, 68 hypertensive patients with AAA and 112 normotensive control subjects. They found a higher frequency of the ‘D’ allele in normotensive AAA patients than in hypertensive AAA patients and concluded that ACE ‘DD’ and ‘ID’ genotypes are independent risk factors of AAAs in normotensive patients. This study also implied that the development of AAA due to this polymorphism is independent of the blood pressure (Pola R 2001). This association between the ACE ‘DD’ genotype and AAA was confirmed by Fatini et al (OR ‘DD’ vs. ‘ID’+’II’=1.9; p<0.0001), and interestingly was maintained after a multivariate analysis adjusted for age, sex and traditional vascular risk factors (OR ‘DD’ vs. ‘ID’+’II’=2.4, 95% CI 1.3-4.2 p=0.003). This article also considered effects on AAA formation from a polymorphism of the angiotensin II receptor (AT1R), but found no association (Fatini C 2005).

A recent meta-analysis combined the above studies and reported a relative risk of 1.33 of this ACE polymorphism and the susceptibility of developing AAA (Thompson AR 2008). A further attempt to clarify the role of ACE and AAA was attempted by Jones et al who recruited 1,226 AAA patients and 1,723 controls, and studied 4 different polymorphisms relating to the renin-
angiotensin system, including the \textit{ACE} \textit{I/D} and the \textit{AT1R} as mentioned before. The study found a strong and repeated association between the \textit{AT1R} allele and susceptibility to AAA (OR 1.6; \textit{P}<0.05), and a weaker effect associated with the \textit{ACE} deletion allele (OR 1.33, \textit{P}<0.02) (Jones GT 2008). More recently, Obukofe et al conducted a large case control study among 1,155 AAA patients and 996 screened controls and found no association between the \textit{ACE} gene insertion/deletion polymorphism and AAA (Obukofe B 2010).

\textbf{Methylenetetrahydrofolate reductase (MTHFR)}

A \textit{MTHFR} polymorphism (C677T) within the fourth exon of the gene was studied by Strauss et al who found the frequency of the ‘T’ allele in the AAA population to be 0.37 as compared to 0.21 in the healthy controls (\textit{P}<0.007). \textit{MTHFR} is required to convert homocysteine to methionine and abnormal function may lead to elevated levels of homocysteine which may in turn disturb the function of the aortic wall and lead to AAA formation (Strauss E 2003).

In contrast, a study conducted by Jones et al with a much larger cohort size did not find any significant differences in the frequency of the \textit{MTHFR} C677T variant between AAA patients and controls (Jones GT 2005). A meta-analysis combining the above two studies along with three others found a relative risk of 1.14 (95\% CI 1.08-1.21) for this polymorphism and AAA development (Thompson AR 2008).
**Interleukins**

Several studies have found an increase in inflammatory cytokines in patients with AAA, (Juvonen J 1997); (Szekanecz Z 1994) with some studies suggesting their use as markers for growth and rupture (Treska V 2000). Comparisons have also been made on the extent of cytokine production between aorto-occlusive disease and aneurysmal disease, albeit in small numbers and have shown that higher levels of IL-10 are seen in AAA, whereas IL-1 beta, TNF-alpha, and IL-6 predominate in aorto-occlusive disease (Davis VA 2001).

Bown et al conducted a study to assess the effect of genetic polymorphisms of interleukin (IL)–1β +3953, IL-6 -174, IL-10 -1082, IL-10 -592, and tumour necrosis factor (TNF) α -308 in 100 patients and 100 controls. The IL-10 -1082 ‘A’ allele was found to be significantly more common in the AAA group than the control group (P= 0.03), with an OR of 1.8 for the allele as a risk factor for the development of AAA (Bown MJ 2003).

Although several other interleukins have been studied, no significant positive relationship has been found (Marculescu R 2005).

**Human Leukocyte Antigens (HLA)**

The link between HLA DRB1 and AAA was shown by Rasmussen et al who found a significantly increased expression in patients with AAA as opposed to controls (Rasmussen TE 1997). Other smaller studies have also found
positive links between HLA complexes and AAA, in both American and Japanese populations (Tilson MD 1996); (Hirose H 1998); (Moñux G 2003).

However, the results of much larger studies are less encouraging. Ogata et al looked at 4 different HLA complexes in 387 patients and 426 controls of Belgian and Canadian origin. Although they found a significant difference in the expression of \( HLA-DQA1^*0102 \) allele, this was only in a subgroup of Belgian males (Ogata T 2006). Another study that recruited 241 AAA patients did not find any significant difference between common alleles of \( HLA-A \), \( HLA-B \) and \( HLA-DR \) between aneurysms and controls (Badger SA Mar 2007).

**Chemokine Receptors (CCR)**

Ghilardi et al studied a common 32 base pair deletion (\( \Delta32 \)) in the \( CCR5 \) gene, comparing genotypes between AAA patients, those with PVD, carotid artery disease, and age and sex matched healthy controls. The deletion mutation occurred more frequently in AAA as opposed to healthy controls (\( P=0.002 \)), and in both patients with PVD and carotid stenosis (Ghilardi G 2004). These results were not however replicated by Sandford et al who examined the same polymorphism in a case-control study among 285 AAA patients and 273 controls. No significant difference between the groups were identified (\( P=0.82 \)) (Sandford B 2009).
**Cathepsins**

The cathepsins are a group of mammalian proteases that are responsible for cell turnover and have therefore been linked to aneurysmal disease because of their role in inflammation and ECM remodelling (Lutgens S 2007). Although the role of cathepsins in AAA is unclear, several mechanisms have been described (Liu J 2006) (Sukhova GK 1998). Apart from their obvious protease activity, Sukhova et al suggested that the involvement of cathepsins in microvessel formation and apoptosis increased their contribution to AAA development and growth (Sukhova GK 2006). More recently, the effect of cathepsins on aortic wall has been studied in animal models but mixed results have been obtained (Bai L 2010) (Sun J 2012). Up until recently, much of the research regarding AAA and cathepsins has been limited to animal studies.

Lv et al used ELISA to determine the cathepsin S levels in plasma samples from 476 AAA patients and 200 age matched controls. The results showed higher plasma levels of cathepsin S in AAA patients than in controls (p<0.001) leading the authors to conclude that cathepsin S could act as a biomarker for AAA (Lv BJ 2012). Lohoefer et al examined the aortic wall of 32 AAA patients and 10 organ donors by immunohistochemistry for expression of cathepsins B, D, K, L and S, and found that luminal endothelial cells of AAA patients were positive for cathepsin D, and medial smooth muscle cells were positive for all cathepsins tested. Expression was much greater in the AAA patients as compared to the controls, although no statistical analysis was performed (Lohoefer F 2012). At present, no polymorphism of the cathepsin gene has been associated with AAA in human subjects.
Other implicated genes

PAI-1 (Plasminogen activator inhibitor-1) was originally studied by Yoon et al and found to have no association with AAA (Yoon S 1999). A subsequent study found no difference in allele frequency between AAA patients and controls, but did notice a trend with familial AAA. However, we must bear in mind that the control samples were from random blood donors with a mean age of 42 years, compared to approximately 70 years of age for the AAA patients (Rossaak JI 2000). These results have not been replicated in further studies (Jones K 2002).

Case control studies

Genetic case control studies are used to compare the frequency of SNP alleles in 2 defined groups; those with the disease (cases) and those without (controls). Occasionally the control group may simply consist of a random selection from the population. Case control studies are usually retrospective and are commonly used when the disease under investigation is uncommon or if there is a long latent period between exposure and disease. They do not carry the same level of evidence as randomised control studies, but are still extremely important. The major difficulty in performing a genetic case control study is determining a good match of the genetic backgrounds between the 2 groups to prevent biased results. This may be achieved by only sampling specific ethnic or geographical groups.
Bias can be a big problem. Sampling (or selection) bias occurs when either of the two groups are selected from a biased cohort. This is commonly seen when one sample of patients are recruited from a specific subgroup of the population, for example cases from hospital clinics and controls from the community. There may be large discrepancies in demographics that may lead to incomparable groups. There are several ways to overcome this including collecting both groups from the same source, matching the controls or by using multiple control groups (Mann CJ 2003). Observational bias occurs when researchers focus the study on an area that is expected to provide positive results. An example of this is asking leading questions during participant interviews. Another source of bias in this study is the diagnostic accuracy of detecting AAA. Whilst our entry criteria into both groups (cases and controls) was documented evidence of aortic size through radiological methods, we did not scan for other aneurysms. Therefore, we cannot be certain that any significant difference found is not just a marker for aneurysmal disease as opposed to specifically for AAA.

Other major problems with association studies are non-replication, false-positive results and inadequate power. For any disease, the literature contains numerous papers from case-control studies showing conflicting results. These ‘incorrect’ associations could be due to chance, bias or error and separating the correct set of results can be impossible.

If the methodology has been correct, poor statistical analysis may also lead to misinterpretation of results. Analysis is usually achieved through contingency
tables, which also allows the incorporation of different genetic models (whether a certain allele is dominant or recessive). Allele specific analysis can also be performed in this way (regardless of genotype), but the genotypes must satisfy the Hardy-Weinberg equilibrium (HWE). Most studies recognise the need for replication and assessment of the positive correlations in a different population. However, most replication series are small and therefore lack statistical power to confirm a significant result.

**The Hardy-Weinberg equilibrium**

The Hardy-Weinberg equilibrium (HWE) states that the gene frequencies and genotype ratios in a randomly breeding population remain constant from generation to generation. This basically means that populations are able to maintain a degree of variability without significantly changing the gene pool unless required. There are several conditions under which the Hardy-Weinberg principle fails to apply. These are mutation, genetic flow, genetic drift, non-random mating and natural selection. The mutation of genes would change the relationship and frequencies of genes and therefore imbalance the equilibrium. Genetic flow alludes to the breeding between different populations. Within a species, breeding normally takes place within local populations that may have completely different characteristics to a distant population of the same species. Breeding between these population may introduce new genes or alter gene frequencies. Genetic drift occurs when the frequency of a certain allele may begin to ‘drift’ towards higher or lower values and can occur due to inbreeding or simply due to chance. HWE must be tested using mathematical models. Control subjects should always maintain
the HWE as they are representative of the ‘normal’ population, whereas the cases may not.

**Genetics and the environment**

Some genetic diseases are caused by a clear pathway, with little contribution from other factors. For example, cystic fibrosis is caused by a deletion mutation (ΔF508) in the cystic fibrosis transmembrane conductance regulator gene (CFTR). This follows an autosomal recessive pattern and is usually predictable. Complex diseases such as AAA follow a much less clear pattern. As yet, no single gene has been identified that ‘causes’ AAA, but many genes have shown an association. The likelihood is that a combination of environmental and genetic causes interact to give susceptibility for a disease.

The risk factors mentioned earlier in this thesis probably play a complex intricate role in disease determination and an individual’s overall risk for the disease is likely to be a multifactorial trait determined by the interaction among genetic and environmental factors. One suggestion is that a certain ‘threshold’ has to be attained before a disease is manifest. Another suggested pathway is that environmental or behavioural factors (smoking, obesity), modify the relationship between polymorphisms and phenotypic traits, such that the presence or absence of an environmental factor would change the genetic effect associated with the polymorphism (Joseph PG 2013).

For example, polymorphisms of the 9p21 chromosomal region have been identified through GWAS to have an association with cardiovascular disease
(McPherson R 2010). This common polymorphism is associated with an increased risk of coronary artery disease. However, despite this clear association, the mechanism of action is unclear. The link between behavioural factors and MI risk associated with 9p21 has been investigated as part of the INTERHEART study in 8,114 individuals (Do R 2011). Whilst there was no association seen with smoking and physical activity, MI risk associated with 9p21 (rs2383206) risk allele was significantly reduced in patients consuming a ‘prudent diet high in raw vegetables’ (OR MI & low fruit and vegetable diet 1.32; 95%CI 1.18-1.48 vs. OR 1.02; 95%CI 0.92-1.14 with high fruit and vegetable diet). These results have been replicated in an independent European cohort comprising of 19,129 patients (FINRISK).

**Smoking, genetics and AAA**

Evidence for a relationship between environmental factors, genetic polymorphisms and association with AAA is extremely limited. Only one quantitative article on this topic regarding smoking has been published by Strauss in 2012 and assessed the link between rs11549465 polymorphism in the *HIF1A* gene (hypoxia inducible factor 1 alpha), smoking and the presence of AAA in a case control study of 535 AAA patients and 525 controls. This study found no significant association of the polymorphism between the 2 groups on initial analysis. However analysis of genetic-environmental interactions showed that the presence of *HIF1A* CT/TT genotypes and exposure to tobacco smoke had strong association with AAA (age and gender
adjusted OR 14.1 for smoking and CT/TT genotype; \( p<0.0001 \) (Strauss E 2012).

Wang et al considered the effect of tobacco-gene interaction on atherosclerosis in polymorphisms of the \( CYP1A1 \), \( GST \), \( p53 \) and \( eNOS \) genes (Wang X 2005). No association was seen with the GST and eNOS genes. The ‘C’ allele of the \( CYP1A1 \) \( MspI \) polymorphism showed an increased risk of coronary artery disease in light smokers (20 pack year history) (OR 3.44, 95%CI 1.46-8.09; \( p=0.0046 \)). No relationship was seen in heavy smokers. The authors have attributed this to the toxic effects of heavy smoking.

Interestingly, whilst 2 variants of the \( p53 \) gene (\( HaeIII \) and \( MspI \)) had no overall association with coronary artery disease, a significant effect was seen in non-smokers (\( p=0.0039 \)).

**Other environmental factors**

Although not specific to AAA, both DM and hypertension have been examined with regard to their effect on genetic polymorphisms. Worley et al conducted a small study (\( n=9 \)) to investigate the relationship between Type 2 DM and \( MMP \) expression, by examining the LDL of both patients and controls (Worley JR 2007). The results showed that Type 2 DM LDL significantly increased the gene expression of \( MMP1 \) (\( p<0.01 \)) and \( MMP9 \) (\( p<0.001 \)), implying that patients with Type 2 DM are more likely to suffer effects of MMP overexpression. This includes exaggerated inflammation and tissue destruction, which could potentially lead to AAA.
Tanner et al randomised 42,418 hypertensive patients from a double blind clinical trial to 4 different hypertensive agents and assessed the risk of coronary artery disease across different polymorphisms of the *MMP9* and *MMP12* genes by assessing the hazard ratios of each genotype with the medication. Interestingly, differing and statistically significant hazard ratios were found with different genotype and medication combinations, implying that the best outcomes could only be achieved by tailored medication (Tanner RM 2011).

**Relationships in genetics**

Relationships between alleles can be described as dominant, recessive or co-dominant. A dominant allele is one that masks the expression of another allele (recessive) at the same locus. This interaction between the alleles leads to the phenotype. Alleles can also be ‘co-dominant’, when the contributions of both alleles are expressed in the phenotype (for example, AB blood group).

The Cochran-Armitage trend test is often used as a genotype based test for association in case control studies. The odds ratio (OR) for a disease is the ratio of allele carriers to non-carriers in cases compared to controls. This provides a value for the increased risk of disease between the 2 groups. Odds ratios can be calculated for the overall genotype or for specific alleles. The allelic odds ratio summarise the magnitude of risk conferred by that particular
allele. The relative risk (RR) can also be calculated but is less commonly used in genetic studies.

**Genetic nomenclature**

Genetic nomenclature refers to the scientific naming of genes that has been facilitated through international committees. This allows worldwide research to be performed in a standardised manner. The HUGO Gene Nomenclature Committee is responsible for naming human genes in such a fashion. Throughout this thesis, genes are referred to by their HUGO ascribed abbreviation. Other online sources exist to simplify and unify genetic nomenclature. The National Centre for Biotechnology Information (NCBI) have developed a SNP database called dbSNP, which aims to contain all identified genetic variations. Every submitted SNP receives an ‘ss’ number. Once accepted, these variations are mapped onto the genome and given a unique ‘reference SNP (rs)’ number. These numbers are commonplace throughout the published literature, and also in this thesis.
**Summary**

Genetic association of AAA have been investigated extensively over the past 50 years through familial, segregation and candidate gene studies. However, whilst modes of inheritance and suspect genes have been identified, confirmation of these through replication studies has proved difficult. One reason for this is the relatively small sample size used in genetic studies, which have led to contradictory results.
Experimental work
Chapter 5. Aims and scope of study

Although extensive research has investigated the pathophysiology of AAA, many of these processes remain unclear. More recently, genetic causes have been suggested and supported by strong evidence. Many different genes have been investigated, using mainly candidate gene analysis but as yet, no single gene has shown a strong association with AAA.

This study will allow me to determine the association between 6 single nucleotide polymorphisms (SNP) across 5 genes and AAA. Single nucleotide polymorphisms are a DNA sequence variation that occurs when a single nucleotide is altered in the genome sequence. Approximately 7 million common (minor allele frequency >5%) SNPs (Botstein D 2003); (Kruglyak L 2001) exist in the human genome and clearly genotyping all of these are outside the scope of this thesis. The selected genes have been chosen because of their scientific and biological plausibility and previous positive associations in underpowered studies. I used well established genotyping techniques but with accurately powered cohort sizes.

The 6 polymorphisms to be studied are: MMP9 cytosine to thymidine transition at position -1562 within the promoter region (rs3918242); TIMP1 cytosine to thymidine change at position +434 (no rs number); TIMP1 guanine to thymidine change (rs2070584); PLA2G7 guanine to thymidine point mutation at position 994 in exon 9 (rs16874954); ESR2 +1730 adenine to
guanine mutation (rs4986938), and a (GT)_n dinucleotide repeat in the HMOX1 gene (no rs number).

The overall aim of this thesis is to investigate a possible association between the 6 genetic polymorphisms listed above and AAA. Understanding the genetics behind AAA will lead to an increased understanding of the pathogenetic mechanisms responsible for AAA formation. This will help in identification and treatment of AAA. At present, apart from risk factor modification, surgical management remains the mainstay of treatment for AAA. Whilst technically feasible, surgery is not always appropriate in elderly, frail patients with multiple comorbidities. The development of non-surgical treatment for AAA is therefore desirable and could have a considerable benefit to the quality of life and overall survival.

The null hypothesis is that the above mentioned polymorphisms of the MMP9, TIMP1, PLA2G7, HMOX1 and ESR2 have no association with AAA.
Chapter 6. Study design

Ethics

There were a number of ethical considerations to appreciate prior to commencement of the study. The majority of these centre around obtaining and storing confidential patient information. After consenting patients into the study, a large amount of personal information was collated from several sources. This initial record is the only piece of information that requires the patient to be identifiable through name and date of birth. It was stored in an enclosed folder in a locked office on the university hospital site. Entry into the building is only allowed with approved access.

All patients were given a unique identifying number that was written on this record. Patient data was also transferred and stored on a stand-alone computer with no network or internet access. This was also kept in the above mentioned office and was not removed. Access to the computer was through several password checks. Blood samples were only identified by the unique patient number. Once processed, they were stored in a locked freezer and kept in the above mentioned building.

The blood samples taken were for genetic analysis which may have implications for patients and their families. If a particular gene is found to be
associated with AAA, this may cause undue stress and anxiety for family members as they may also possess the gene and therefore be susceptible for the disease. Secondly, DNA would be stored and could potentially be used for future research not aligned to this study. Patients were counselled regarding the above matters and reassured that the genetic analysis performed would be completely anonymous. They were consented specifically for the polymorphisms under investigation in this study but were also questioned regarding future research. Prior to the start of this project, plans were submitted to, and approved by the Leicestershire Northamptonshire Rutland Research Ethics Committee 1 (LNR REC).

**Clinical Methods**

A case-control association study was performed, whereby people with a disease (cases) are compared to a group of people without the disease (controls). As mentioned above, bias can be a huge problem in this type of study. To minimise this, the same radiological methods were used to identify cases and controls and recruitment therefore took place through the same routes. Observational bias occurs when the variable in question is assessed unfairly by the patient or investigator because of their presumed effect. For example, false answers by patients' at interview. In our study this was overcome by using recorded data from patient records. Other possible solutions such as ‘blinding’ participants and investigators was not practical.
**Patient Recruitment**

All patients (cases and controls) were recruited from the same sources in an attempt to minimise bias. These included outpatient hospital clinics, inpatient hospital wards, and community AAA screening clinics in Leicester, Leicestershire and Rutland. Entry criteria into the study was with radiological evidence proving or refuting an AAA. Both ultrasound and computed tomography methods of imaging were used. A minimum aortic diameter of 3cm was used to represent aneurysmal disease and a maximum diameter of 2.5cm was used for control patients with a ‘normal’ aorta.

Once patients had been identified from their scan reports, they were approached and educated about the study. After a period of two-way discussion, they were given an information leaflet on the study and if desired, consented into the study (Appendices 1&2). Patient demographics were collected via an interview with the patient and family members on a standardised pro forma, which was used for cases and controls (Figure 26).
**Figure 26: Patient demographics record**

<table>
<thead>
<tr>
<th>Date:</th>
<th>PATIENT ID LABEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood sample taken:</td>
<td>PATIENT ID LABEL</td>
</tr>
<tr>
<td>Consented:</td>
<td>PATIENT ID LABEL</td>
</tr>
</tbody>
</table>

**Aneurysm size**

- **Date of Scan:**
- **Type of Scan:**
- **Size at diagnosis:**
- **Date of operation:**

<table>
<thead>
<tr>
<th>Expansion:</th>
<th>Date</th>
<th>Size.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>...../.....</td>
<td></td>
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<td></td>
<td>...../.....</td>
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</tr>
</tbody>
</table>

**Co morbidity:**

- **Smoking:** Yes/no/ex | No. | Start date | End date: |
- **Cholesterol:** Yes/No | Duration |
- **BP:** Yes/No | Duration |
- **MI:** Yes/No. | Details |
- **Angina:** Yes/No. | Details: |
- **Cor Angio:** Yes/No | Details: |
- **CABG:** Yes/No | Details: |
- **Claudication:** Yes/No |
- **Lower limb:** Bypass | Yes/No. |
| Angioplasty | Yes/No | Amputation | Yes/No. |
- **CVA:** Yes/No. | Details: |
| Carotid Endarterectomy: | Yes/No. |
- **DM:** Yes/No. | Duration: |
| Diet controlled | Oral Hypoglycaemic | Insulin |
- **COPD:** Yes/No. | Duration: |
| Inhalers | Nebulisers | Hospitalised |
- **Malignancy:** Yes/No. | Type: |
| Current: | <3/12 | >3/12 |
- **Others:** ………………………………

**Family History:** Yes/No | Details: |

**Medication:**

- **Aspirin** Yes | No. |
- **B-blocker** Yes | No. |
- **Statin** Yes | No. |
- **Nitrate** Yes | No. |
- **Clopidroge** Yes | No. |
- **Digoxin** Yes | No. |
- **Warfarin** Yes | No. |
- **Diuretic** Yes | No. |
- **ACEI** Yes | No. |
- **Others:** ………………………………

- **Reason for admission:**

- **Family History:** Yes/No | Details: |
Measurements of aortic diameter (either USS or CT) were recorded from patient records. Details on cardiovascular risk factors including smoking exposure, hypertension, ischaemic heart disease (IHD), hyperlipidaemia, peripheral vascular disease (PVD), cerebrovascular accidents (CVA), diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD) and malignancy were recorded. Any major interventions for these illnesses such as coronary artery bypass grafts were also recorded. Family history for AAA and a full drug history were also noted.

The hospital records of all the patients were consulted after the interview to increase the accuracy of the data obtained by filling in any missing data from the interview process and by verifying patient claims. This also helped to minimise any possible recall bias and also to reduce any differences in data collection between cases and controls.

**Laboratory methods**

After obtaining informed, written consent to participate in the study from the patient, a peripheral blood sample was taken. In most circumstances this was via direct venous puncture of the median cubital vein using a standard 21 gauge (green) needle and a 20ml syringe. The blood was then transferred into 3 sterile vacuumed 6ml plastic bottles (BD Vacutainer) that contained EDTA (ethylenediaminetetraacetic acid) as an anticoagulant.
The 3 bottles were then labelled using the patient’s identification number before being stored in the blood collection box at room temperature. At the end of each collection session (approximately 3 hours), the blood samples would be taken to the laboratory for processing.

**Extraction and storage of the buffy coat**

In blood, DNA is only contained in the nucleated white blood cells. DNA can be extracted from whole blood using commercially available kits which involve separating the white blood cells from the rest of the blood constituents such as red blood cells (which have no nucleus and hence no genomic DNA) and plasma, before the DNA can be extracted. The ‘buffy coat’ refers to a small layer (approximately 1% of the total volume of blood in the tube) that contains the white cells and some platelets. After centrifugation, three distinct layers, representing the differing blood constituents can be seen (Figure 27). The plasma (55% of total volume) sits above the buffy coat, which separates the plasma from the erythrocytes (44% of total volume).
The method for extracting the buffy coat is detailed in Appendix 3.

**DNA extraction and purification protocol**

Due to the large numbers of samples that needed to be prepared, a commercial kit was used (Gentra Systems PureGene DNA purification kit). The PureGene system works via alcohol and salt precipitation. The first step is to lyse cells with an anionic detergent in the presence of a DNA stabilizer. The proteins are digested and removed along with other contaminants by salt precipitation. The DNA is then alcohol precipitated and dissolved in a DNA stabilizer. This protocol can be performed relatively quickly but also accurately and consistently. The exact protocol is detailed in Appendix 4. The concentrations of each individual DNA sample obtained vary enormously.
They must be quantified prior to dilution and final storage at the desired quantity and concentration.

**Quality assessment**

Once the DNA has been isolated, it must be quantified before it can be used for genomic analysis. This determines the concentration of DNA in the sample and allows correct dilution and analysis.

The commonest method of quantifying DNA is by using a spectrophotometer, and taking conventional absorbance measurements (Figure 28).

**Figure 28:** Spectrophotometer used in the assessment of DNA purity

This works on the simple principle that since nitrogenous bases absorb UV light, the more concentrated the DNA solution, the more UV light that will be
absorbed. A260 and A280 are the spectrophotometer measurement of absorbance at the wavelengths of 260nm and 280nm respectively. A260 is frequently used to measure DNA concentration and A280 is used to measure protein concentration. Although nucleic acids have an absorption maximum at 260nm, most samples will contain contaminants such as proteins and single stranded DNA that absorb maximally at 280nm. Therefore both these values are recorded and a ratio obtained to calculate DNA purity in the presence of contaminants:

\[
\frac{A[260]}{A[280]} = \text{pure DNA concentration}
\]

The higher the ratio, the more pure the DNA sample and a value of >1.8 suggests little protein contamination in a DNA sample.

In practical terms, 5µL of the DNA solution is taken and diluted to a final volume of 500µL using distilled water. This allows accurate measurement and prevents wasting DNA. However, in doing this, the DNA solution is more dilute than the original sample by a factor of 100. Therefore the absorbance value obtained (A260) is of the diluted solution. To determine the concentration of the original sample, the measured A260 value must be multiplied by the same factor 100 and by a constant of 50. This constant arises from the knowledge that the concentration of pure double-stranded DNA with an A260 of 1 is 50ng/µL.

\[\text{Unknown ng/µL} = A260 \times 50 \text{ ng/µL} \times 100\]

(or simply multiply the A260 by 5000, to give a concentration in ng/µL)
Genotyping of polymorphisms

Many different genotyping methods exist for the determination of polymorphic sites. Five out of the six polymorphisms under evaluation in this study were single nucleotide polymorphisms (SNP) and one was a dinucleotide repeat, all of which require polymerase chain reaction (PCR) amplification of DNA prior to genotyping. Identification of the SNP can be performed in a number of different ways but for the majority of the SNPs in this study, I opted to use a technique known as restriction fragment length polymorphism (RFLP) analysis. In this technique, DNA is divided or ‘digested’ by restriction enzymes that cleave the DNA at specific short sequences, leaving ‘restriction fragments’ that can be separated according to their lengths using gel electrophoresis (Figure 29). An RFLP occurs when there are differing lengths of fragment between populations, as the fragments are considered to represent different alleles. RFLP analysis is a simple, cost-effective method of genotyping. However, this method can be slow and laborious and ultimately does require a specific restriction enzyme and ‘cut site’ to be effective. Another technique used for genetic discrimination in this study was allele specific PCR analysis. This involves discrimination of the genetic variations through allele specific primers.
**Figure 29:** Examples of 2 common restriction enzymes Eco RI and HaeIII with the effects of 'cleavage' (Reproduced from Alberts R 2004). The large red arrows represent the cleavage site, with the resulting products shown.

**Polymerase Chain Reaction (PCR)**

Polymerase Chain Reaction (PCR) is a technique used to amplify DNA by in vitro enzymatic replication and allows millions of copies of a small segment of DNA to be produced from a very small amount of template DNA. As the PCR progresses, the formed DNA is also used as a template for replication which therefore allows exponential replication. Importantly, the new strands of DNA are limited to the copied sequence between the two primers, which includes the segment under investigation. At the end of the procedure, the original template of DNA forms a negligible part of the total DNA available (Figure 30).
The key elements in a PCR reaction are:

DNA template, including the area that is to be amplified; 2 primers (reverse and forward) that complement each DNA strand at the 5’ and 3’ regions. These can be considered as the start and stop sequences; a DNA polymerase (usually Taq polymerase); deoxynucleoside triphosphates (dNTPs); buffer solution, which provides an environment for optimum activity and stability of the DNA polymerase and magnesium.
PCR takes place in a thermal cycler which is a machine with a heated block that heats and cools the samples to allow the different stages of the PCR reaction to take place (Figure 31).

Figure 31: The G-Storm PCR thermal cycler. The 96 well plate with prepared pre-PCR reagents is placed in the heating block. The lid is closed and the desired PCR programme is run.
**PCR Procedure**

The PCR procedure consists of a varying number of cycles (usually ranging between 20 and 40) of varying temperature stages (usually 3), which govern the quality of DNA amplification. The temperatures used in the PCR stages differ according to the melting point of the primers, the concentration of the reagents, and the enzyme used. The various stages of the PCR cycle are discussed below (Figure 32):

**Initialisation**

The reaction mix is heated to a high temperature (approx 95°C) for a period of time (approx 1-9 minutes). This allows initial denaturation of the DNA template and the primers.

**The cycle**

*Denaturation*

This is the first step in the cycle and involves heating the mix to approx 95°C for 20-30secs. It disrupts the hydrogen bonds between complementary bases of the DNA strands yielding single strands of DNA that can be replicated.

*Annealing*

In the second step of the cycle, the temperature is lowered to usually between 50-65°C for 20-40 seconds. This allows annealing of the primers to the
single-stranded DNA template. As a rough estimate, the annealing temperature is about 3-5°C below the melting point of the primers in the reaction mix. The reason for picking specific primers is that stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence and to ensure that only specific regions are amplified. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Elongation

This is the final step in the PCR cycle. During this step, the DNA polymerase enzyme synthesises a new DNA strand complimentary to the DNA template strand by adding dNTPs. The temperature of the step is subsequently chosen depending on the optimum temperature of the enzyme used; for Taq polymerase, this is usually between 75-80°C. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, the DNA polymerase will polymerize a thousand bases per minute.

Final Elongation

This single post-cycling step is usually performed at roughly the same temperature as the elongation step but for 5-15 min, to ensure that any remaining single-stranded DNA is fully extended.
**Storage**

At the end of the reaction, the mixture can be kept at a temperature of 4°C.

**Figure 32:** Schematic representation of the PCR process. Note how the extension of the newly synthesised DNA is limited by the primers (Reproduced from Cowrie Genetic Database Project)

---

**Restriction endonucleases**

A restriction endonuclease or restriction enzyme is an enzyme that ‘cuts’ double-stranded DNA following its specific recognition of short nucleotide sequences, known as restriction sites. Whilst many different restriction endonuclease are available and are tailored to individual reactions, the basic methodology remains the same. The PCR product is added to the restriction
enzyme, along with buffer and water and is left in a waterbath (usually at 37°C for 4 hours). At the end of this period, the enzyme will have digested the DNA at the polymorphic site if the polymorphism is present. If not, the DNA remains undigested. The products now will have different sizes and can therefore be determined by gel electrophoresis.

**Gel electrophoresis**

Agarose gel electrophoresis is a technique used to separate DNA fragments using an electric current applied across a gel matrix, to assess the size of the DNA products. As nucleic acids have a negative charge, they migrate towards the positive end of the gel when an electrical charge is applied. Shorter, lighter products migrate faster and further than longer, heavier ones. Other factors that affect migration include the voltage across the gel as well as the gel concentration.

Several dyes are added to the gel and DNA to allow visualisation of the products. A loading buffer, which is essentially a coloured dye, is added to the DNA sample prior to loading it onto the gel. This allows visualisation of the sample to be loaded (rather than loading a colourless liquid onto a colourless gel) and helps it to sink into the well. As the DNA sample migrates across the gel, the coloured band will also move allowing assessment of progress. Ethidium bromide (EtBr) is added to the gel prior to it being cast. This fluoresces under UV light when intercalated into DNA. Therefore by running DNA through an EtBr treated gel, it can be visualised under a UV light to show
distinct bands of DNA (Figure 33). The process of making and running an agarose gel is described in Appendix 5.

**Figure 33:** Schematic representation highlighting the method of gel electrophoresis. 1. A gel ready for loading, 2. Loading the ladder, 3. Loading the samples, 4. Passing an electric current through the gel, 5. Migration of the samples. (Reproduced from http://www.wikidoc.org/images/1/11/Agarose-Gelelektrophorese.png)

**PCR Contamination**

PCR contamination can be a huge problem, mainly because of the sensitive and specific nature of the PCR technique. The following methods were adopted to prevent PCR contamination:
1. DNA sample preparation, PCR sample preparation and PCR was all performed in different areas. PCR reaction mixes were prepared in a different room to where PCR was conducted.

2. Gloves were worn at all times when PCR was performed.

3. Dedicated PCR pipettes were used for the preparation of PCR reaction mixes.

4. Pipette tips with aerosol filters allowed the prevention of microdroplets being injected into the PCR mixture, and thus prevented contamination of PCR reaction mixtures.

5. Negative controls were performed (reaction mixture without any DNA). If bands are present after PCR in these samples it is either contamination or primer dimerisation (annealing of 2 primers due to partially complimentary parts).

Quality control is of particular importance when performing PCR, and several measures were put in place to highlight erroneous results. For each genotyping reaction (plate of 96 samples), a control sample was placed in the final well (bottom right corner). This contained the generic mastermix but no genomic DNA, and therefore should not give a result. Each plate either contained AAA or control samples, and were genotyped in an alternating fashion, to exclude methodological bias. During digestion reactions, samples with known results were included on the analysis as positive controls.
Current knowledge

A summary of the 6 polymorphisms under investigation in this thesis, and previous positive association study details are given in Table 4. The rationale for re-examination of these polymorphisms was that the primary studies, whilst showing positive associations, have numerous limitations and small cohort sizes. This may lead to inaccurate results and misinterpreted conclusions.

Table 4. Summary of all 6 polymorphisms investigated

<table>
<thead>
<tr>
<th>Gene</th>
<th>chromosome</th>
<th>rs number</th>
<th>wild type allele</th>
<th>mutant allele</th>
<th>primary author</th>
<th>date</th>
<th>n AAA</th>
<th>n controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9</td>
<td>20</td>
<td>3918242</td>
<td>C</td>
<td>T</td>
<td>Jones</td>
<td>2003</td>
<td>414</td>
<td>203</td>
</tr>
<tr>
<td>TIMP1</td>
<td>X</td>
<td>-</td>
<td>C</td>
<td>T</td>
<td>Ogata</td>
<td>2005</td>
<td>387</td>
<td>425</td>
</tr>
<tr>
<td>TIMP1</td>
<td>X</td>
<td>2070584</td>
<td>G</td>
<td>T</td>
<td>Ogata</td>
<td>2005</td>
<td>387</td>
<td>425</td>
</tr>
<tr>
<td>ESR2</td>
<td>14</td>
<td>4986938</td>
<td>A</td>
<td>G</td>
<td>Massart</td>
<td>2004</td>
<td>99</td>
<td>225</td>
</tr>
<tr>
<td>PLA2G7</td>
<td>6</td>
<td>16874954</td>
<td>G</td>
<td>T</td>
<td>Unno</td>
<td>2002</td>
<td>131</td>
<td>106</td>
</tr>
<tr>
<td>HMOX1</td>
<td>22</td>
<td>-</td>
<td>short</td>
<td>long</td>
<td>Schillinger</td>
<td>2002</td>
<td>70</td>
<td>61</td>
</tr>
</tbody>
</table>

Matrix Metalloproteinase 9 (MMP9)

A common cytosine to thymidine transition at position -1562 in the promoter region of the MMP9 gene had been identified by Zhang et al in 1999 (Zhang B 1999), and was found to cause a 1.5 times increase in promoter activity. This would cause an overexpression of MMP9 and was speculated by Jones et al to be associated with AAA formation by causing an accelerated degradation of the aortic wall. This study recruited 414 AAA patients and 203 control subjects from a single centre in New Zealand. Subjects were included in the study as cases if they had a radiological proven AAA of greater than or equal to 5cm diameter, meaning that small aneurysms were ignored. Controls were
recruited from the community and had to be above the age of 55 years with ‘current good health’ (for which no specific criteria were set). No mention regarding imaging the control group is made. Demographic details from both groups on age, gender, and general cardiovascular risk factors were collected. The samples were genotyped using RFLP PCR for the polymorphism described above. Results showed a ‘significant difference in genotype frequency between groups, with more ‘CT’ heterozygotes or ‘TT’ homozygotes observed in patients with AAA compared with control subjects’ (P = 0.025, adjusted OR 2.41) (Jones GT 2003). The authors’ concluded that their study showed enough evidence to support the role of \textit{MMP9} in the pathogenesis of AAA.

\textbf{Heme Oxygenase 1 (HMOX1)}

Schillinger et al (Schillinger M 2002) are the only group to study an association between the \textit{HMOX1} gene and AAA in humans. A dinucleotide (‘GT’) repeat polymorphism that is classified as ‘short’ (<25 repeats) or ‘long’ (≥25 repeats) was examined by this group in 70 patients with AAA and 61 ‘healthy atherosclerotic free’ individuals in a single centre in Austria. A patient was deemed to be ‘free of atherosclerosis’ based on clinical history, absence of symptoms of atherosclerotic disease or atherosclerotic risk factors (DM, hypertension), and absence of atherosclerotic plaques in the extra-cranial carotid vessels on DUSS. Limited demographics were included for analysis. Smokers were only labelled as such if they smoked more than 3 cigarettes per day. Aneurysms were defined radiologically as a ‘permanent dilatation of
the aorta, with a diameter of at least 50% greater than the proximal neck’. The median AAA diameter was 4.8cm. Inflammatory aneurysms as defined on CT were excluded from the study. Inclusion criteria for the control group were ‘atherosclerotic free individuals’ without AAA. This was determined both clinically and radiologically using USS, CT or angiography. Genotyping of DNA was performed using PCR and results were sequenced to determine the number of ‘GT’ repeats. They identified that the short repeat was more commonly seen in control patients (P=0.04), and concluded that patients with AAA had a higher frequency of the long repeat, which may be associated with a greater inflammatory response. However the authors do acknowledge that the study is small and may be subject to error and bias. The results also show a wide 95% CI for the OR, which casts some doubt on the strength of the results. The need for confirmation studies using large cohorts and different ethnic groups is echoed.

**Estrogen Receptor β (ESR2)**

Massart et al (Massart F 2003); (Massart F 2004) hypothesised a protective effect of the female sex hormones in the formation of AAA, and studied several sex steroid genes amongst 99 AAA patients and 225 controls, all recruited from a single Italian centre. AAA was positively identified using radiological imaging, although the actual size that constituted an AAA is not described. Recruitment criteria for the control group is not discussed. Genotyping was conducted using RFLP PCR techniques. Although the demographic data is not given in the paper, it states that both groups were
well matched in terms of age, height, weight, BP, blood glucose, and lipid levels. Several hormone receptors were examined but in particular, a +1730 (A-G) Alu RFLP of the ESR2 3’ untranslated region using PCR. This study reported a statistically significantly higher incidence of the Alu restriction site (homozygous or heterozygous for the polymorphism) amongst AAA patients compared to the wild type (P<0.05). The authors of the study also acknowledge the need for replication studies among different populations.

**Platelet Activating Factor Acetylhydrolase (PAFAH/PLA2G7)**

Platelet activating factor (PAF) is a potent phospholipid activator and mediator of many leukocyte functions of inflammation. It is produced by a variety of cell types, including neutrophils, macrophages and platelets. PAF is degraded by PAFAH, which protects low density lipoproteins (LDL) against oxidative modification, a process thought to be important in preventing atherosclerosis. Therefore a lack of the enzyme could potentially accelerate the atherosclerotic process and possibly predispose to AAA formation.

A single point missense mutation at position 994 (G-T) in exon 9 of the PAFAH/PLA2G7 gene has been shown to cause a deficiency of the enzyme. Unno et al observed this genotype in a single centre study in Japan among 131 AAA patients and 106 controls (Unno N Feb 2002); (Unno N Jul 2002). Cases were determined using radiological evidence of an AAA of 3cm or greater and those with connective tissue disorders such as Marfan’s were excluded from the study. Controls were recruited from the same geographical
area and matched for age and sex with AAA patients. Recruitment was from patients in gastrointestinal screening programmes (using endoscopy) who did not have a history of CVA, IHD or PVD. Both groups were demographically well matched although the AAA group did have higher rates of hypertension and hyperlipidaemia. Whilst the classification of co-morbidities were based on WHO criteria, smokers were only considered as such if they were actively smoking and had a smoking index of >100 (years smoked x amount).

Genotyping was conducted through allele specific PCR methods using 4 primers. They found the frequency of the mutant ‘T’ allele to be significantly higher in the AAA population than in the controls (P=0.015). Whilst the authors concluded that this polymorphism of the PLA2G7 gene was an independent risk factor for AAA, they did appreciate the need for larger studies among different ethnic groups. Although most of the data on AAA and this polymorphism is among the Japanese population, Stafforini et al examined a subgroup of 108 Caucasian North Americans and could not identify the polymorphism (Stafforini DM 1996).

**Tissue inhibitor of metalloproteinase 1 (TIMP1)**

The TIMPs were studied as they are major inhibitors of the MMPs. Downregulation of TIMPs could lead to an increase in the activity of MMPs and subsequently increase degradation of the aortic wall, resulting in AAA. Ogata et al (Ogata T Jun 2005); (Wang X 1999) identified 2 SNPs of the TIMP1 gene to be associated with AAA. The first was a ‘C’ to ‘T’ change at position +434 and the second, a ‘G’ to ‘T’ change, ascribed the rs2070584
This study recruited 812 unrelated Caucasian patients (387 AAA and 425 controls) from 2 centres in Belgium and Canada. AAA were defined as a minimum infrarenal aortic diameter of 3cm. Recruitment was made from both sexes (male 81.7%), and from patients treated conservatively (old age and/or small aneurysms), or operatively (both elective and emergency AAA repair). Control patients were recruited from spouses of AAA patients and other admissions to the above institutions, the data regarding which is not given. Data regarding inclusion or exclusion criteria, imaging and demographics is vague. The samples were genotyped for 14 different genes including the 2 TIMP1 variants using different PCR methods. The TIMP1 (nt+434) SNP was analysed using PCR based restriction endonuclease assays, whilst the rs2070584 SNP was genotyped using Taqman assay. None of the polymorphisms were associated significantly with AAA, after the initial analysis. Positive relationships were only seen in male subjects without a family history in both of the above SNPs (nt +434, P=0.0047 and rs2070584, P= 0.015).

**Need for further research**

The overwhelming problem with the above studies is the sample sizes used in the genotyping. As mentioned above, huge sweeping conclusions have been made regarding the genetics of AAA based on very small cohorts. Whilst the majority of authors have addressed this in their discussion, replication of these results in adequately powered cohorts has not been performed. The study designs also vary dramatically in terms of recruitment criteria, demographics and analysis. Basic criteria such as definition of aortic
aneurysm size is not consistent. Many of the papers do not comment on the size of the aorta in the control groups, which to lead to inaccurate results. Some do not list demographics, making comparison among studies difficult.

This study aims to address these points and provide a more robust examination of the SNPs in question through correct patient recruitment, methodology and analysis.

**Specific genotyping methods**

PCR remains hugely variable between different machines and reagents and therefore needs optimising prior to genotyping. The main variables that can be modified are the magnesium, annealing temperature and number of cycles in the PCR reaction. Magnesium is a co-factor for *Taq* polymerase activity and this can be modified through its concentration. Annealing temperature varies according to the primers used and with individual PCR machines. Before the full genotyping could be performed, the experiment had to be ‘optimised’ for each of the variables.
**Matrix Metalloproteinase 9 (MMP9) -1562 C–T (rs3918242)**

**Method 1**

Methods were adapted from 2 sources; Jones et al (Jones GT 2003) and Yoon et al (Yoon S 1999). The *MMP9* polymorphism was genotyped first because it represents a typical RFLP. A PCR reaction was run prior to digestion of the products.

Once the primers, mastermix constituents and PCR conditions were established, the reaction would need optimisation to allow successful genotyping. Initially this required adjustment of the annealing temperature and/or the magnesium concentration. For the purposes of this study, the temperature was always adjusted first. A temperature gradient was assessed based around a previously published annealing temperature to identify the optimum temperature for our specific PCR thermal cycler. The initial reaction details are given below:

**Primers**

Forward: 5’- CAA CGT AGT GAA ACC CCA TCT CT -3’  (23 bases)

Reverse: 5’- TCC AGG CCC AAT TAT CAC ACT TAT -3’  (24 bases)
A 12 x 25μL mastermix was made up using the following constituents; water 19μL, buffer (10x) 2.5μL, MgCl₂ (1.5mM) 0.75μL, dNTP (200µM) 0.5µL, DNA (100ng) 1µL, forward and reverse primer (10pM) 0.5µL each, Taq DNA polymerase 0.25μL. The PCR conditions were: initial denaturation at 94°C for 1min, annealing 30 cycles of 94°C for 25 secs, chosen temperature gradient (50°C to 72°C, automatically set by the machine) for 25 secs, and 72°C for 30 secs, followed by a final extension at 72°C for 3 mins.

All the frozen reagents required in the mastermix apart from the Taq DNA polymerase (Taq) were left to thaw on ice. The Taq was kept in the freezer and only removed when required to prevent it from becoming unstable. The mastermix was assembled in a suitable tube (eppendorf), mixed thoroughly using a vortex and distributed equally (25μL) across 12 different PCR tubes. PCR was then performed on the 12 tubes using the reaction detailed above. The PCR products were run on a 2% agarose gel using a standard 1kb DNA ladder (Invitrogen, Grand Island, NY, USA).

Bands were seen in lanes 1-8 at the expected size of 379bp, with the strongest bands (representing the ideal temperature) seen in lanes 6-7 (60.6°C and 63.1°C) (Figure 34). Good strength bands were also seen in lanes 1-5, but this was accompanied by a degree of ‘ghosting’. This is the presence of excessive bands due to inadequate optimisation, or non specific primer annealing.
Figure 34: Typical results from gel electrophoresis. The gels run from top to bottom. Lanes run from left to right with the ladder furthest left. Each mark on the ladder represents 100bp. Results show markings at approx 400bp with single bands representing ideal annealing temperature seen in lanes 6 and 7.

The above experiment was repeated but with a focused temperature gradient, between 60°C and 63°C across 12 wells to identify a specific temperature. This was determined by a strong clear band at 379bp at 62.1°C. For convenience and without affecting the results, an annealing temperature of 62°C was set as the optimum annealing temperature. In this instance, there was no need to optimise for magnesium concentration as a single strong band at the correct size was seen.

The next stage was to run multiple samples to verify the reaction and to proceed with digestion. Five different DNA templates (both cases and
controls) were chosen at random in an attempt to obtain different genotypes.

The above PCR reaction was used and digestion methods were adapted from published literature and the manufacturer’s guidelines (Promega, Madison, WI, USA). The restriction enzyme *BbuI* was used in the following mix: water 16.3µL, restriction enzyme buffer (x10) 2µL, BSA (10µg/µl) 0.2µL, PCR product variable (see below), *BbuI* (10u/µl) 0.5µL.

Although the manufacturer’s guidelines recommended only 1µL of PCR product to be added to the digestion mix, it was felt that this represented only a small volume of the total and may lead to inaccurate results. Variable volumes of PCR product (2, 5 and 10µL) were used and the total volume for the mix was maintained at 20µL by adjusting the volume of water used. The samples were then incubated in a water bath at 37°C for 4 hours, prior to being run on a 2% agarose gel.

Poor results were obtained as the bands were very faint. It was thought that this may be due to low volumes of PCR product used, so the experiment was repeated with water being completely omitted from the reaction mix and substituted with PCR product instead. The digestion reaction mix was therefore changed as follows: Buffer 2µL, BSA 0.2µL, PCR product 17.3µL, *BbuI* 0.5µL.

Only one sample showed evidence of digestion. It is highly likely that the other samples used in the reaction were ‘undigestable’, meaning they are homozygous for the wild type allele. However, to confirm that the reaction is
fully operational all 3 variants of the genotype need to be visualised. Multiple 
samples were therefore genotyped to try and visualise all genotypic variants, 
i.e. fully digested (band at 320 only – homozygous for the polymorphism), 
partially digested (band at 320 and 379 - heterozygote) and undigested (band 
at 379 only – homozygous for the wild type allele). Complete digestion was 
ensured by running control samples.

Digestion results are shown below (Table 5 & Figure 35):

**Table 5:** Digestion results across 11 samples chosen at random showing all 3 
genotypic variations of the *MMP9* polymorphism.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A997</td>
<td>weak band at 379</td>
</tr>
<tr>
<td>2</td>
<td>A998</td>
<td>strong band at 379</td>
</tr>
<tr>
<td>3</td>
<td>A991</td>
<td>2 bands at 320 and 379</td>
</tr>
<tr>
<td>4</td>
<td>A987</td>
<td>2 bands at 320 and 379</td>
</tr>
<tr>
<td>5</td>
<td>A986</td>
<td>strong band at 379</td>
</tr>
<tr>
<td>6</td>
<td>C824</td>
<td>2 strong bands at 320 and 379</td>
</tr>
<tr>
<td>7</td>
<td>C825</td>
<td>strong band at 379</td>
</tr>
<tr>
<td>8</td>
<td>C820</td>
<td>strong band at 320</td>
</tr>
<tr>
<td>9</td>
<td>C829</td>
<td>2 strong bands at 320 and 379</td>
</tr>
<tr>
<td>10</td>
<td>C808</td>
<td>2 strong bands at 320 and 379</td>
</tr>
<tr>
<td>11</td>
<td>C626</td>
<td>strong band at 379</td>
</tr>
</tbody>
</table>
Figure 35: Results showing digestion of MMP9 polymorphism PCR products with Bbul. The gel shows all three variations after digestion. Sequences that are homozygous for the ‘C’ allele remain undigested and have a band at 379bp (lane 2), homozygous for the ‘T’ allele have a fully digested band at 320bp (lane 8) and partially digested sequences have bands at both 320 and 379bp (lane 10).

Ladder    1     2     3     4     5    6     7     8     9     10    11    12

wells

band at 379

band at 320
**Method 2**

Upon scaling up the *MMP9* genotyping to process 96 well plates, the assay failed and no bands were seen implying that the PCR process was no longer working.

In an attempt to assess the potential problems, DNA samples that had given positive results previously were re-run using the same PCR methods and mastermix criteria as described. This failed to produce any visible bands. New reagents were then ordered in case some of the mastermix constituents had degraded, and the same experiment was repeated using results from 12 known samples, whilst keeping the PCR conditions and mastermix constant. This also failed to produce any visible bands on gel electrophoresis.

The reaction was then re-optimised in case the new reagents required slightly different conditions. Again the annealing temperature was examined first by using a temperature gradient whilst keeping the mastermix constituents the same. A gradient was run across 12 wells between 55°C and 70°C. This range was chosen as it encompassed the previous annealing temperature of 62°C. A DNA sample was chosen that had consistently produced good quality results. Bands were seen at the correct position in lanes 2-6, with the brightest band representing a temperature of 56.4°C. This represented a drop in annealing temperature of 6°C for the new reagents. No bands were seen at the previous temperature of 62°C. To confirm this change, the experiment was repeated using this new annealing temperature (56°C) across 12 selected DNA samples. Bands were confirmed at the correct position of
379bp in all but 2 lanes. However, the bands were not particularly strong. As the new annealing temperature was significantly lower than the original temperature and due to a lack of clarity of the bands, a temperature gradient was re-examined and identified bands between temperatures of 55°C to 65°C, but with some smearing. The clearest single band was seen at a temperature of 60°C, and this temperature was therefore adopted as the new annealing temperature for the PCR reaction. This was then tested across 12 further randomly selected DNA samples but again did not provide any results. The experiment was repeated but again failed. The old and new primers were then tested using the same reaction and temperature gradient to confirm the integrity of the primers and the correct annealing temperature. Clear bands were seen at temperature of 60°C at the correct size. However, again on testing multiple samples, the reaction failed to produce any results.

During this procedure, there had been several occasions where the reaction had been optimised and on up-scaling the procedure, it had failed. Clearly this was not ideal for running thousands of samples. The key aspects of the reaction mix had been altered several times, new reagents and enzymes had been tested in case the old ones had degenerated and multiple DNA samples had been tried. The PCR reaction had also been changed many times but none of these had been consistent enough to produce regular results. The PCR machine was also changed at this time and instead of the new G storm machine, the older Perkin Elmer machines were tested in the lab. None of the above measures led to a robust reproducible reaction.
A literature search was conducted to find different methodology for the \textit{MMP9} (rs3918242) polymorphism. An article from Woo et al (Woo M 2007) had used different methods and a different restriction enzyme to genotype the same polymorphism in patients with colorectal cancer. The following method was therefore attempted:

Primers

\textbf{Forward:} 5’- GCC TGG CAC ATA GTA GGC CC -3’ (20 bases)
\textbf{Reverse:} 5’- CTT CCT AGC CAG CCG GCA TC -3’ (20 bases)

A 50\textmu L mastermix reaction was setup that consisted of water 39\textmu L, buffer 5 \textmu L (10x), MgCl$_2$ 1.5\textmu L (1.5mM), dNTP 1\textmu L (200\textmu M), DNA 1\textmu L (100ng), forward and reverse primers 1\textmu L each (10pM), Taq DNA polymerase 0.5\textmu L. The PCR conditions were: denaturation at 94\degree C for 4 mins, annealing cycles of 35; 94\degree C for 20 secs, 63\degree C for 20 secs and 72\degree C for 30 secs, with a final extension phase at 72\degree C for 7 mins. Pre-digestion bands were expected at 435bp with post digestion bands seen at 247bp and 188bp. The reaction was optimised around the annealing temperature given above (63\degree C). Pre-digestion bands were seen immediately at the correct size with an optimum annealing temperature of 67\degree C. Digestion was therefore performed and optimised using the restriction enzyme \textit{SphI}. \textit{SphI} and \textit{Bbul} are isoschizomers, or pairs of restriction enzymes that are specific to the same recognition sequence (GCATG/C). This led to strong post-digestion bands with all 3 genotypic variants identified. The experiment was then repeated for all the DNA samples. The final genotyping methods are given in appendix 6.
**Platelet Activating Factor AcetylHydrolase (PLA2G7) 994 G-T (rs16874954)**

Methods were adapted from Unno et al (Unno N Feb 2002); (Unno N Jul 2002). This method used an allele specific PCR method (Figure 36) where 3 independent PCR reactions were run to determine a single genotype.

**Figure 36:** Diagrammatic representation of allele specific PCR (Reproduced by Twyman RM 2005)

Four separate primers were used as listed below. The set of primers A and B amplifies the entire exon 9 of the PLA2G7 gene, a positive control for the PCR reaction. Primers A and C are specific for the normal ‘G’ allele, and primers A and D are specific for the mutant ‘T’ allele (Stafforini DM 1996).
A: 5’- CTA TAA ATT TAT ATC ATG CTT -3’ (21 bases)
B: 5’- TTT ACT ATT CTC TTG CTT TAC -3’ (21 bases)
C: 5’- TCA CTA AGA GTC TGA ATA AC -3’ (20 bases) \{ difference of 1 base only \}
D: 5’- TCA CTA AGA GTC TGA ATA AA -3’ (20 bases)

The mastermix constituents used in a 25µL reaction were: water 19µL, buffer 2.5µL (10x), MgCl$_2$ 0.75µL (1.5mM), dNTP 0.5µL (200µM), DNA template 1µL (100ng), forward and reverse primer 0.5µL each (10pM), Taq DNA polymerase 0.25µL. PCR was performed with an initial denaturation phase at 94°C for 5min. Two annealing cycles were required in each reaction. The first lasted for only 5 cycles at 94°C for 60 secs, 56°C for 60 secs and 72°C for 60 secs. The second annealing phase lasted for 25 cycles and required temperatures of 94°C for 30 secs, 52°C for 30 secs, 72°C for 30 secs. There was also a final extension phase at 72°C for 5 mins.

To find our optimal annealing temperature using primers A and B, the 1$^{st}$ annealing temperature was kept at 56°C and the 2$^{nd}$ was run as a gradient between 50°C and 65°C. The reason behind not altering the first annealing temperature is that the cycle is very small and altering this is unlikely to have a demonstrable effect on the overall result. A product size of 160bp was expected. This initial experiment did not produce any results. I then consulted the original genotyping methods published by Stafforini et al (Stafforini DM 1996) that had been used and adapted by Unno et al. Minor modifications were appropriately made to the mastermix so that it consisted of: water 16.25µL, buffer 2.5µL (10x), MgCl$_2$ 1.5µL (3mM), dNTPs 0.5µL (200µM each),
DNA 1µL (100ng), forward and reverse primers 1.5µL each (10pM), Taq DNA polymerase 0.25µL. In essence, the concentration of magnesium was doubled. The same temperature gradient was run and the experiment produced results at the correct size in lanes 1-7. The strongest band corresponded to a temperature of 52.8°C (Figure 37).

**Figure 37**: An example of gel electrophoresis during optimisation of the PLA2G7 gene polymorphism for annealing temperature. Bands of different intensities can be seen at 160bp across the lanes. The brightest band (lane 4; 52.8°C) would correspond to the ideal annealing temperature of the reaction.

As the bands were clear with no excessive bands, the magnesium concentration was not altered and an annealing temperature of 53°C was set for the second annealing phase. I then tested the reaction successfully across multiple samples (n=12), revealing bands at the correct size (160bp). Positive genotyping at this stage, using primers A & B means that the correct position in the PLA2G7 gene has been amplified.
The remaining two primers (C & D) were subsequently analysed individually with primer A, using the above PCR conditions (annealing temperatures of 56°C and 53°C as already determined) and mastermix to determine the patient’s genotype. Six DNA samples were tested for each of the primers giving 12 genotyping reactions in total. Positive genotyping is represented by a band at 108bp. The possible results are: homozygous for the wild type (band in AC only), homozygous for the mutant allele (band in AD only) or heterozygote (bands in both AC & AD). The reaction worked and gave good preliminary genotyping results (Figure 38).

**Figure 38:** Typical results from gel electrophoresis of the PLA2G7 polymorphisms. Here 6 DNA samples are examined, each with 2 sets of primers; AC (odd numbered lanes) which is present if the patient has the wild type and AD (even numbered lanes) which is seen if the patient has the polymorphism. Bands are visible at the correct position (108bp) in the lanes using AC primers only implying that these 6 samples do not have the polymorphism.
Finalised methodology is described in appendix 7.
**Estrogen Receptor β (ESR2) +1730 A-G (rs4986938)**

Methods were adapted from 2 articles from Massart et al (Massart F 2003); (Massart F 2004). The following reagents and PCR settings were used:

**Primers**
- **Forward:** 5’- CAA GTC CAT CAC GGG GT -3’ (17 bases)
- **Reverse:** 5’- AGA TGA ACC CAG GCT GGT G -3’ (19 bases)

**Mastermix constituents (50µL):** water 37.8µL, buffer (10x) 5µL, MgCl$_2$ (2.5mM) 2.5µL, Triton X-100 (1%) 0.5µL, dNTP (200µM) 1µL, DNA (100ng) 1µL, forward and reverse primers (0.4pM) 1µL each, Taq DNA polymerase 0.2µL. PCR conditions were: denaturation at 94°C for 10 mins, annealing 35 cycles, 94°C for 30 secs, 60°C for 30 secs, 72°C for 90 secs, extension at 72°C for 3 mins. Pre-digestion PCR products were expected to be 168bp.

From the published data, the given annealing temperature was 60°C. The annealing temperature was first adjusted over a gradient between 55°C and 65°C to identify the optimum annealing temperature for our thermocycler. This did not yield any results. On further examining the published methods, the primer concentration was very small. This was increased from 0.4pM to 10pM, which produced results but at the wrong size. Bands were seen at 350bp, which is virtually double the size of expected results. This could potentially be due to primer dimerisation due to a gross excess of primer used. The primer concentration was then examined using the same
mastermix as above. A temperature gradient was set between 50°C to 70°C and the primer concentration was varied at 2pM, 4pM, 6pM, 8pM. Variable bands were seen at all concentrations but none at the correct size of 168bp. Instead, as seen previously, they were at 350bp. Ghosting was also seen at higher primer concentrations (6pM and 8pM). An optimal primer concentration of 4pM was chosen as this represented the clearest band (albeit at the wrong size) and the experiment was repeated with a reduction in the denaturation time from 10 mins to 1 min over the same temperature gradient. Again this experiment worked very well and produced clear bands, but in the wrong position (350bp), with the brightest band at 51°C.

Next, the magnesium concentration was adjusted to see if the correct result could be obtained. For this, I reverted back to the original experiment and used the same mastermix and PCR reagents, at an annealing temperature of 51°C. Five reactions were tested each with varying concentrations for MgCl₂, ranging from 1.5pM to 3.5pM in 0.5pM incremental steps. Bands were seen in all lanes at approximately 350bp, with the optimum concentration at either 1.5pM or 2pM. However, the product sizes were still not correct. Digestion of these products with Alul was attempted with a view to achieve the correct digestion products but no digestion was seen. As consistent results at 350bp were obtained, it would appear that the reaction is working well. Several crucial factors have been altered but the product size remains unchanged. This suggests that the primers that are used (published in the above mentioned studies) may be incorrect.
The authors of the key articles mentioned above were contacted and questioned on the use of the primers as well as the PCR reaction. An email was received from the primary author Francesco Massart stating the experiment conditions and reagents were correct, but that they had also struggled to make the reaction work. Correspondence was also received from Maria Brandi, the senior author, who informed us that both the primers were incorrect by 3 bases. The correct primers should be as follows (changes in bold type):

Forward: 5’- CAA GTC **CTC** CAT CAC GGG GT -3’  (20 bases)
Reverse: 5’- AGA TGA **AGC** CCA GGC **TCC** TG -3’  (20 bases)

There were also some minor changes suggested for the PCR settings that were incorporated for future experiments. The above primers were run at 10pM concentration with a randomly chosen DNA sample over a temperature gradient for optimisation as shown below:

Mastermix constituents (25µL): water 18.9µL, buffer (10x) 2.5µL, MgCl₂ (2.5mM) 1.25µL, Triton X-100 (1%) 0.25µL, dNTP (200µM) 0.5µL, DNA (100ng) 0.5µL, forward and reverse primers (10pM) 0.5µL each, Taq DNA polymerase 0.1µL. PCR conditions were: denaturation 94°C for 3 min, annealing 35 cycles; 94°C for 30 secs, temperature gradient 55-70°C for 30 secs, 72°C for 60 secs, final extension at 72°C for 5 mins.
Pre-digestion PCR bands were expected at 168bp. After digestion with Alul, final results should fall into one of the following 3 genotypes:

‘AA’ – band at 168bp
‘AG’ – bands at 44bp, 124bp, 168bp
‘GG’ – bands at 44bp, 124bp

Sharp bands were seen at 168bp in all lanes with no smearing or excess of primer seen (Figure 39). Strongest bands were identified between lanes 6-8 (61.4°C to 65.4°C), allowing the annealing temperature to be set at 64°C. As the bands were so well defined, the magnesium concentration was not altered. Multiple samples were run, with a view to digestion as previously described, but using the Alul restriction enzyme. The digestion reaction required optimisation by adjusting the volume of restriction enzyme and template to give a final digestion mix of: post PCR DNA 8µL, H₂O 0.5µL, Alul 0.5µL and buffer 1µL (Figure 40). Complete digestion was ensured by running control samples.
**Figure 39:** A typical gel showing results from genotyping of the *ESR2* polymorphism. Twelve different samples are examined across the 12 lanes and pre-digestion bands are evident at 168bp in all lanes.

![Image of gel with bands at 168bp](image)

**Figure 40:** Typical gel showing final genotyping results of the *ESR2* gene after digestion with *AluI* restriction enzyme. Variable bands can be seen at positions (44, 124, 168bp) highlighting the various genotypes. A single band at position 168bp (lane 2) demonstrates that the sample is homozygous for the wild type, a band at 44bp and 124bp (not seen on this gel) demonstrates that the sample is homozygous for the wild type and bands at 44bp, 124bp and 168bp demonstrate that the patient is heterozygous (lane 6).

![Image of gel with bands at 44bp, 124bp, and 168bp](image)

Finalised genotyping methods can be seen in appendix 8.
Tissue inhibitor of Metalloproteinases 1 (TIMP1 nt+434 C-T)

Methods were adapted from Ogata et al (Ogata T 2005) and Wang et al (Wang X 1999). This genotyping involves 2 PCR reactions whereby the products from the first reaction are used as the template for the second, instead of original DNA. These secondary PCR products then undergo digestion with a restriction enzyme. This technique is known as nested PCR and is used to reduce product contamination caused by amplification of unexpected primer binding sites. The following methodology was used:

Primers
Forward: 5’- GAC AAA TCA TCT TCA TCA CC -3’ (20 bases)
Reverse: 5’- TGG GGA CAC CAG AAG TCA AC -3’ (20 bases)

Mastermix constituents in a 30µL reaction were water 22.8µL, buffer 3µL (10x), MgCl₂ 1.5µL (1.5mM), dNTP 0.6µL (200µM), DNA 0.6µL (50ng), forward and reverse primer 0.6µL each (10pM), Taq DNA polymerase 0.3µL. PCR conditions were denaturation at 94°C for 10 mins, annealing at 20 cycles; 94°C for 30 secs, temperature gradient (50°C to 60°C) for 30 secs, 72°C for 30 secs, and final extension at 72°C for 3 mins. The expected product size was 653bp.

A temperature gradient, as described above, was used in the first instance to optimise the reaction. Although bands were visualised, they were very faint and in completely the wrong position (700-800bp) (Figure 41).
In the first instance, I reduced the denaturation time from 10 mins to 1 min, at the same annealing temperature. This did not produce any bands. Next, I altered the number of PCR cycles from 20 to 35 (which is in keeping with many of the other PCR settings from previous experiments). Again an annealing temperature gradient as described above was run (50°C to 60°C). This experiment produced bands at 700-800bp, which is not at the expected band size but in an identical position to the initial experiment. There were also excessive bands seen throughout the gel which may be due to a high primer concentration. For the next experiment, the primer concentration was halved to 5pM and the experiment was re-run across an annealing temperature gradient of 55°C to 70°C. This produced identical results. More importantly, no bands were seen at the correct size of 653bp. It was thought at this stage that changing the magnesium concentration would not change the product sizes, but instead optimise an incorrect experiment. As the reaction consistently produced results at the wrong size, it was thought that
maybe the published primers were incorrect. The senior authors of the primary paper (Ogata et al 2005) were contacted to confirm the primer sequences. After some delay, the response informed us that the primers quoted in the original published article were incorrect. New primer sequences as shown below were ordered and the reaction re-optimised.

**Primers**

A  Forward: 5’- TAA GCT CAG GCT GTT CCA GG -3’  (20 bases)

B  Reverse: 5’- TGG GGA CAC CAG AAG TCA AC -3’  (20 bases)

Although the reverse primer is identical to that which is published, the forward primer is completely different.

Using the above primers, the following PCR settings were applied across a temperature gradient between 50°C and 70°C: Denaturation at 94°C for 1 min, annealing at 35 cycles; 94°C for 30 secs, temperature gradient (50°C to 70°C) for 30 secs, 72°C for 30 secs, and final extension at 72°C for 3 mins. The expected product size was 653bp. Mastermix constituents in a 30µL reaction were water 22.2µL, buffer 3µL (10x), MgCl 1.5µL (1.5mM), dNTP 0.6µL (200µM), DNA 1.2µL (100ng), forward and reverse primer 0.6µL each (10pM), Taq DNA polymerase 0.3µL.

This yielded results at the correct size of 653bp with an optimal annealing temperature of 64°C (Fig 42). Ghosting of bands was seen at the lower temperatures.
**Figure 42:** A typical gel showing optimisation reactions for the *TIMP1* (nt+434) polymorphism across a temperature gradient from 50°C to 70°C (left to right) in the 12 lanes. Multiple bands can be seen in lanes 1-6, including at 653bp, indicating that the annealing temperature is not specific for the reagents. However, the clearest bands at 653bp were in lanes 7-12, with the sharpest band in lane 8 which corresponds to a temperature of 64°C.

As good results had been obtained, the magnesium concentration was not altered and the second PCR reaction was run using PCR products from the primary reaction and optimised using the conditions below. A band was expected at 339bp.

**Primers**

C  Forward: 5’- AGG CTG TTC CAG GGA GTC GC -3’  (20 bases)

D  Reverse: 5’- CCG CCA TGG AGA GTG TCT GC -3’  (20 bases)

The above primers were used in an identical PCR reaction and mastermix to the previous experiment. An annealing temperature gradient between 50°C and 70°C was applied. This experiment also produced good results with bands at 339bp. Multiple bands were seen in all lanes except lane 7, which was chosen as the optimum temperature (64°C) (Figure 43). A common
annealing temperature of 64°C had therefore been determined by the optimisation process implying that the same PCR settings could be used for both reactions. The digestion reaction was optimised using the restriction enzyme NruI in the following mastermix: PCR product 20µL, Buffer 2µL, BSA 0.2µL, NruI 0.5µL. Identical conditions for digestions to those described in previous experiments were used. Complete digestion was ensured by using control samples with known digestion results.

**Figure 43:** Optimisation of the secondary reaction in the genotyping of the TIMP1 (nt+434) polymorphism. PCR products from the first reaction were used as the template for this second reaction. The samples here are run across a temperature gradient from 50°C (lane 1) to 70°C (lane 12). There are multiple bands seen in each lane including at the expected position of 339bp with a clear isolated band in lane 7 (indicating the optimum temperature).

A band was visible at 339bp after digestion. This could represent that the digestion process has failed or that the sample in question is not digestible. The next stage was to run the same series of experiments across multiple samples. Seventeen DNA templates were selected at random and run using the same mastermix as above. The PCR settings were also kept identical.
and an optimum annealing temperature for both PCR reactions was set at 64°C. Both experiments worked well and produced bands at the correct sizes. Digestion was then performed on the samples using the following mastermix: 

*NruI* 1µL, buffer 1µL, DNA 8µL. Unfortunately, no digestion products were seen. Rather than changing the experiment, a larger DNA sample base was tested (96 templates). After digestion, multiple bands were seen and 3 clear results were identified, which are in the correct position according to previously published data:

A single band at 339bp  (homozygous for wild type)
2 bands at 19 and 320bp  (homozygous for mutant allele)
3 bands at 19, 320, 339bp (heterozygous)

The band at 19bp was difficult to visualise, as it is so small. I attempted to see this band more clearly by running the samples for longer on the agarose gel and trying higher density gels (3%), but unfortunately, this did not make any difference. In effect, visualisation of the band at 19bp was not essential as it could be inferred by the position of the other bands. If only one band was seen at 339bp, this corresponded to a homozygous wild type genotype and if a single band was identified at 320bp, this corresponded to a homozygous mutant genotype. If two bands were identified, this was obviously heterozygous (Figure 44).
**Figure 44:** A typical gel showing digestion of samples with *NruI* for the *TIMP1* (nt+434) genotype. Evidence of digestion can be seen with 2 bands present in lanes 2, 4 and 7 at sizes 379bp and 320bp.

<table>
<thead>
<tr>
<th>Ladder</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
</table>

Whilst the above 3 genotypic variants may be determined in female patients, male patients should not have a heterozygous genotype. These patients only have one ‘X’ chromosome (location of the *TIMP1* gene) and therefore could only be of a wild type or mutant genotype.

The finalised methods are given in appendix 9.
**Heme Oxygenase-1 (HMOX1 (GT)\textsubscript{n}, repeat)**

Methods were adapted from published data from Schillinger M (Schillinger M 2002) and Exner M (Exner M 2001). The *HMOX1* gene polymorphism is a dinucleotide repeat and in previous research has been divided into either short (\(\leq 24\)) or long (>24) repeats. This initially proved to be problematic because there is no given product size in the literature as previous centres used sequencing to group their results. The author of the original article (Schillinger) was contacted regarding product sizes but no correspondence was received. To identify the correct size, we found the coding area of the gene and identified the attachment sites of the primers. As previous papers have quoted a ‘cut-off’ of 24 ‘GT’ repeats, we were able to count the numbers of base pairs between the primers assuming that 24 repeats represented the wild type and anything above this was the mutant allele. Twenty-four ‘GT’ repeats represents 48bp. The number of base pairs before and after the repeat sequence is 36 and 30 respectively. If we total these together we should acquire a cut-off size, which appeared to be 114bp.

The following primers and PCR conditions were used:

**Primers**

Forward: 5’- AGA GCC TGC AGC TTC TCA GA -3’ (20 bases)
Reverse: 5’- ACA AAG TCT GGC CAT AGG AC -3’ (20 bases)
Denaturation for 10 mins at 95°C, followed by 41 cycles of annealing at 93°C for 40 secs, 51°C for 40 secs and 70°C for 30 secs. A final extension phase at 72°C for 5 mins followed. The mastermix consisted of a 50µL reaction of water 39µL, buffer 5µL (10x), MgCl$_2$ 1.5µL (1.5mM), dNTP 1µL (200µM), DNA 1µL (100ng), forward and reverse primer 1µL each (10pM), Taq DNA polymerase 0.5µL.

The above experiment was conducted and no results were seen. Optimisation of the reaction was commenced by identifying the ideal annealing temperature, by setting up a gradient between 50°C and 65°C. The reaction worked but only faint bands were seen in all lanes at 100-200bp, with the brightest band at 64.9°C. Additional bands were also identified at the higher temperatures at 300bp and 800bp. I continued to optimise the reaction by modifying the magnesium concentration. Using an annealing temperature of 65°C, the magnesium concentration was altered across 5 wells starting between concentrations of 0.25mM and 1.25mM (in 0.25mM increments). Results revealed bands in all lanes at a size less than 100bp, with lanes 1, 2 and 4 also containing bands between 100bp and 200bp as previously seen. The experiment was repeated but with higher concentrations of magnesium. Again 5 concentrations were tested using the same reaction starting at 0.5mM and rising in 0.5mM increments to 2.5mM (Figure 45) Again multiple bands were seen in all the lanes. The strongest band at 100-200bp was seen using a concentration of 2mM. In an attempt to reduce excess bands (at <100bp), the concentration of primer was modified. Using an annealing temperature of 65°C and a magnesium concentration of 2mM, primer concentrations of 1pM,
2pM, 4pM, 5pM and 8pM were run. Poor results were obtained, with no results seen at the lower concentrations, and smearing seen with concentrations of 5pM and 8pM. Several repeats of the experiment produced similar results. It was thought that some of the reagents had become contaminated, and new reagents were ordered.

**Figure 45:** An example gel from the optimisation stage for the *HMOX1* gene showing varying Mg concentrations between 0.5mM to 2.5mM in 0.5mM increments. A band is seen at <100bp in each lane and additional bands are seen between 100bp and 200bp in lanes 1, 2 and 4. The brightest band is seen in lane 4 which represents a concentration of 2mM.

The reaction proved variable and temperamental and initial runs failed to work. The experiment was re-optimised by individually changing the temperature and magnesium concentration as detailed above. After several experiments, I concluded that the optimum annealing temperature had changed to 50°C with an optimum magnesium concentration of 1mM. The
experiment now consistently produced good results with variable bands appearing in 3 distinct sites between 100-200bp, which may represent the different genotypes depending on whether the samples are homozygous or heterozygous. The high band was therefore denoted ‘H’, the middle band ‘M’ and the low band ‘L’. Another band was intermittently seen at approx 250bp, but no band was seen at <100bp. This categorisation made interpretation and recording of the results easier as shown (Figure 46).

**Figure 46:** Typical results after optimising for the *HMOX1* polymorphism. The yellow line is highlighting the middle band. Bands are identified in three distinct areas labelled middle and high bands (M&H-lane 1), middle and low bands (M&L-lane 2) and middle band only (M-lane 4).

The methodology now seemed robust enough to obtain results from running all the samples. Whilst the products obtained seemed consistent, we needed some way of corroborating these sizes with the number of ‘GT’ repeats so that
all samples could be imaged on gel electrophoresis. The best way was to sequence the results and this work was carried out at the Protein Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester.

Three samples that had produced consistent but individual results from genotyping were chosen (Table 6). Prior to sequencing, the samples required to be ‘cleaned’ to avoid incorrect results. This was achieved using a commercially available kit from GenElute (GenElute clean up kit, Sigma-Aldrich, St. Louis, USA). After ‘cleaning’, the sample concentration was checked using a spectrophotometer and diluted to 20ng/µL. This was then sent along with 8-10µL of the original DNA template and small volumes of both forward and reverse primers for sequencing.

**Table 6**: Results from genotyping of 3 samples for the *HMOX1* polymorphism prior to sequencing.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample ID</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A1032</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>A1051</td>
<td>M&amp;L</td>
</tr>
<tr>
<td>3</td>
<td>A1048</td>
<td>M&amp;H</td>
</tr>
</tbody>
</table>

The sequencing results were displayed as a string of bases present in the sample. Any base that was not identified was highlighted and a generic symbol was entered in the sequence. The sequencing results obtained were not perfect and there was a proportion of the result that was missing (no base identified). Overall however, it showed good correlation with the results from
the gel electrophoresis. In theory, all samples could be sequenced to assess the number of ‘GT’ repeats. In practice, this method would need to be more robust and give clearer results to be of any use. Sequencing thousands of samples would also be extremely expensive and difficult to perform. However, because the results of the gel electrophoresis and sequencing were well matched, we adopted to use the former approach.

To further test the stability of the reaction, genotyping was conducted for a whole DNA plate (96 samples). Once again, good results were produced and as the results from one whole plate would produce a full array of results, it is easy to determine where the bands lie in relationship to each other (high, middle or low). To make this clearer, a straight line was marked on the gel using a surgical vicryl 2/0 ligature at the middle band which represented 114bp. Any low and middle band therefore represented a short repeat (≤24 GT repeats) and the remainder (high bands) represented a high repeat (>24 GT repeats). The results could now easily and accurately be classified.

The methods were now used for full genotyping and the finalised methods are detailed in appendix 10.
Tissue inhibitor of Metalloproteinases 1 (TIMP1 rs2070584)

We attempted to genotype the rs2070584 SNP using methods from Ogata et al (Ogata T 2005). However, this polymorphism proved the most difficult to genotype. The above study used a 5'-nuclease assay (Taqman) which was not available in our laboratory. Alternative published methodology was sought but very little was found. It was clear that we had to adopt an alternative method to the standard PCR used previously.

The three main alternatives considered were Taqman, GoldenGate and DASH (dynamic allele-specific hybridisation). Taqman analysis, which was used in the original article, requires real time PCR but with an increased specificity due to specialist hydrolysis probes. This is an excellent well established technique but does not allow fast throughput of samples, and can be time consuming. The GoldenGate assay is a commonly used technique that uses beadchip arrays to allow multiple SNPs to be examined per sample. Dynamic allele-specific hybridisation (DASH) uses the fact that mismatched base pairs are less chemically stable than matched base pairs, meaning that mismatched pairs have a lower melting point. The DASH technique therefore uses relatively simple techniques to identify differences in temperature, and can also use this to detect other mutations. Advice was sought from the University of Leicester Genetics department and based on my requirements. Essentially I required a readily available fast throughput technique, with an appropriate cost-profile, that produced high quality results. DASH would need to be outsourced and was therefore excluded from further discussions. The
University of Leicester Genetics department had a lot of experience with the GoldenGate assay and I was advised that this would be the most productive and allow genotyping of many other SNPs as an additional experiment. This GoldenGate assay has already been proven and was used as part of the HapMap project.
GoldenGate genotyping assay

In order to genotype the TIMP1 (rs2070584) SNP, a GoldenGate genotyping assay using tag SNPs was conducted.

A tag SNP is defined as a representative SNP in a region of the genome with high linkage disequilibrium. There are approximately 7 million known common SNPs in the human genome (Botstein D 2003); (Kruglyak L 2001) and it has been suggested that alleles of SNPs within close proximity are correlated (Halperin E 2005). The theory is that haplotypes within an area of reasonable linkage disequilibrium (the non-random association of alleles at two or more loci) can be represented by a small fraction of the haplotypes. This in theory allows large areas of the genome to be studied by genotyping a small number of significant SNPs (tag SNPs), therefore making the overall process of genotyping more efficient.

Methods

Patient selection

The same patient database that was collected for the above experiment was used. The most recent samples with high quality DNA were selected for this study.
SNP selection

The SNPs were identified from two main sources, published articles via Pubmed or medline, or Human SNP websites (http://www.ensembl.org; www.ncbi.nlm.nih.gov/SNP).

I primarily wanted to assess the rs2070584 SNP that could not be assessed in our lab. As an additional experiment, due to the capacity on the GoldenGate assay, I was also able to examine other SNPs from the above mentioned genes. Tag SNP’s within 10kb of our chosen genes were identified using the Tagger software (version 2) using an $R^2$ threshold of 0.8, and were included in the analysis. Across the 5 genes, 34 tag SNPs were identified in the relevant area of the genome (HMOX1 [6 tag SNPs]; MMP9 [3]; TIMP1 [4]; ESR2 [13]; PLA2G7 [8]). Those SNPs with low design scores (high failure rates) were removed. The above SNPs were added onto an assay being run in the laboratory, which included other many other SNPs, mainly involving the cathepsin gene.

Genotyping

Genotyping was performed using the Illumina GoldenGate assay system on an Illumina Beadstation 500 G (Illumina Inc., San Diego, USA), according to the manufacturer’s protocol by the Leicester Institute of Genetics and Genome Sciences at the University of Leicester.

The GoldenGate assay makes use of novel bead array technology developed by Illumina. This is essentially a 3-day process whereby in the initial phases
the DNA undergoes a period of hybridization. Three assay oligonucleotides are designed for each SNP locus. Two oligonucleotides are specific to each allele of the SNP site (Allele Specific Oligonucleotide (ASO)), whereas the third hybridises between 1 and 20 bases downstream from the ASO, and is hence termed the Locus Specific Oligonucleotide (LSO). All three contain regions of genomic complementarity, as well as universal PCR primer sites. In addition, the LSO contains a unique address sequence complementary to a specific bead type. Once hybridised, a polymerase fills the segment between the ASO and LSO, and a DNA ligase seals this extended segment. This now forms PCR templates that can be amplified with universal PCR primers. The primers are labelled with Cy3 and Cy5 dyes, and after the PCR reaction the dye labelled products are hybridised to their complimentary bead type. A BeadArray Reader is used to analyse the fluorescence signal. This process can be divided into 8 simplified steps (Figure 47 & 48) (GoldenGate assay workflow 2007):

1. The DNA sample is activated for binding to paramagnetic particles. This requires a minimum amount of DNA (250ng at 50ng/µL).
2. The activated DNA is combined with the assay oligonucleotides, hybridisation buffer, and paramagnetic particles. (hybridisation).
3. After hybridisation, several washes are performed to remove excess and mis-hybridised oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO.
4. The joined, full length products provide the template for PCR using universal primers P1, P2 and P3.
5. Universal primers P1 and P2 are Cy3- and Cy5- labelled.

6. The single stranded, dye labelled DNA are hybridised to their complement bead type via their unique address sequences.

7. Wash and dry the array matrix, the bead array reader is used to analyse fluorescent signal on the array.

8. This is then interpreted by a computer for automated genotyping clustering and calling.

The optimised reaction was then run on all samples for the 34 SNPs involved.

**Figure 47:** Schematic representation of the GoldenGate assay (Reproduced from GoldenGate assay workflow 2007)
Figure 48: GoldenGate assay genotyping protocol (Reproduced from GoldenGate assay workflow 2007)

![Figure 2: Protocol Time Requirements](image)

<table>
<thead>
<tr>
<th>Protocol Step</th>
<th>One Plate</th>
<th>Two Plates</th>
<th>Three Plates</th>
<th>Four Plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hands</td>
<td>Total</td>
<td>Hands</td>
<td>Total</td>
</tr>
<tr>
<td>Make activated DNA</td>
<td>2:00</td>
<td>2:00</td>
<td>2:00</td>
<td>2:00</td>
</tr>
<tr>
<td>Add DNA and hybridize to oligonucleotides</td>
<td>0:30</td>
<td>3:00</td>
<td>0:30</td>
<td>3:00</td>
</tr>
<tr>
<td>Extend, ligate, and clean up</td>
<td>1:30</td>
<td>2:00</td>
<td>2:00</td>
<td>2:00</td>
</tr>
<tr>
<td>PCR cycle</td>
<td></td>
<td>3:00</td>
<td></td>
<td>3:00</td>
</tr>
<tr>
<td><strong>Day 1 Totals</strong></td>
<td>4:00</td>
<td>10:00</td>
<td>5:00</td>
<td>11:00</td>
</tr>
<tr>
<td>Bind PCR product, aluA dye-labelled strand, prepare for hybridization</td>
<td>1:30</td>
<td>2:00</td>
<td>2:15</td>
<td>3:15</td>
</tr>
</tbody>
</table>
| Hybridize to Array Matrix
| **Day 2 Totals**                                  | 2:00      | 3:00       | 2:45         | 3:45        | 3:30         | 4:30        | 4:30         | 5:30        |
| Wash and dry Array Matrix                          | 0:10      | 0:20       | 0:10         | 0:30        | 0:15         | 0:45        | 0:15         | 0:45        |
| Image Array Matrix                                 |            |            | 0:20         | 4:00        | 0:50         | 5:00        |              | 5:00        |
| **Day 3 Totals**                                  | 0:10      | 2:30       | 0:10         | 4:30        | 0:15         | 6:45        | 0:15         | 8:45        |
| **Total genotypes (1536-plex)**                   | 147,456   | 204,912    | 442,388      | 589,824     |

1 Requires two technicians
2 Times listed are for processing on the Sentrix Array Matrix; analysis can also be carried out on the Sentrix BeadChip format.
**Statistical analysis**

Statistical analysis was required prior to commencement of the study to determine the optimum cohort sizes required to show an effect. The following table gives genotypic sample size calculations for each of the polymorphisms at three significance levels and two power levels (Table 7). Sample sizes shown are for the number of individuals in each of the case and the control groups. These were calculated using the genotype frequencies determined in previous relevant studies outlined above to calculate relative risk values for each genotype which were, in turn, used to determine sample sizes (Purcell S 2003).

**Table 7**: Estimated sample sizes at 2 power levels and 3 significance levels for 4 of the considered polymorphisms.

<table>
<thead>
<tr>
<th>Power</th>
<th>Alpha</th>
<th>80%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>PLA2G7</td>
<td>138</td>
<td>205</td>
<td>300</td>
</tr>
<tr>
<td>ESR2</td>
<td>438</td>
<td>648</td>
<td>948</td>
</tr>
<tr>
<td>MMP9</td>
<td>44</td>
<td>65</td>
<td>95</td>
</tr>
<tr>
<td>HMOX1</td>
<td>103</td>
<td>153</td>
<td>224</td>
</tr>
</tbody>
</table>

We proposed to use the sample size figures for 90% power, alpha=0.001 with a 10% increase for genotyping errors. The largest sample required would be for the ESR2 polymorphism at 1276 cases and 1276 controls. For the
allele/AAA association the number is for individuals rather than total alleles as shown in the sample size profile below (Table 8).

**Table 8:** Number of patients needed to study per polymorphism at 90% power and alpha at 0.001

<table>
<thead>
<tr>
<th>Candidate Gene</th>
<th>Sample size for allele/AAA association</th>
<th>Sample size for genotype/AAA association</th>
<th>Maximum sample size required</th>
<th>+10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2G7</td>
<td>174</td>
<td>367</td>
<td>367</td>
<td>404</td>
</tr>
<tr>
<td>ESR2</td>
<td>389</td>
<td>1160</td>
<td>1160</td>
<td>1276</td>
</tr>
<tr>
<td>TIMP-1 (nt+434)</td>
<td>276</td>
<td>276</td>
<td>276</td>
<td>304</td>
</tr>
<tr>
<td>(rs2070584)</td>
<td>344</td>
<td>344</td>
<td>344</td>
<td>378</td>
</tr>
<tr>
<td>MMP9</td>
<td>404</td>
<td>117</td>
<td>404</td>
<td>444</td>
</tr>
<tr>
<td>HMOX1</td>
<td>54</td>
<td>274</td>
<td>274</td>
<td>301</td>
</tr>
</tbody>
</table>

All SNPs were assessed for deviation from Hardy-Weinberg equilibrium. Odds ratio (OR), 95% confidence intervals (95% CI), and P values were calculated using a Cochran Armitage trend test. Binary logistic regression analyses and other statistical analyses were conducted using SPSS (version 14). Logistic regression analysis was only conducted for positively associated SNPs that had previously been examined, either in this study or others preceding this. This therefore included any positively associated SNP from the first part of the study (individual candidate gene assays), or any replicated SNP in the second half of the study (GoldenGate assays). For all statistical tests, the significant level was set at 0.05.
Results
Chapter 7. Results

Six genetic polymorphisms across five genes were primarily examined in this study. These were chosen as previous studies have shown them to have a positive association with AAA. Whilst *MMP9* has many polymorphic sites, a cytosine (‘C’) to thymidine (‘T’) transition at -1562 within the promoter region has been shown to increase promoter activity by 1.5-fold and lead to aneurysmal disease (Jones GT 2003). Two polymorphisms of the tissue inhibitor of metalloproteinase 1 (*TIMP1*) have been positively associated with AAA. The first is again a ‘C’ to ‘T’ transformation at +434 and the second is a guanine ‘G’ to thymidine ‘T’ switch (rs2070584) (Ogata 2005); (Wang 1999). A ‘G’ to ‘T’ mutation in the Platelet activating factor acetylhydrolase (*PLA2G7*) gene at position 994 in exon 9 has also shown to be associated with AAA, albeit only in a Japanese population (Unno N 2002). The Heme oxygenase 1 (*HMOX1*) gene does not have a point mutation but instead has a variable number of dinucleotide (‘GT’) repeats in its promoter sequence. Patients with AAA had longer ‘GT’ repeats (25 or more) when compared to patients with atherosclerosis and/or healthy controls (Schillinger 2002). A +1730 adenine (‘A’) to guanine (‘G’) *Alu*I restriction fragment length polymorphism of the Estrogen receptor β (*ESR2*) 3’ untranslated region in exon 8 has also been linked to AAA (Massart 2004).

The above polymorphisms were studied with respect to their association with AAA using the various techniques mentioned above.
Patient demographics

A total of 2,261 patients were recruited into the study, of which 1,202 patients had radiological evidence of an AAA (cases) and 1,059 had a normal sized aorta (controls). Ninety percent of cases were men (n=1,077), with this figure rising to 98% (n=1,035) of controls. Patients in the AAA group were slightly older (median age 72), compared to the control group (median age 66). Other comorbidities are listed in Table 9.

Table 9: Patient demographics. (Percentages given in parentheses, unless stated)

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=1202)</th>
<th>Controls (n=1059)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>1077 (90)</td>
<td>1035 (98)</td>
</tr>
<tr>
<td>Median age</td>
<td>72 (range 47-96)</td>
<td>66 (range 51-98)</td>
</tr>
<tr>
<td>Smoking-never</td>
<td>112 (9)</td>
<td>328 (31)</td>
</tr>
<tr>
<td>Smoking-current</td>
<td>300 (25)</td>
<td>160 (15)</td>
</tr>
<tr>
<td>Smoking-ex</td>
<td>790 (66)</td>
<td>571 (54)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>760 (63)</td>
<td>424 (40)</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>310 (26)</td>
<td>105 (14)</td>
</tr>
<tr>
<td>Diabetes Mellitus type I</td>
<td>45 (4)</td>
<td>40 (4)</td>
</tr>
<tr>
<td>Diabetes Mellitus type II</td>
<td>82 (7)</td>
<td>79 (7)</td>
</tr>
</tbody>
</table>
**MMP9 (rs3918242)**

A total of 1,163 cases and 1,043 control patients (total 2,206) were successfully genotyped (success rate 97.5%). There was no evidence of deviation from Hardy-Weinberg equilibrium in either the cases or control group (AAA, P=0.08; controls, P=0.612; Pearson Chi Squared). Genotype and allele frequencies are shown in Tables 10 & 11. The overall distribution of the genotypes was not significantly different between the cases and controls (Odds Ratio for ‘T’ allele was 0.99; P=0.82).

**Table 10:** Case and control genotype frequencies for the MMP9 polymorphism using the Cochran Armitage trend test

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=1163)</th>
<th>Controls (n=1043)</th>
<th>Common odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>CC</td>
<td>865</td>
<td>74</td>
<td>788</td>
<td>76</td>
</tr>
<tr>
<td>CT</td>
<td>284</td>
<td>24</td>
<td>235</td>
<td>23</td>
</tr>
<tr>
<td>TT</td>
<td>14</td>
<td>2</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>1163</td>
<td></td>
<td>1043</td>
<td></td>
</tr>
</tbody>
</table>

**Table 11:** Comparison of allele frequencies between cases and controls for the MMP9 polymorphism

<table>
<thead>
<tr>
<th>Allele</th>
<th>AAA (n=1163)</th>
<th>Controls (n=1043)</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2014</td>
<td>87</td>
<td>0.05</td>
<td>0.82</td>
</tr>
<tr>
<td>T</td>
<td>312</td>
<td>13</td>
<td>0.05</td>
<td>0.82</td>
</tr>
</tbody>
</table>
These results contradict those from a study by Jones et al, but agree with a study by Ogata et al, both of which were smaller in size. The discrepancy in results was assessed by Ogata and explained by differences in ethnic variation.
**TIMP1 (nt+434)**

A total of 2,213 patients (1,171 AAA and 1,042 controls) were successfully genotyped for the TIMP1 nt+434 ‘C’ to ‘T’ polymorphism (success rate 97.9%). Both the AAA and controls showed significant deviation from HWE (AAA & controls, P<0.05, Pearson’s Chi squared). As the TIMP1 gene is located on the X chromosome, to allow correct analysis, all female subjects were excluded from the analysis. Male patients therefore only had one allele for analysis. The genotype frequencies (Table 12) are given below:

**Table 12: Genotyping frequencies for the TIMP1 (nt+434) polymorphism using Cochran Armitage trend test**

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=1171)</th>
<th>Controls (n=1042)</th>
<th>Common odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>C</td>
<td>607</td>
<td>52</td>
<td>523</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>564</td>
<td>48</td>
<td>519</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>1171</td>
<td></td>
<td>1042</td>
<td></td>
</tr>
</tbody>
</table>

There was no association found between this SNP and AAA. The TIMP1 (nt+434) has previously been examined by Ogata et al and showed significant results only after subgroup analysis.
**PLA2G7 (rs16874954)**

A total of 2,197 patients were successfully genotyped of which 1,162 were cases and 1,035 were controls (success rate 97.2%). There was no deviation from HWE. Genotyping (Table 13) and allele (Table 14) frequencies are given below:

**Table 13:** Genotyping frequencies in the PLA2G7 gene polymorphism using a Cochran Armitage trend test

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=1162)</th>
<th>Controls (n=1034)</th>
<th>Common odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>GG</strong></td>
<td>1162</td>
<td>100</td>
<td>1034</td>
<td>100</td>
</tr>
<tr>
<td><strong>GT</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>TT</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1162</td>
<td>100</td>
<td>1035</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 14:** Allele frequencies in the PLA2G7 gene polymorphism

<table>
<thead>
<tr>
<th>Allele</th>
<th>AAA (n=1162)</th>
<th>Controls (n=1035)</th>
<th>$\chi^2$</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>G</strong></td>
<td>2324</td>
<td>100</td>
<td>2069</td>
<td>100</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Whilst published data regarding the PLA2G7 polymorphism shows a significant association between Japanese males and AAA, data from this study barely shows deviation from the wild type alleles in UK Caucasian patients. Only one patient from the whole cohort of 2,197 enrolled in the study showed heterogeneity for the polymorphism, representing a frequency of 0.05%. This correlates with findings from Stafforini et al who did not identify any genotypic variants from the wild type on assessment of 108 North Americans (Stafforini DM 1996).

Several online databases exist where common SNPs are examined and genotype frequencies in large studies are published. We searched the National Center for Biotechnology Information SNP database (NCBI dbSNP) for the PLA2G7 SNP and examined data from the HapMap project. This is an international project that collates data from Canada, China, Japan, Nigeria, the United Kingdom and the United States of America to develop a public resource that helps researchers find genes associated with human disease (http://hapmap.ncbi.nlm.nih.gov). Data from the HapMap project seems to mirror our results (Table 15). The allele frequencies quoted for the Caucasian European population are 0.008 for the ‘G’ allele and 0.992 for the ‘T’ allele. Data for the Japanese population is also given and shows an increase in the proportion of patients with the ‘G’ allele to 0.178 (‘T’ allele frequency 0.822).
**Table 15:** A comparison of the allele frequencies in the *PLA2G7* SNP between data from this study and that from the HapMap project.

<table>
<thead>
<tr>
<th></th>
<th>Frequency ‘G’ allele</th>
<th>Frequency ‘T’ allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>HapMap Caucasian European</td>
<td>0.992</td>
<td>0.008</td>
</tr>
<tr>
<td>HapMap Japanese</td>
<td>0.822</td>
<td>0.178</td>
</tr>
</tbody>
</table>
**ESR2 (rs4986938)**

A total of 1,170 AAA patients and 1,041 controls (total 2,211 patients) were genotyped for the *ERβ* +1730 A-G polymorphism (success rate 97.8%). The cases were in HWE (P=0.606, Pearson chi squared) but the controls were not (P<0.05, Pearson Chi squared). There was a significant difference of the genotypes between the cases and controls with a higher proportion of the ‘A’ allele seen in the AAA population (OR 1.33, 95% CI 1.18-1.50, P<0.05). The full results are shown in Tables 16 & 17.

**Table 16:** Genotype frequencies of the *ESR2* polymorphism using a Cochran Armitage trend test

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=1170)</th>
<th>Controls (n=1041)</th>
<th>Common odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>AA</strong></td>
<td>179</td>
<td>15</td>
<td>82</td>
<td>8</td>
</tr>
<tr>
<td><strong>AG</strong></td>
<td>568</td>
<td>49</td>
<td>523</td>
<td>50</td>
</tr>
<tr>
<td><strong>GG</strong></td>
<td>423</td>
<td>36</td>
<td>436</td>
<td>42</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1170</td>
<td>1041</td>
<td>1.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.18-1.50)</td>
<td></td>
</tr>
</tbody>
</table>
Table 17: Allele frequencies of the ESR2 gene polymorphism

<table>
<thead>
<tr>
<th>Allele</th>
<th>AAA (n=1170)</th>
<th>Controls (n=1041)</th>
<th>$\chi^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>A</td>
<td>926</td>
<td>40</td>
<td>687</td>
<td>33</td>
</tr>
<tr>
<td>G</td>
<td>1414</td>
<td>60</td>
<td>1395</td>
<td>67</td>
</tr>
</tbody>
</table>

The demographic details were entered into a logistic regression model to determine which of these were independently associated with AAA (Table 18). Factors that were independently associated with AAA, in order of declining odds ratio were male gender, previous MI, hypertension, smoking history and age. The presence of diabetes mellitus was found to have a reverse association with AAA (diabetes appears to be a negative risk factor). Both the ‘AG’ (OR 1.99, P<0.001) and ‘AA’ (OR 1.90, P<0.001) genotypes were entered individually into the model and compared to the ‘GG’ genotype, and were also found to be associated with AAA.
Table 18: Results from logistic regression analysis of the ESR2 gene

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR2 genotype (vs. GG)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>&lt;0.001</td>
<td>1.99</td>
<td>1.65 to 2.32</td>
</tr>
<tr>
<td>AA</td>
<td>&lt;0.001</td>
<td>1.90</td>
<td>1.57 to 2.22</td>
</tr>
<tr>
<td>Previous MI</td>
<td>&lt;0.001</td>
<td>2.65</td>
<td>2.38 To 2.91</td>
</tr>
<tr>
<td>Hypertension</td>
<td>&lt;0.001</td>
<td>1.97</td>
<td>1.77 to 2.16</td>
</tr>
<tr>
<td>Smoking history</td>
<td>&lt;0.001</td>
<td>1.50</td>
<td>1.37 To 1.61</td>
</tr>
<tr>
<td>Age (per year)</td>
<td>&lt;0.001</td>
<td>1.13</td>
<td>1.11 to 1.14</td>
</tr>
<tr>
<td>Male Gender</td>
<td>&lt;0.001</td>
<td>3.41</td>
<td>2.91 to 3.91</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>0.01</td>
<td>0.79</td>
<td>0.61 to 0.97</td>
</tr>
</tbody>
</table>

Model statistics: $\beta = 7.54, SE \beta = 0.67, P < 0.001$

This SNP was originally investigated by Massart et al (Massart F 2004) who hypothesised that as fewer AAA are seen in women, female sex hormones may offer a protective effect. The study assessed both estrogen and progesterone hormones and found an association between the ‘GG’ genotype and AAA. This is the complete opposite of the results determined by this study. We found the ‘AA’ genotype to be positively associated with AAA. Whilst the functional significance of the polymorphism is yet to be determined, it is known that there is no change in amino acid sequence in the ESR2 protein. Despite this, it is possible that this polymorphism is in linkage disequilibrium with other regulatory sequence variations that may affect gene expression or function (Yaich L 1992).
This is the largest in a small group of studies that examines the effect of this polymorphism and its association with AAA. Despite having contrasting results to the above-mentioned study by Massart et al (Massart F 2004), positive results in a large cohort are encouraging. However, we did observe deviation from the HWE that casts some element of doubt on the strength of the results. The Hardy-Weinberg principle states that both allele and genotype frequencies in a population remain constant, or in equilibrium across generations unless specific disturbing influences are introduced. These include non-random mating (consanguinity), genetic drift (whereby a population is isolated with a fixed number of mating possibilities), migration, or new mutations (Attia J 2009 (i)). This has been discussed in more detail in Chapter 4. It is unlikely that any of the above reasons are the cause for deviation in our dataset. Both cohorts were selected from a random sample of a Western population, where the above factors are minimal. The other reasons for failure to maintain the equilibrium is an error in genotyping (which is the most likely explanation in our dataset), or a chance finding. Whilst the cases do not have to observe the HWE as they are usually selected, the controls must be in equilibrium as they represent the ‘general population’ (Attia J 2009 (ii)).

Hosking et al examined 107,000 genotypes generated by Taqman, RFLP, sequencing and mass spectrometry from 443 SNPs and across 2,750 patients (Hosking L 2004). SNPs with MAF >5% showed an 11.5% deviation from HWE (36/313 SNPs). The causes for this were explored and reported as genotyping errors in 21 SNPs (58%), non-specific assays in 5 SNPs (14%)
and interestingly, no cause was found in 10 SNPs (28%). The authors recognised that failure to meet HWE could occur as a chance finding, but reiterated the fact that in a randomly mating large population, genotyping error is the most likely cause.

To try and confirm a genotyping error, we looked to examine genotyping data for this SNP in other large published studies. We examined data from the European Caucasian HapMap population with reference to the ESR2 +1730 A-G (rs4986938) SNP. The quoted genotype and allele frequencies are shown in Table 19. Whilst the AAA cohort from this study loosely resembles the HapMap data, the control cohort was grossly different especially in the ‘AA’ group, which could represent a potential source of genotyping error.

**Table 19:** Genotype frequencies from this study compared to the HapMap project for the ESR2 (rs4986938) polymorphism

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>‘A’ allele</th>
<th>‘G’ allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study (cases)</td>
<td>0.15</td>
<td>0.48</td>
<td>0.36</td>
<td>0.62</td>
<td>0.38</td>
</tr>
<tr>
<td>This study (controls)</td>
<td>0.08</td>
<td>0.50</td>
<td>0.41</td>
<td>0.66</td>
<td>0.33</td>
</tr>
<tr>
<td>HapMap</td>
<td>0.14</td>
<td>0.48</td>
<td>0.38</td>
<td>0.60</td>
<td>0.40</td>
</tr>
</tbody>
</table>
**HMOX1**

The analysis of this polymorphism differed from previous results because the *HMOX1* polymorphism was not based on a single point mutation but rather changes in ‘GT’ dinucleotide repeat segments. As is documented widely in published data, the polymorphism can be divided by the length of the repeat segment into the ‘short (S)’ allele; consisting of less than 25 repeats, or ‘long (L)’ allele; which is 25 repeats or more (Yamada N 2000); (Exner M 2001); (Schillinger M 2002). These have found that the presence of the ‘S’ allele exhibited a reduced inflammatory response after vascular injury and therefore the heterozygous class ‘S’ were grouped together with homozygous class ‘S’ and compared to homozygous class ‘L’. For the purposes of this thesis we maintained this classification but whereas this is clinically useful, it makes assessment of validity through Hardy-Weinberg analysis difficult as only 2 genotypic groups were recorded.

A total of 2,191 patients were successfully genotyped, of which 1,163 were AAA patients and 1,028 were controls (success rate 96.9%). Genotyping results are shown in Table 20.
The results show a statistically significant association for the short repeat in patients with AAA when compared to our control population. The results were entered into logistic regression analysis as shown in Table 21. The association of the short allele with AAA was maintained after regression analysis (OR 2.13, P<0.001). Factors that were independently associated with AAA, in order of declining odds ratio were male gender, smoking history, previous MI, hypertension, and age. The presence of diabetes mellitus was not found to be independently associated with AAA.
Table 21: Logistic regression analysis of the HMOX1 gene

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMOX1 genotype short</td>
<td>&lt;0.001</td>
<td>2.13</td>
<td>1.73 to 2.61</td>
</tr>
<tr>
<td>Male gender</td>
<td>&lt;0.001</td>
<td>4.69</td>
<td>2.93 to 7.52</td>
</tr>
<tr>
<td>Smoking history</td>
<td>&lt;0.001</td>
<td>3.61</td>
<td>2.81 to 4.63</td>
</tr>
<tr>
<td>Previous MI</td>
<td>&lt;0.001</td>
<td>2.73</td>
<td>2.11 to 3.54</td>
</tr>
<tr>
<td>Hypertension</td>
<td>&lt;0.001</td>
<td>2.22</td>
<td>1.84 to 2.69</td>
</tr>
<tr>
<td>Age (per year)</td>
<td>&lt;0.001</td>
<td>1.16</td>
<td>1.14 to 1.18</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>0.39</td>
<td>0.75</td>
<td>0.74 to 2.37</td>
</tr>
</tbody>
</table>

Model statistics: $\beta = 9.46$, $SE \beta = 0.67$, $P <0.001$

These results also contradict other published data. This is the largest and only the second published study to examine the effect of the HMOX1 (GT)$_n$ repeat polymorphism on human AAA. The previous study was conducted by Schillinger et al who genotyped 70 patients with AAA and 61 controls for the polymorphism and found that patients with AAA were more likely to have the long repeat. Short repeats are associated with increased upregulation of HMOX1 and consequently a potent anti-inflammatory effect. It has been proposed by Schillinger that this would be protective in the development of an AAA. However, this study has shown the opposite, and that increased upregulation of HMOX1 seems to be associated with AAA disease. The actual mechanism of action remains unknown.
A summary of all the genotyping results is shown in Table 22.

**Table 22: Genotyping frequencies of all 5 polymorphisms studied**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total samples</th>
<th>Common Odds Ratio</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HMOX1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>433 730</td>
<td>1163</td>
<td>1.99</td>
<td>52.42</td>
</tr>
<tr>
<td>Control</td>
<td>236 792</td>
<td>1028</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ESR2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>AA AG GG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>423 568 179</td>
<td>1170</td>
<td>1.42</td>
<td>20.56</td>
</tr>
<tr>
<td>Control</td>
<td>436 523 82</td>
<td>1041</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MMP9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>CC CT TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>865 284 14</td>
<td>1163</td>
<td>0.99</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>788 235 20</td>
<td>1043</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PLA2G7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>GG GT TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>1162 0 0</td>
<td>1162</td>
<td>0.76</td>
<td>0.003</td>
</tr>
<tr>
<td>Control</td>
<td>1034 1 0</td>
<td>1035</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TIMP1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>C T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAA</td>
<td>607 564</td>
<td>1171</td>
<td>0.94</td>
<td>0.53</td>
</tr>
<tr>
<td>Control</td>
<td>523 519</td>
<td>1042</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**GoldenGate genotyping**

A total of 932 male patients were used in the study, of which exactly half were patients with aneurysms (cases) and half were those with scanned normal aortas (controls). The most recent samples that met the quality control criteria were extracted from the databank and used as part of this study. The AAA group had a median age of 73 years (range 47 years to 95 years) and a median aortic diameter of 6cm (range 3cm to 9.5cm). Demographics of both groups of patients are listed in the Table 23. In general, patients in the AAA group were older and had statistically significant higher rates of co-morbidities. Interestingly, there were almost identical rates of diabetes mellitus among both groups in this selected sample.

**Table 23:** Patient demographics for the GoldenGate study (percentages in parentheses, unless stated).

<table>
<thead>
<tr>
<th></th>
<th>AAA (n=466)</th>
<th>Controls (n=466)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age</td>
<td>73 [range 47-95]</td>
<td>66 [range 51-98]</td>
</tr>
<tr>
<td>Current or ex-smoker</td>
<td>433 (93)</td>
<td>333 (71)</td>
</tr>
<tr>
<td>Previous MI</td>
<td>137 (29)</td>
<td>53 (11)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>286 (61)</td>
<td>190 (41)</td>
</tr>
<tr>
<td>COPD</td>
<td>56 (12)</td>
<td>35 (8)</td>
</tr>
<tr>
<td>Type 2 Diabetes</td>
<td>35 (8)</td>
<td>36 (8)</td>
</tr>
<tr>
<td>Family history of AAA</td>
<td>49 (11)</td>
<td>26 (6)</td>
</tr>
</tbody>
</table>
A total of 96 SNPs were assessed on the assay, including the 34 tag SNPs identified for the 5 genes already genotyped. Thirty SNP’s out of the total 96 failed genotyping quality control which was identified by a call rate of less than 80 percent threshold. Unfortunately this included the TIMP1 rs2070584 SNP, meaning that this SNP was not analysed as part of the study. The cluster plots of the remaining 66 SNPs were further checked and re-analysed. This left a total of 66 SNPs that were included in further analysis (Appendix 11).

Seven SNP’s (rs1415148; rs4576; rs12568757; rs217120; rs9999631; rs8019787, rs10888395) out of the 66 that were analysed reached statistical significance (Table 24 & 25). None of these were within the 5 genes in the primary study. Five of the SNPs are located in the Cathepsin genes, whereas the remaining 2 are located in the Actinin and Aryl Hydrocarbon Receptor Nuclear Translocator gene. All SNPs except rs217120 (cases & controls P<0.05) and cases in the rs1415148 (P=0.04, Pearson chi squared) were in HWE. Logistic regression analysis was not performed as the positively associated SNPs were all discovery SNPs, and had not been previously identified.

The rs217120 SNP within the Cathepsin C gene is the SNP that shows the most significance with a common odds ratio of 1.55 (P=0.005) for the ‘AA’ genotype amongst the AAA population. There was a statistical significance of the ‘A’ allele with the AAA cohort compared to the controls (OR 1.33, P=0.01). Two polymorphisms in the Cathepsin S gene also showed a significant association with odds ratios of 1.3 (P=0.03) for the ‘GG’ genotype.
Chapter 7. Results

(rs1415148) and 1.24 (P=0.04) for the ‘AA’ genotype (rs12568757) respectively. The other Cathepsin genes to show a positive association were the Cathepsin O (rs9999631; Odds ratio 1.23, P=0.03 for the ‘GG’ genotype) and the Cathepsin G (rs8019787; Odds ratio 1.23, P=0.04 for the ‘CC’ genotype. The remaining 2 genes that showed positive results were the actinin gene (rs4576) polymorphism (Odds ratio 1.29, P=0.03 for the ‘AA’ genotype), and the ARNT gene (rs10888395) polymorphism with an odds ratio of 1.25 (P=0.05 for the ‘GG’ genotype).
### Table 24: Genotyping results of the positive SNPs seen from GoldenGate assay

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>AAA (n=466)</th>
<th>Controls (n=466)</th>
<th>OR (95%CI)</th>
<th>P value</th>
<th>Gene</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1415148</td>
<td>AA</td>
<td>43</td>
<td>68</td>
<td>1.30</td>
<td>0.03</td>
<td>CTSS</td>
<td>Cathepsin S</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>190</td>
<td>196</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>130</td>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4576</td>
<td>AA</td>
<td>267</td>
<td>251</td>
<td>1.29</td>
<td>0.03</td>
<td>ACTN3</td>
<td>Actinin</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>128</td>
<td>156</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>18</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12568757</td>
<td>AA</td>
<td>129</td>
<td>127</td>
<td>1.24</td>
<td>0.04</td>
<td>CTSS</td>
<td>Cathepsin S</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>211</td>
<td>215</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>69</td>
<td>106</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs217120</td>
<td>AA</td>
<td>222</td>
<td>197</td>
<td>1.55</td>
<td>0.005</td>
<td>CTSC</td>
<td>Cathepsin C</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>170</td>
<td>192</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>7</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10888395</td>
<td>AA</td>
<td>41</td>
<td>66</td>
<td>1.25</td>
<td>0.05</td>
<td>ARNT</td>
<td>Aryl Hydrocarbon Receptor Nuclear Translocator</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>190</td>
<td>204</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>181</td>
<td>177</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9999631</td>
<td>CC</td>
<td>192</td>
<td>235</td>
<td>1.23</td>
<td>0.03</td>
<td>CTSO</td>
<td>Cathepsin O</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>189</td>
<td>172</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>40</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs8019787</td>
<td>CC</td>
<td>106</td>
<td>80</td>
<td>1.23</td>
<td>0.04</td>
<td>CTSG</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>211</td>
<td>237</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>110</td>
<td>127</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 25: Allele frequencies for the positively associated SNPs from GoldenGate genotyping

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>AAA (n=466)</th>
<th>Controls (n=466)</th>
<th>$\chi^2$ (1d.f.)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1415148</td>
<td>A</td>
<td>276 38</td>
<td>332 43</td>
<td>4.38</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>450 62</td>
<td>434 57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4576</td>
<td>A</td>
<td>662 80</td>
<td>658 76</td>
<td>5.00</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>164 20</td>
<td>212 24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12568757</td>
<td>A</td>
<td>469 57</td>
<td>469 52</td>
<td>4.30</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>349 43</td>
<td>427 48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs217120</td>
<td>A</td>
<td>614 77</td>
<td>586 71</td>
<td>6.30</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>184 23</td>
<td>234 29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10888395</td>
<td>A</td>
<td>272 33</td>
<td>336 38</td>
<td>3.92</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>552 67</td>
<td>558 62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9999631</td>
<td>C</td>
<td>573 68</td>
<td>642 73</td>
<td>4.30</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>269 32</td>
<td>242 27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs8019787</td>
<td>C</td>
<td>423 50</td>
<td>397 45</td>
<td>4.07</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>431 50</td>
<td>491 55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Summary

A total of six genetic polymorphisms were examined in five genes in a population of 2,261 patients (1,202 cases and 1,059 controls). Two polymorphisms, \textit{ESR} +1730 A-G and \textit{HMOX1} (GT)$_n$, showed an association with AAA, whereas three polymorphisms (\textit{MMP9}, \textit{TIMP1}, and \textit{PLA2G7}), did not. Unfortunately one polymorphism (rs2070584) could not be genotyped.

A different method of genotyping rs2070584 SNP was attempted with the GoldenGate assay, but again, this failed. Seven SNP’s (rs1415148; rs4576; rs12568757; rs217120; rs9999631; rs8019787, rs10888395) did however reach statistical significance in the GoldenGate assay, and show a potential association with AAA. Five of these SNPs are located in the Cathepsin genes.
Discussion
Chapter 8. Discussion

According to recent data, mortality rates from ruptured AAA in England and Wales represent a significant cause of death in men each year (AAAQIP 2011). Elective repair of AAA is beneficial as it is associated with lower rates of mortality and improved quality of life. However, as AAA are commonly asymptomatic, detection is mainly a chance finding. Several strategies are under investigation to help with identification and management of AAA. Screening has now been widely adopted in the UK and will attempt to identify smaller aneurysms in younger patients to allow surveillance or operative treatment in healthier patients. Research has focused on identifying factors influencing growth rates, pathogenic causes and mechanisms behind AAA. This enhanced understanding may lead to easier detection and potentially the identification of pharmacological sites of therapy.

Another focus of research has been genetics as AAA is thought to represent a complex disease comprising of genetic and environmental factors (Majumder PP 1991); (Verloes A 1995); (van Vlijmen-van Keulen CJ 2002); (Kuivaniemi H 2003). Research into susceptibility genes has mainly been conducted using candidate gene approaches, with many of the implicated variants having the potential to cause disruption to the aortic wall and therefore lead to aneurysm formation. Whilst this has provided us with a vast amount of data on numerous genes, much of the research has been conducted on small cohorts and results have been confusing and contradictory. Identifying a
gene or group of genes would allow us to better understand the pathogenetics behind AAA and identify possible pathways for investigation.

**Individual genotyping and AAA**

Six genetic polymorphisms across five genes were examined for a relationship with AAA. One polymorphism (rs2070584) could not be genotyped, despite using 2 different methods. Two polymorphisms showed a potential association with AAA, the ESR2 +1730 A-G and the HMOX1 (GT)$_n$ repeat. The 3 other polymorphisms that were examined (MMP9, TIMP1 and PLA2G7) did not show any association with AAA.

**GoldenGate genotyping and AAA**

Seven SNPs (rs1415148; rs4576; rs12568757; rs217120; rs9999631; rs8019787, rs10888395) showed a potential association with AAA. Five of these SNPs are located within the cathepsin gene, whereas the remaining 2 are novel associations identified with AAA (actinin and ARNT genes). The strongest association was seen with the rs217120 SNP with the cathepsin C gene (P=0.005).

Whilst the initial genotyping identified 2 potential associations with AAA, these were not replicated in the GoldenGate assay. This is a common finding throughout the literature in association genetics, and unfortunately the cause
is merely speculative. In our dataset, HWE was not met for the ESR2 polymorphism and could not be assessed for the HMOX1 repeat. Again this could be due to a multitude of reasons, with the simplest being genotyping error. The GoldenGate assay therefore could be argued as a more accurate assessment tool, as it uses current high throughput technology. However, we must remember from this study that approximately a third of SNPs failed genotyping quality control, which may have included the abovementioned SNPs, meaning that they were not analysed. This is a significant failure rate across such an established technique. The discrepancy in results across the 2 techniques could also be explained merely due to chance. Any technique used will have positive and negative points (as described in Chapter 4. Genetic association studies) and using different techniques is beneficial as it provides more accurate results. Realistically, the actual technique used depends on the availability of technologies and expertise in their use.

Although we were unable to positively replicate our results, the use of 2 different techniques has led to the identification of several novel SNPs and AAA, which requires a new line of investigation. The strengths of this study lie in the large correctly powered numbers genotyped for each SNP. We have maintained stringent recruitment criteria, with all patients (especially controls) having an aortic measurement and demographics correctly recorded. Genotyping has been performed with quality control throughout the whole process, from DNA processing to digestion reactions. Statistical analysis has been performed with the help of a genetic statistician. Much of the above was lacking in previous studies and may have led to incorrect results.
Limitations

A large study using quality controlled DNA samples and a mixture of established and modern high throughput genotyping with correctly powered cohort sizes was adopted to minimise error. However, human and computer error cannot be completely excluded. The main problems with case control studies have already been discussed in the methods chapter of this thesis, and usually lie in the control group. Both populations were recruited from the same areas (hospital related activities) and underwent the same radiological methods of identification of AAA. In our opinion, this allows for closer matching of cohorts, but some may argue that this infringes on selection bias. One possible solution for this is blinding of participants and investigators, but for the purposes for the study, this was impractical.

The results found in this study are completely different to those published in previous literature although similar methodology was used. An inability to replicate results has led to some questioning the value of genetic analysis. Colhoun et al assessed the potential causes for this lack of replication and suggested that the most important factors were publication bias, failure to attribute the results to chance and inadequate sample sizes, all of which are potentially reversible (Colhoun HM 2003). We have addressed these problems in our study design.
Results from thesis

In summary the main results from the thesis are as follows:

1. The ESR2 +1730 ‘AA’ genotype appears to be associated with AAA, although there is deviation from HWE.

2. The ‘short’ (<24 GT) repeat of the HMOX1 polymorphism is associated with AAA.

3. Polymorphisms of the MMP9, TIMP1, and PLA2G7 gene were not associated with AAA.

4. Seven SNPs showed an association with AAA after genotyping with the GoldenGate assay. Five of these are located on the Cathepsin gene.
**Future work**

We feel that this study forms a platform on which future studies could be planned. Whilst we have some positive results, it is clear that repeat studies of these genes would be required, preferably in a large sample before a true association could be inferred. The TIMP1 (rs2070584) polymorphism failed to genotype with the 2 methods that were used in this thesis, and it would be appropriate to attempt re-genotyping of this SNP, possibly using a Taqman assay.

At present, several centres are involved in a genome wide association study (GWAS), as the ‘gold standard’ assessment of the human genome that endeavours to reveal any significant associations. Results have only recently been published from two large studies. The first involved Dutch and Icelandic populations (1,292 AAA cases and 30,503 controls with a follow-up of top markers in 3,267 AAAs and 7,451 controls) and found the ‘A’ allele of rs7025486 on 9q33 within the DAB2IP (coding for an inhibitor of cell growth and survival) to be associated with AAA (OR 1.21 and P = 4.6 × 10⁻¹⁰) (Gretarsdottir S 2010). The second, conducted by Bown et al recruited 1,866 AAA patients and 5,435 controls from centres across the UK, Australia and New Zealand, with replication (2,871 cases and 32,687 controls), and a follow-up study (1,491 cases and 11,060 controls). This study found the rs1466535 SNP, located within the low-density-lipoprotein receptor-related protein 1 (LRP1) demonstrated significant association with AAA (P=0.0042).
This association was confirmed in the follow-up study (P=0.035) (Bown MJ 2011).

Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. There is evidence to suggest that environmental factors may promote complex diseases by stimulating a variety of epigenetic mechanisms (Goldberg AD 2007). Some authors believe that epigenetics may have a role in AAA development, although in a recent review, the evidence for this was limited (Krishna SM 2010). Nonetheless, this current development may provide a new direction of focus between environmental factors and the development of AAA.
**Conclusion**

The null hypothesis that the examined polymorphisms have no association with AAA is not true. Whilst we have shown an association between polymorphisms of the *ESR2* and *HMOX1* genes, further work needs to be conducted to verify this association.

As already mentioned in this thesis, the pathogenic mechanisms that lead to AAA are likely to have complex environmental and genetic components. It is unlikely that a single gene will ‘cause’ AAA, like in other diseases such as cystic fibrosis. Our negative associations are therefore as important as the positive results. We have not found any association in the *MMP9* gene polymorphism, although this is continually cited in the literature as a significant SNP in aneurysmal disease. If a new causative factor for AAA was identified, it would help in the understanding of the mechanisms behind aneurysm formation and possibly lead to early detection or pharmacological intervention that may prevent the large numbers of deaths caused by ruptured AAA. Serum or urine examination to assess and stratify for aneurysmal disease is therefore still very much a future target. As yet, genetic examination has not revealed a potential target that is both sensitive and specific to be used as a marker for AAA and much of the knowledge gained cannot be directly related to clinical practice. However, this represents an important area of continued research and may in the future unlock the mysteries behind aneurysmal disease.
Appendices
An investigation into candidate genes for abdominal aortic aneurysms

Patient information sheet version 4 (cases aneurysm non-operative) June 2006

For patients with aneurysms.

INFORMATION SHEET 4

Principal Investigator

Mr RD Sayers MD MRCS
Reader in surgery
University Hospitals of Leicester NHS Trust
Contact telephone number: 0116 2523135

You are invited to participate in the above study, which will improve our understanding of aneurysms and hopefully lead to improvements in their future treatment. We are comparing a group of patients with aneurysms to a group who do not have aneurysms. Your participation as someone who has an aneurysm would be greatly appreciated.
1. What is the purpose of the study?

Arteries are blood vessels that take blood away from the heart. The aorta is the main artery in the body. An aneurysm occurs when the wall of the artery becomes weak and it stretches. Although aneurysms usually affect the aorta, they can also occur in other blood vessels such as the leg arteries.

We do not know why some people develop aneurysms and others do not. We do know however, that aneurysms are more common in some families. This may suggest a genetic cause. We also know that different people produce different amounts of certain types of protein in the body that can result in an aneurysm.

Protein levels in the blood are controlled by genes (chemicals that contain genetic information). We know that some people have genes that cause high or low levels of circulating proteins. A recent study has suggested that patients who produce a low level of one protein may be more likely to develop aneurysms. We aim to do a similar study in more detail to clearly see whether there is a link between aneurysms, proteins and genes. We will compare a group of patients with aneurysms to a group without aneurysms.

You have been invited to participate in this study because you have been diagnosed by your doctor as having an aneurysm.
2. **What will be involved if I take part in the study?**

The study looks at the levels of naturally occurring proteins in the blood and the genes that control the amount of proteins produced. In order to do this we need to take a blood sample from a vein in your arm, you will probably have had blood tests before as part of medical care. We will only need 15-20ml of blood. From this we can analyse the protein levels and the genes. Your genes will be examined by extracting and analysing DNA (which contains genetic information) from your blood sample. As new potential genes that cause aneurysms are discovered all the time the sample taken from you may be used in future studies of these newer genes.

3. **Will the information obtained in the study be confidential?**

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study.

4. **What if I am harmed by the study?**

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS ie compensation is only available if negligence occurs.

5. **What happens if I do not wish to participate in this study or wish to withdraw from the study?**

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.
PATIENT CONSENT FORM

An investigation into candidate genes for abdominal aortic aneurysms

Principal Investigator:
Mr Robert D Sayers MD MRCS
Reader in Surgery
Clinical Sciences Building / Leicester Royal Infirmary

This form should be read in conjunction with the Patient Information Leaflet, version no 4 dated June 2006

I agree to take part in the above study as described in the Patient Information Sheet.

I understand that I may withdraw from the study at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

For drug studies if appropriate: At the termination of this trial I understand that there is no guarantee that the drug treatment received during this trial will continue.

Trust Indemnified studies:
I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs. I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with .......................................................... and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

Signature of patient .......................................................... ........ Date...........................................
(Name in BLOCK LETTERS) ....................................................................................................

I confirm I have explained the nature of the Trial, as detailed in the Patient Information Sheet, in terms which in my judgement are suited to the understanding of the patient.

Signature of Investigator ............................................. Date.................................
(Name in BLOCK LETTERS) .............................................................................................
Appendix 2. Information sheet and consent forms for controls recruited

An investigation into candidate genes for abdominal aortic aneurysms

Study information sheet version 5 (controls) June 2006

For people without aneurysms.

STUDY INFORMATION SHEET 5

Principal Investigator

Mr RD Sayers MD MRCS

Reader in surgery

University Hospitals of Leicester NHS Trust

Contact telephone number: 0116 2523135

You are invited to participate in the above study, which will improve our understanding of aneurysms, a condition that you do not have and hopefully lead to improvements in their future treatment. We are comparing a group of patients with aneurysms to a group who do not have aneurysms. Your participation would be greatly appreciated.
1. What is the purpose of the study?

Arteries are blood vessels that take blood away from the heart. The aorta is the main artery in the body. An aneurysm occurs when the wall of the artery becomes weak and it stretches. Although aneurysms usually affect the aorta, they can also occur in other blood vessels such as the leg arteries.

We do not know why some people develop aneurysms and others do not. We do know however, that aneurysms are more common in some families. This may suggest a genetic cause. We also know that certain types of chemical in the body called matrix metalloproteinases (MMPs) can weaken the walls of blood vessels and cause them to stretch, resulting in an aneurysm. It is likely that cytokines (naturally occurring chemicals in the blood) can make MMPs more or less active and may therefore alter the chance of an aneurysm forming.

Protein levels in the blood are controlled by genes (chemicals that contain genetic information). We know that some people have genes that cause high or low levels of circulating proteins. A recent study has suggested that patients who produce a low level of one protein may be more likely to develop aneurysms. We aim to do a similar study in more detail to clearly see whether there is a link between aneurysms, proteins and genes. We will compare a group of patients with aneurysms to a group without aneurysms.

You have been invited to participate in this study since your doctor has found that you do not have an aneurysm and we would like to compare people such as yourself with patients with aneurysms. Your help would be greatly appreciated.
2. **What will be involved if I take part in the study?**

The study looks at the levels of naturally occurring chemicals in the blood and the genes that control the amount of cytokines produced. In order to do this we need to take a blood sample from a vein in your arm, you will probably have had blood tests before as part of medical care. We will only need 15-20ml of blood. From this we can analyse the cytokine levels and the genes. Your genes will be examined by extracting and analysing DNA (which contains genetic information) from your blood sample. **As new potential genes that cause aneurysms are discovered all the time the sample taken from you may be used in future studies of these newer genes.**

3. **Will the information obtained in the study be confidential?**

All details recorded in the study will be in the strictest confidence. No details that could be used to identify you will be used in any records relating to the study.

4. **What if I am harmed by the study?**

Medical research is covered for mishaps in the same way as for patients undergoing treatment on the NHS ie compensation is only available if negligence occurs.

5. **What happens if I do not wish to participate in this study or wish to withdraw from the study?**

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future clinical encounters will not be affected.
CONSENT FORM

An investigation into candidate genes for abdominal aortic aneurysms

Principal Investigator:
Mr Robert D Sayers MD MRCS
Reader in Surgery
Clinical Sciences Building / Leicester Royal Infirmary

This form should be read in conjunction with the Study Information Leaflet, version no 5 dated June 2006

I agree to take part in the above study as described in the Patient Information Sheet.

I understand that I may withdraw from the study at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

For drug studies if appropriate: At the termination of this trial I understand that there is no guarantee that the drug treatment received during this trial will continue.

Trust Indemnified studies:
I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs. I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with ............................................................and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

Signature of patient .................................................. ........ Date..............................
(Name in BLOCK LETTERS) ....................................................................................................

I confirm I have explained the nature of the Trial, as detailed in the Patient Information Sheet, in terms which in my judgement are suited to the understanding of the patient.

Signature of Investigator .......................................... Date..............................
(Name in BLOCK LETTERS) ....................................................................................................
Appendix 3. Method for extraction of the buffy coat from whole blood

1. Centrifuge the samples for 10 minutes at 4°C at 2000rpm
2. Label a cryotube per patient with corresponding ID numbers
3. Transfer all samples to a Class II microbiological safety cabinet with appropriate waste disposal facilities via clinical waste route.
4. Remove the plasma from each sample using a sterile transfer pipette and dispose of in clinical waste bin.
5. Remove 500µL of buffy coat per tube using a sterile transfer pipette and store in a cryotube.
6. Repeat steps 5 & 6 for the remaining 2 EDTA collection tubes per patient collecting the buffy coat in the same correctly labelled cryotube
7. Snap freeze in liquid nitrogen
8. Store the buffy coats in a minus 80°C freezer.
Appendix 4. Detailed method of DNA extraction and storage

Cell lysis

1. Rapidly thaw frozen ‘buffy coats’ in a water bath at 37°C
2. Prepare 15ml Falcon tubes with corresponding ID numbers of the buffy coat samples to be thawed
3. Add 4.5ml of RBC Lysis solution to each tube
4. Add between 500µl to 1ml of buffy coat to the RBC Lysis solution
5. Incubate at room temperature for 10 minutes, occasionally inverting the tubes to mix
6. Centrifuge for 10 minutes at room temperature at 2000G
7. Decant the liquid into a clinical waste area
8. Vortex the remainder of the tube, and a large white cell pellet should be visible. If not, repeat steps 3-7
9. Add 4ml Cell Lysis solution to the white cell pellet
10. Using a pipette break up the pellet using direct force and by pipetting up and down to lyse the cells
11. Incubate at 37°C for the rest of the day (minimum 4-6 hours) and then leave at room temperature for as long as necessary for the solution to become homogenous (usually overnight).
**Protein precipitation**

1. Sample must be at room temperature
2. Add 1ml Protein Precipitation solution. This will sink to the bottom of the tube.
3. Vortex for 20 seconds
4. Centrifuge for 10 min at room temperature and 2000G, to produce a pellet

**DNA precipitation**

1. Prepare clean tubes containing 3ml isopropanol
2. Transfer supernatant containing DNA to isopropanol
3. Invert gently 50 times. The strands of DNA precipitating out will become visible
4. Centrifuge for 3 min at room temperature and 2000G, to produce a pellet
5. Pour off supernatant and drain by inverting the tubes
6. Add 3ml 70% ethanol (as a wash)
7. Invert gently several times
8. Centrifuge for 1 min at room temperature and 2000G, to produce a pellet
9. Pour off ethanol carefully ensuring that the pellet remains in the tube
10. Drain for 10-15 min by inverting the tube
**DNA hydration**

1. Add 250µL of DNA hydration solution to the tube if a pellet is visible. If no pellet can be seen, we assume the pellet is small with a small amount of DNA. Therefore only 50µL of DNA hydration solution is added, to avoid hugely reducing the DNA concentration.

2. Incubate at 65°C for 1 hour, tapping the tubes periodically.

3. Leach at room temperature overnight (12 hours).

**DNA Storage**

Transfer DNA to eppendorf tube and store at -20°C.
Appendix 5. Making and running an agarose gel

Requirements:

Agarose, buffer TBE x1, ethidium bromide (10mg/ml), gel forming trays and spacers, fume cupboard.

1. For 2% small gels, 1g agarose with 50ml buffer is used. This is basically doubled for larger gels. A small gel will only process a maximum of 8 samples.

2. The above mixture is collated in a beaker. The two constituents will not mix together and the agarose will simply collect at the bottom of the beaker. The solution is then heated using a microwave until the agarose dissolves, and the solution becomes clear.

3. The beaker with warm solution is then carefully transferred to a fume cupboard where 5µl of ethidium bromide is added.

4. The gel is then allowed to cool in the beaker to approximately 60°C. During this period of cooling, the block is prepared.

5. Commercially available masking tape is applied to both open sides of the block. An appropriate 'comb', with the correct number of wells (after accounting for markers and ladders) is then added to the block. Make sure that if at all avoidable, samples must be kept away from the edges as they may run in the meniscus of the gel and cause irregularities.

6. The block is then moved to the fume cupboard and the gel poured in the prepared block slowly.

7. Bubbles must be removed from the gel by either combing them out or by gently and superficially moving them to one side.

8. Allow the gel to set for 20 minutes or until firm.
9. Carefully remove the masking tape and the combs and place the hardened gel in an electrophoresis tank containing buffer so that the gel is fully immersed with buffer.

10. Before the PCR products can be effectively loaded into the wells, 2µL of blue dye is added to each sample.

11. 25µL of each PCR product is then loaded into a well on the gel using a pipette. This is then repeated for the remaining samples so that each well corresponds to a different sample. A ladder is conventionally loaded on the left hand side.

12. Once all the samples are loaded, a lid is placed on the gel tank and it is connected to a power supply so that there is a negative charge at the end containing the samples and a positive charge at the other end.

13. Set the power supply 150 volts and start.

14. The ladder will migrate the quickest and the power supply should be switched off when the ladder has reached the end of the gel.

15. The gel is then removed and viewed in a dark room under a UV light.
Appendix 6. Finalised genotyping methods for the *MMP9* polymorphism

**Primers**
Forward: 5'- GCC TGG CAC ATA GTA GGC CC -3'  (20 bases)
Reverse: 5'- CTT CCT AGC CAG CCG GCA TC -3'  (20 bases)

**Mastermix constituents**

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**PCR conditions**

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**Results**

Pre-digestion products expected at 435bp
Post digestion products expected at:

435bp (homozygous wild type)
188bp, 247bp (homozygous polymorphism)
188bp, 247bp, 435bp (heterozygous)
Appendices

Appendix 7. Finalised genotyping methods for the PLA2G7 polymorphism

Primers
A: 5’- CTA TAA ATT TAT ATC ATG CTT -3’ (21 bases)
B: 5’- TTT ACT ATT CTC TTG CTT TAC -3’ (21 bases)
C: 5’- TCA CTA AGA GTC TGA ATA AC -3’ (20 bases)  
D: 5’- TCA CTA AGA GTC TGA ATA AA -3’ (20 bases)  

Mastermix constituents
25µL reaction
Water
Buffer x10
MgCl₂ 3mM
dNTP 200µM
DNA 100ng
Forward Primer 10pM
Reverse Primer 10pM
Taq DNA polymerase 0.25µL

PCR conditions
Denaturation 94°C 5min
94°C 60 sec
1st Annealing 56°C 60 sec  
72°C 60 sec  
94°C 30 sec
2nd Annealing 53°C 30 sec  
72°C 30 sec
Extension 72°C 5 min

Results
Expected product size using primers A&B 160bp
Expected product size using primers A&C or A&D 108bp

Band using primers A&C – homozygous for wild type
Band using primers A&D – homozygous for polymorphism
Bands using both A&C and A&D – heterozygote
Appendices

Appendix 8. Finalised genotyping methods for the ESR2 polymorphism

**Primers**
Forward: 5’- CAA GTC CTC CAT CAC GGG GT -3’ (20 bases)
Reverse: 5’- AGA TGA AGC CCA GGC TCC TG -3’ (20 bases)

**Mastermix constituents**
25µL reaction
- Water
- Buffer 10x
- MgCl₂ 2.5mM
- Triton X-100 1%
- dNTP 200µM
- DNA 100ng
- Forward Primer 10pM
- Reverse Primer 10pM
- Taq DNA polymerase 0.1µL

**PCR conditions**
- Denaturation: 94°C 3min, 30 secs
- Annealing: 64°C 30 secs, 35 cycles
- Extension: 72°C 60 secs, 5 mins

**Digestion mix**
- Post PCR DNA 8µL
- Water 0.5µL
- AluI 0.5µL
- Buffer 1µL

**Results**
Pre-digestion products expected at 168bp
Post digestion products expected at:
- 168bp (homozygous wild type)
- 44bp, 124bp (homozygous polymorphism)
- 44bp, 124bp, 168bp (heterozygous)
Appendix 9. Finalised genotyping methods for the TIMP1 (nt+434) polymorphism

**Primers**

A Forward: 5’- TAA GCT CAG GCT GTT CCA GG -3’ (20 bases)

B Reverse: 5’- TGG GGA CAC CAG AAG TCA AC -3’ (20 bases)

C Forward: 5’- AGG CTG TTC CAG GGA GTC GC -3’ (20 bases)

D Reverse: 5’- CCG CCA TGG AGA GTG TCT GC -3’ (20 bases)

**Mastermix constituents**

30μL reaction

- Water
- Buffer 10x
- MgCl₂ 1.5mM
- dNTP 200μM
- DNA 100ng
- Forward Primer 10pM
- Reverse Primer 10pM
- Taq DNA polymerase 0.3μL

**PCR conditions**

- Denaturation: 94°C 1 min
- 94°C 30 secs
- Annealing: 64°C 30 secs
- 72°C 30 secs 20 cycles
- Extension: 72°C 3 mins

**Digestion mix**

- NruI 0.5μL
- BSA 0.5μL
- Buffer 1μL
- PCR product 8μL

**Results**

- Bands expected at 653bp using primers A&B
- Bands expected at 339bp using primers C&D

Post digestion products expected at:

- 339bp (homozygous wild type)
- 19bp, 320bp (homozygous polymorphism)
- 19bp, 320bp, 339bp (heterozygous)
Appendix 10. Finalised genotyping methods for the *HMOX1* polymorphism

**Primers**
Forward: 5’- AGA GCC TGC AGC TTC TCA GA -3’ (20 bases)
Reverse: 5’- ACA AAG TCT GGC CAT AGG AC -3’ (20 bases)

**Mastermix constituents**

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**PCR conditions**

- **Denaturation**
  - 95°C: 3min
  - 93°C: 40 secs
- **Annealing**
  - 50°C: 40 secs
  - 70°C: 30 secs
- **Extension**
  - 72°C: 5 mins

- **40 cycles**

**Results**
Short repeat (<24GT) bands up to 114bp
Long repeat (>24bp) bands larger than 114bp
**Appendix 11.** The 66 successfully genotyped SNPs with their chromosomal location & associated gene

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