Thesis submitted for the degree of Doctor of Med

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by

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Statement of originality

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

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Abbreviations

-	subtraction
+	addition
x	multiplication
=	equals
A	Adenine
AAA	Abdominal aortic aneurysm(s)
ACE	angiotensin converting enzyme
Ang	Angiotensin
ApoE	Apolipoprotein-E
bp	base pairs
C	Cytosine
CHD	Coronary Heart Disease
CRP	C-reactive protein
CT	Computed Tomography Scan
DES	desmosine
df	degrees of freedom
DNA	Deoxyribose nucleic acid
dNTP	deoxy-Nucleic acid Tri-phosphate
DPD	deoxypyridinoline
ECG	ElectroCardioGram
ECM	ExtraCellular Matrix
EDP	Elastin-Degradation Products
EDTA	Ethylene Di-amine Tetra-acetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay

EVAR	EndoVascular Aneurysm Repair
g	gram(s)
G	Guanine
HDL-C	High Density Lipoprotein Cholesterol
HG	Heteroduplex Generator
HO-1	Heme Oxidase-1
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IHG	Induced Heteroduplex Genotyping
IL	Interleukin
isoDes	isodesmosine
mg	milligram(s)
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
MIF	macrophage migration inhibitory factor
ml	millilitre(s)
MMP	Matrix MetalloProteinase
ng	nanogram(s)
NOS	Nitric Oxide Synthase
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
PAI	Plasminogen Activator Inhibitors
PDGF	platelet derived growth factor
PCR	Polymerase Chain Reaction
pg	pictogram(s)
PVD	Peripheral Vascular Disease

PYR	pyridinoline
RAAA	Ruptured Abdominal Aortic Aneurysm(s)
RCF	Radial Centrifugal Force
RFPL	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
SMC	Smooth Muscle Cell
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
T	Thymine
Taq	Thermus Aquaticus
Th	T helper
TNF	Tumour Necrosis Factor
t-PA	tissue-type Plasminogen Activator
TIMP	Tissue Inhibitor of MetalloProteinase
u-PA	urokinase-type Plasminogen Activator
US	Ultrasound Scan
V	Volts
µg	microgram(s)
µg	microlitre(s)

Publications and presentations arising from this thesis

Publications

Feasibility of preoperative computer tomography in patients with ruptured abdominal aortic aneurysms: A time to death study in patients without operation
Lloyd GM, Bown MJ, MGA Norwood, Deb R, Fishwick G, Bell PRF, Sayers RD
The Journal of Vascular Surgery 2004; 39 (4): 788-791.

Patients with abdominal aortic aneurysm: are we missing the opportunity for cardiovascular risk reduction?
Lloyd GM, Newton JD, Norwood MGA, Franks SC, Bown MJ, Sayers RD
The Journal of Vascular Surgery 2004; 40 (4); 691-697.

Published abstracts

The IL-10 -1082 gene polymorphism: A candidate gene for abdominal aortic aneurysms
GM Lloyd, MJ Bown, RD Sayers
Br J Surg 2005; 92: 1304

Missing the opportunity to improve survival in patients with abdominal aortic aneurysms
Lloyd GM, Newton JD, Norwood MGA, Franks SC, Bown MJ, Sayers RD
Br J Surg 2005; 92 (suppl 1): 126

Presentations to learned societies

The Feasibility of CT scans for Patients with Ruptured Abdominal Aortic Aneurysms: A Time to Death Study in Patients Treated Without Operation
Lloyd GM, Bown MJ, Norwood MGA, Fishwick G, Bell PRF, Sayers RD.
Vascular Anaesthesia Society of Great Britain and Ireland
9/9/2003 Leicester (Poster and oral presentation)

The Feasibility of CT scans for Patients with Ruptured Abdominal Aortic Aneurysms: A Time to Death Study in Patients Treated Without Operation
Lloyd GM, Bown MJ, Norwood MGA, Fishwick G, Bell PRF, Sayers RD.
East Midlands Surgical Society Meeting
17/10/2003 Doncaster

The Feasibility of CT scans for Patients with Ruptured Abdominal Aortic Aneurysms: A Time to Death Study in Patients Treated Without Operation
Lloyd GM, Bown MJ, Norwood MGA, Fishwick G, Bell PRF, Sayers RD.
Midland Vascular Surgical Society Meeting
12/3/04 Shrewsbury

Patients with abdominal aortic aneurysm: Are we missing the opportunity for cardiovascular risk reduction?
Lloyd GM, Newton JD, Norwood MGA, Bown MJ, Sayers RD
Midland Vascular Surgical Society Meeting
12/3/04 Shrewsbury

The interleukin 10 -1082 polymorphism: A candidate gene for abdominal aortic aneurysms
Lloyd GM, Bown MJ, Sayers RD
Society of Academic and Research Surgery
January 2005 Newcastle (Patey Prize session)

Missing the opportunity to improve survival in patients with abdominal aortic aneurysms
Lloyd GM, Newton JD, Norwood MGA, Franks SC, Bown MJ, SayersRD
Association of Surgeons of Great Britain and Ireland
April 2005 Glasgow

Interleukin 10 genotype: Associated with AAA formation but not growth
Lloyd GM, Bown MJ, Thompson J, Sayers RD
The Vascular Society of Great Britain and Ireland
November 2005 Bournemouth

The IL-10 -1082 gene polymorphism: A candidate gene for abdominal aortic aneurysms
Lloyd GM, Bown MJ, Sayers RD
The Vascular Society of Great Britain and Ireland
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Chapter 1

Abdominal Aortic Aneurysms

Contents

The abdominal aorta

The aorta enters the abdomen through the aortic hiatus of the diaphragm at the level of the 12th thoracic vertebra. It descends in the retroperitoneum, inclining slightly to the left, and gives large branches to the gut and kidneys; the coeliac trunk at the level of the 12th thoracic vertebra, the superior and inferior mesenteric arteries at the levels of the 1st and 3rd lumbar vertebrae respectively and the renal arteries at the level of the 2nd lumbar vertebra. The splenic vein and the body of the pancreas intervene between the celiac trunk and the superior mesenteric artery and the left renal vein, the uncinate process of the pancreas and the third part of the duodenum lie between the origins of the superior and inferior mesenteric arteries. The other smaller branches of the aorta are the paired suprarenal, gonadal and inferior phrenic arteries along with the four lumbar arteries, and the median sacral artery.

The aorta terminates at the level of the 4th lumbar vertebra where it bifurcates to form the paired common iliac arteries (Figure 1.1). There is a gradual tapering of the aorta in an anterior-posterior diameter as it passes from the aortic hiatus to the bifurcation (Webster MW 1991). Conventionally the abdominal aorta is divided into supracoeliac, suprarenal and infrarenal sections, on the basis of the origins of the coeliac and renal arteries. Table 1.1 displays the reported range of diameter and standard deviation of the different sections of the abdominal aorta measured by abdominal radiological imaging.

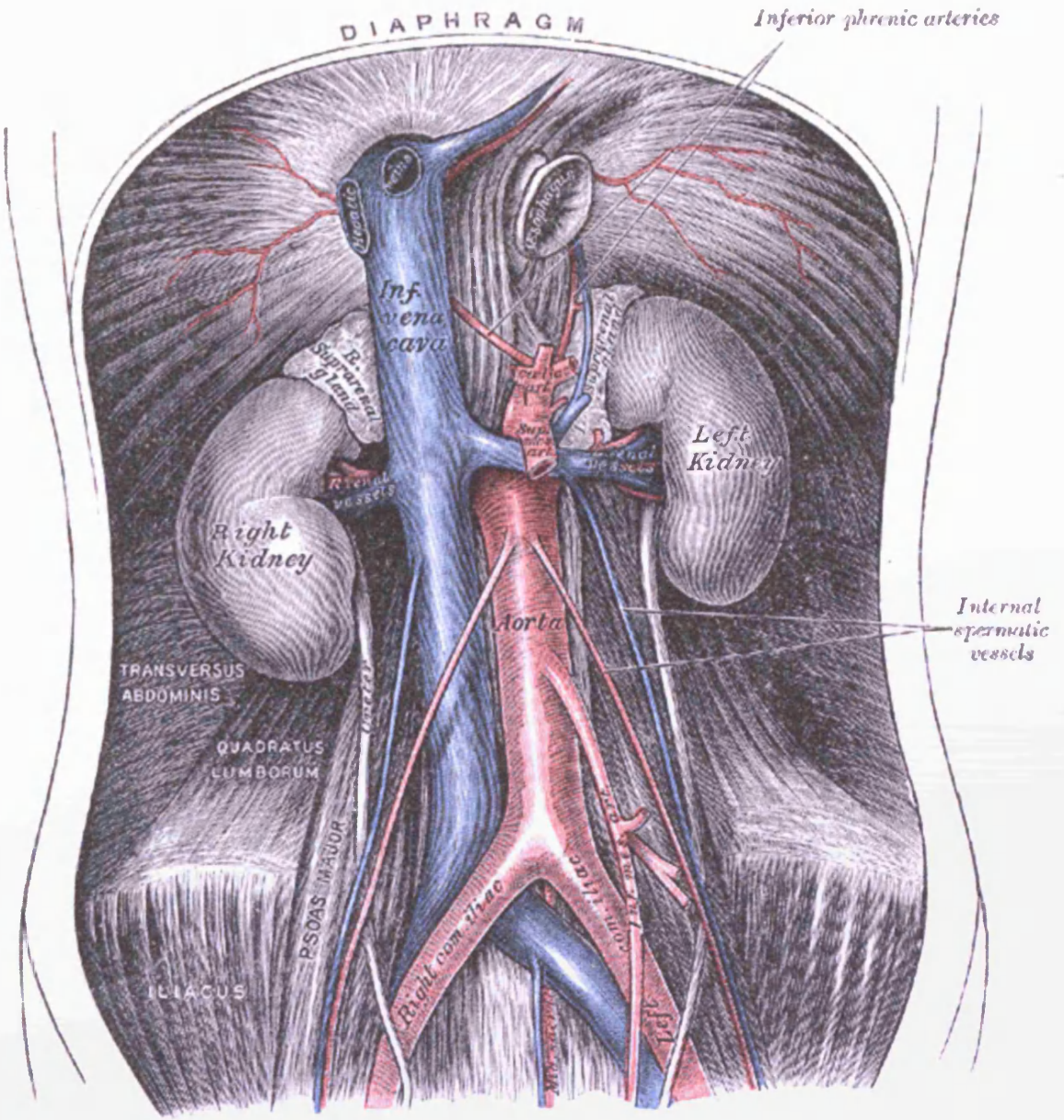


Figure 1.1. Diagram of abdominal aorta, its relations and its main branches. (Gray H 2000)

Section of abdominal aorta	Sex	Range of reported mean diameter (cm)	Range of reported standard deviation of diameter (cm)	Assessment method
Supracoeliac	Male	2.5 – 2.72	0.24 – 0.35	CT
	Female	2.1 – 2.31	0.27	CT
Suprarenal	Male	1.98 – 2.27	0.19 – 0.23	CT
	Female	1.86 – 1.88	0.09 – 0.21	CT
Infrarenal	Male	1.99 – 2.39	0.30 – 0.39	CT
		1.41 – 2.05	0.04 – 0.37	CT, US, arteriography
	Female	1.66 – 2.16	0.22 – 0.32	CT, arteriography
		1.19 – 1.87	0.09 – 0.34	CT, US, arteriography

Table 1.2. Reported diameter range and standard deviation of supracoeliac, suprarenal and infrarenal sections of abdominal aorta in men and women. (Abbreviations: CT, computed tomography. US, ultrasound) (Johnston KW 1991)

Structure of the aortic wall

The aorta is classified as an elastic artery because of the large amount of elastic tissue in its wall causing high compliance. The structure of the aorta follows that of other arteries in that it consists of three concentric layers. The intima, the inner layer is composed of endothelium that rests on a layer of connective tissue. The middle layer, the media consists of a large amount of elastin, a compliant connective tissue that forms multiple concentric lamellae along with smooth muscle cells, collagen and extracellular matrix components. The adventitia, the outer layer is thin and is largely made up of the strong connective tissue, collagen, along with fibroblasts and some elastin.

Definition of an abdominal aortic aneurysm

A true arterial aneurysm is a localised dilatation of an artery involving all three layers of the vessel wall. In 1991, an ad hoc committee of the Joint Councils of the Society for Vascular Surgery and the North American Chapter of the International Society for Cardiovascular Surgery recommended that an arterial aneurysm is defined as 'a permanent localised (i.e. focal) dilatation 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). Applying this definition to the abdominal aorta leads to a diameter of (approximately) ≥ 3 cm being defined as aneurysmal. However, a problem with this definition is that comprehensive data describing normal aortic dimensions depending on patient age, sex and body size do not exist (demonstrated by the large standard deviation in Table 1.1). Between 90 and 95 % of abdominal aortic aneurysms (AAA) occur in the infrarenal aorta (Blanchard JF 1999) and an infrarenal to suprarenal aortic diameter ratio > 1.5, or even > 1.2 are alternative definitions of AAA (Sterpetti AV 1987) (Alcorn HG 1996). These definitions serve to prevent over diagnosis of AAA in physically larger individuals, who have proportionately larger aortas, however they are not appropriate for the 5-10 % of patients with suprarenal involvement. In the world literature, no consistent definition has been applied to AAA, leading to a degree of inconsistency in comparing reports.

Prevalence

The prevalence, or total numbers of AAA cases at a given moment of time, in a given population is difficult to accurately ascertain because the majority of small AAAs are asymptomatic and clinical examination is neither sensitive nor specific. Determination of AAA prevalence has largely depended upon radiological population screening and autopsy studies. Overall prevalence of between 1.3 and 12.7% has been reported in the population aged over 65, with screening and autopsy studies reporting comparable figures (Lederle FA 1997) (Boll AP 1998) (Collin J 1988) (Smith FC 1993) (Lucarotti M 1993) (Scott RA 1995) (Pleumeekers HJ 1994). The wide variation in the reported prevalence is probably a consequence of the varying definitions of AAA, the age of patients studied and possibly the geographic areas covered. Lederle et al reported a 4.6% prevalence of AAA if an aortic diameter $\geq$ 3 cm is used to define an AAA, but a prevalence of only 1.4% in the same patient group if a diameter of $\geq$ 4 cm is used as the definition of an AAA (Lederle FA 1997). The prevalence of AAA increases progressively with age, with patients aged 75-79 years being twice as likely as those aged 65-69 years to have an AAA (Vardulaki KA 2000). There is a male predominance to all aneurysmal disease, and the prevalence of AAA is between 3 and 6 times higher in men than women (Powell JT 2003) (Vardulaki KA 2000).

Incidence

The reported incidence or number of new cases of AAA in a specific population over a given period of time varies between 11.3 and 117.2 per 100 000 person years in men and between 3 and 33.9 per 100 000 person years in women (Fowkes FGR 1989) (Pleumeekers HJCM 1994) (Wilmink ABM 1998). Determining the incidence of AAA is subject to the same difficulties as determining prevalence, leading to wide

variation in reported figures. Vardulaki et al reported an incidence of 0.08% in men aged 50, rising to 0.67% at the age of 65, before falling with increasing age, in contrast to the incidence cardiovascular disease which rises progressively with age (Vardulaki KA 1999). Importantly, the incidence of AAA appears to be increasing (Naylor AR 1988) (Samy AK 1994) (Melton L 1984). Fowkes et al showed the age standardised mortality increased 20-fold in men and 11-fold in women between 1950 and 1984 and also demonstrated an increase in hospital admissions for AAA between 1968 and 1983 (Fowkes FGR 1989). In Australia, an increase in AAA incidence of 100% in men and 200% in women was reported between 1971 and 1981 (Castleden WM 1985) and Pleumeekers et al estimated a 4.2% annual increase in AAA in men and a 14% annual increase in women (Pleumeekers HJCM 1994). There has been debate as to whether a real increase in incidence has occurred, or whether the apparent increase is, in part, a reflection of the ageing population and / or greater diagnostic awareness (Collin J 1990).

Risk factors

While a small percentage of AAA are caused by genetic disorders such as Marfan syndrome or Ehlers-Danlos syndrome, the majority appear to occur sporadically and are classified as acquired or non-specific. Commonly cited risk factors for AAA are male sex (Vardulaki KA 2000) (Singh K 2001), advanced age (Scott RA 1991) (Norman PE 1991) (Pleumeekers HJCM 1994) (Gillum RF 1995), smoking, hypertension (Vardulaki KA 2000), family history of AAA, history of previous vascular disease and hyperlipidaemia (Bengtsson H 1989) (Allardice J 1988) (MacSweeney ST 1993). It is likely that a number of environmental factors combine and in patients with an underlying susceptibility, AAA formation occurs.

Sex

AAAs are between 3 and 6 times more prevalent in men than women and a different age distribution seems to be present with women being affected approximately 10 years later than men (Scott RA 2002). With increasing age, the male to female ratio of AAA decreases (Semmens JB 2000). There is also evidence that in addition to an overall increase in incidence of AAA over the last few decades, the gender differences are decreasing, with AAA incidence increasing proportionately more rapidly in women (Pleumeekers HJCM 1994).

Smoking

Smoking was first identified as a risk factor for AAA in the Framingham Study and has since been shown to be a strong independent risk factor for AAA formation, with a relative risk of between 2.6 and 9 (Blanchard JF 1999). Ninety percent of patients with AAA have a history of cigarette smoking (Hammond EC 1969) (Lederle FA 1997). Interestingly, the association between smoking and AAA appears to be even stronger than the association between smoking and coronary or cerebral vascular disease (Lederle FA 2003).

Racial differences

Although limited data exists, AAA appear to be predominantly a disease of white populations, with a white to black ratio of between 2 and 6 to 1 (Johnson G 1985) (Costa M 1986). AAA occur very infrequently in the United Kingdom Asian population (Spark JI 2001). There is also some evidence that the sex distribution is race dependent, and an equal male to female ratio has been demonstrated in black

populations (Costa M 1986). These racial differences may support a genetic component to AAA formation.

Genetics

In 1977, Clifton reported the first familial clustering of AAA, in 3 brothers with ruptured abdominal aortic aneurysms (RAAA), and since then there have been numerous reports of high familial incidence of AAA (Clifton MA 1977). Bengtsson et al performed abdominal ultrasound examinations of siblings of patients with AAA and found that 29% of the brothers and 6% of the sisters had an AAA (Bengtsson H 1989). Men with a first degree relative with an AAA have been shown to have approximately a ten fold increased risk of developing an AAA (Johansen K 1986) (Pleumeekers HJCM 1994). This suggests a familial and probably genetic cause of AAA in some patients.

A number of isolated genetic defects have been identified in members of individual families in whom AAA is prevalent, including a variation on chromosome 16, which is associated with increased alpha-2-haptoglobulin activity and elastin degradation (Powell JT 1990), and a mutation on the type 3 collagen gene (Kontusaari S 1990). However, no consistent isolated genetic defect has been isolated in patients with AAA, and a multifactorial or polygenic mechanism may underlie genetic susceptibility to AAA.

Hypertension

Hypertension is commonly regarded as a risk factor for AAA, however contradictory evidence for this exists. A number of prospective and case-control studies have

demonstrated a convincing association between hypertension and AAA (Blanchard JF 1999) (LaMorte WW 1995) (Reed D 1992) and the increased haemodynamic load on the aortic wall in hypertensive patients is a plausible mechanism for increased risk of AAA formation. The demonstration in some studies that hypertension increases the rate of expansion in established AAA supports this theory (Cronenwett JL 1990). Screening studies however, as a rule, have not demonstrated a strong association between hypertension and AAA and it has been suggested that the design of these studies may be less subject to patient selection bias and therefore may give a more accurate indication of the risk of hypertension (Lederle FA 1997) (Pleumeekers HJ 1995) (Blanchard JF 1999).

Serum lipids

Similarly conflicting evidence exists for the association between elevated lipids and AAA. Reed et al in a study of 8000 patients found that high serum cholesterol was associated with AAA formation (Reed D 1992). However, other studies have cast doubt on a clear link between elevated lipids and it seems certain that if an association between lipids and AAA exists, it is far weaker than that between lipids and occlusive manifestations of atherosclerosis.

Atherosclerosis

In the past, AAA were frequently termed atherosclerotic aneurysms because of the presumed causal role of atherosclerosis. There is a strong association between AAA and atherosclerosis; aneurysmal change frequently occurs in aortas affected by atherosclerosis, and hypertension, smoking, hyperlipidaemia, male sex and advanced age are risk factors common to both conditions. Generalised atherosclerosis is a

frequent finding in patients with AAA and the reported prevalence of AAA in patients with peripheral vascular disease is double that of similarly aged patients in the general population (Allardice JT 1988).

However, important differences exist between AAA and atherosclerosis, casting doubt on a causal relationship. Atherosclerosis is primarily a disease of the arterial intima and media whereas the key pathological changes in aneurysmal disease occur in the media and adventitia (Ailawadi G 2003). Only a small percentage of patients with aortic atherosclerosis develop AAA, and the severity of atherosclerosis has not been shown to be a predictor of AAA formation (Deak SB 1992) (Lederle FA 2003). Age and sex differences exist between atherosclerosis and AAA; AAA almost exclusively affects the elderly whereas occlusive arterial disease affects younger age groups, with a peak incidence at around 55 years (Collin J 1990). Tilson et al compared patients with AAA to those with occlusive aorto-iliac disease and demonstrated patients with AAA were > 9 times more likely to be male and were on average 11 years older than patients with occlusive arterial disease (Tilson MD 1992). If atherosclerosis is indeed the cause of AAA, such a long latent period between onset of atherosclerosis and AAA formation would be unusual. The incidence of coronary artery disease and stroke, clinical manifestations of atherosclerosis, are both decreasing (Gillum RF 1987) (Sarti C 2000) whereas the incidence of AAA appears to be increasing (Reed D 1992). Differences in risk factors also exist; diabetes is strongly correlated with atherosclerotic occlusive arterial disease but a negative association between AAA and diabetes has been reported (Lederle FA 1997) and although hypertension is frequently cited as a risk factor for AAA, it is much more strongly associated with atherosclerosis. There is a strong familial and probably

genetic role in AAA formation that has not been observed in atherosclerosis. In addition, it has been shown that some patients with AAA have a generalised arterial dilating diathesis involving arteries not commonly affected by atherosclerosis (Ward AS 1992).

AAA formation

Although the precise cause of AAA is unknown, the pathological changes that occur in the aortic wall are well described. The principal pathological features of AAA are loss of elastin and smooth muscle cells from the aortic media, increased collagen turnover and a chronic inflammatory infiltrate in the media and adventitia consisting of macrophages and lymphocytes (Lopez-Candales A 1997). A destructive remodelling process is initiated resulting in progressive structural change and continuing aneurysm expansion. It is thought that loss of elastin is the cause of initial arterial dilatation, and failure of the strong collagen fibres is the mechanism by which further dilatation and rupture occurs.

There is convincing evidence that matrix metalloproteinases (MMPs), a family of zinc dependent proteases with substrate specificity for elastin and collagen play a key role in AAA formation and rupture (Patel MI 1996). MMPs are secreted by macrophages and smooth muscle cells, and in the normal arterial wall regulate wall remodelling in combination with their inhibitors, tissue inhibitors of MMPs (TIMPs) (Patel MI 1996) (Wills A 1996). In AAA, the remodelling balance is shifted towards collagen and elastin degradation. Macrophages and lymphocytes secrete cytokines and it is thought that cytokines are responsible for MMP control; pro-inflammatory cytokines activate MMPs while anti-inflammatory cytokines have an inhibitory effect on MMPs. At

present it appears that MMPs 2 and 9 are the most important in AAA formation (Ailawadi G 2003).

AAA expansion

There is some evidence that an age related increase in the diameter of the abdominal aorta occurs even in patients without AAA. Singh et al reported a median abdominal aortic diameter of 1.75 cm in men aged 25 and a median diameter of 2.25 cm in men aged 55 (Singh K 2001). However other studies have reported that only a small percentage of aortas expand with age (Wilmink AB 2001). In contrast, although a small percentage of AAA remain the same size for a number of years, the natural history of AAA is progressive expansion and eventual rupture unless death from another cause supervenes. AAA expansion rate is of importance as it is established that the rate of expansion correlates with the risk of rupture and knowledge of the expected rate of expansion allows prediction of the future need for elective repair and the appropriate timing of periodic screening in patients with small AAA (Limet RJ 1991). Larger diameter AAAs have a greater rate of expansion, indicating a nonlinear and possibly exponential growth pattern (Bernstein EF 1976) (Bernstein EF 1984) (Cronenwett JL 1985) (Cronenwett JL 1990) (Limet RJ 1991). Sterpetti et al calculated that AAA of 3.5 to 3.9 cm expand by 0.18 cm / year whereas AAA of 5 to 5.4 cm expand by 0.68 cm / year (Sterpetti AV 1985). Limet et al reported similar expansion rates, AAA < 4cm expand at a rate of 0.53 cm / year, 4 to 4.9 cm AAA expand at a rate of 0.69 cm / year and AAA > 5 cm expand at a rate of 0.74 cm / year (Limet R 1991). Collin et al reported that AAA of 2.5 to 2.9 cm expand by 0.11 cm/ year, those of 3.0 to 3.4 cm expand by 0.19 cm / year and AAA of 3.5 – 3.9 cm expand by 0.29 cm / year (Collin J 1991). An expansion rate of > 1cm / year has been

used as an arbitrary indicator of risk of rupture and therefore often regarded as one of the indications for elective AAA repair (UK Small Aneurysm Trial Participants 1998) (Lederle FA 2002) (Scott RA 2002). Rapid rate of expansion has been independently associated with hypertension, advanced age, smoking and cardiac disease (Chang JB 1997).

Risk of rupture

AAA diameter is the most reliable predictor of risk of rupture. Lederle et al in a prospective study of 198 patients with AAA > 5.5 cm in whom surgery was not performed because of medical contraindications or patient refusal demonstrated the one year incidence of rupture for AAA between 5.5 and 5.9 cm was 9.4%, for AAA between 6 and 6.9 cm it was 10.2% and for AAA > 7 cm was 32.5% (Lederle FA 2002). Reed et al found that AAA < 4cm had an annual rupture rate of 0%, AAA between 4.0 and 4.9 cm had a rupture risk of 1%, AAA between 5.0 and 5.9 cm had an 11% risk and AAA between 6.0 and 6.9 cm had an annual risk of 26% (Reed WW 1997). The UK Small Aneurysm Trial demonstrated similar results; AAA < 4 cm had a 0.3% annual rupture rate, AAA 4.0 to 4.9 cm had a rupture rate of 1.5% and AAA 5 to 5.9 cm had an annual rupture rate of 6.5% (Brown LC 1999). Table 1.2 demonstrates reported rupture risk depending on AAA size. Importantly, the risk of rupture increases markedly between 5 and 6 cm.

AAA diameter (cm)	Rupture risk (% / year)
< 4	0
4 – 5	0.5 – 5
5 – 6	3 – 15
6 – 7	10 – 20
7 – 8	20 – 40
> 8	30 – 50

Table 1.2. Annual risk of AAA rupture depending on aortic diameter (Brewster DC 2003).

Although of lesser importance than AAA size; hypertension, smoking, female sex and chronic obstructive pulmonary disease (COPD) appear to increase the relative risk of AAA rupture (Brown LC 1999). Significantly, women have a higher risk of rupture for a given AAA size than men. This probably reflects the greater proportional increase in aortic diameter in women, who have smaller aortas than men, and it has been debated whether the 5.5 cm criteria for AAA repair in women is too high (United Kingdom Small Aneurysm Trial Participants 2002).

Ruptured AAA

Ruptured abdominal aortic aneurysms (RAAA) are the eighth commonest cause of death in the UK, and accounted for 2.1% of male deaths and 0.75% of female deaths in patients over the age of 65 and approximately 9500 deaths overall in England and Wales in the year 2001 (Figure 1.2) (Multicentre Aneurysm Screening Study Group 2002) (Office for national statistics 2001). RAAA classically presents with abdominal

and / or back pain, an expansile abdominal mass and hypovolaemic shock. Approximately half of patients with RAAA reach hospital alive and in the patients undergoing surgery the mortality rate is approximately 50% (Figure 1.3) (Bown MJ 2002). Rupture can be prevented by elective AAA repair, which carries a mortality of approximately 5% (Hallin A 2001).

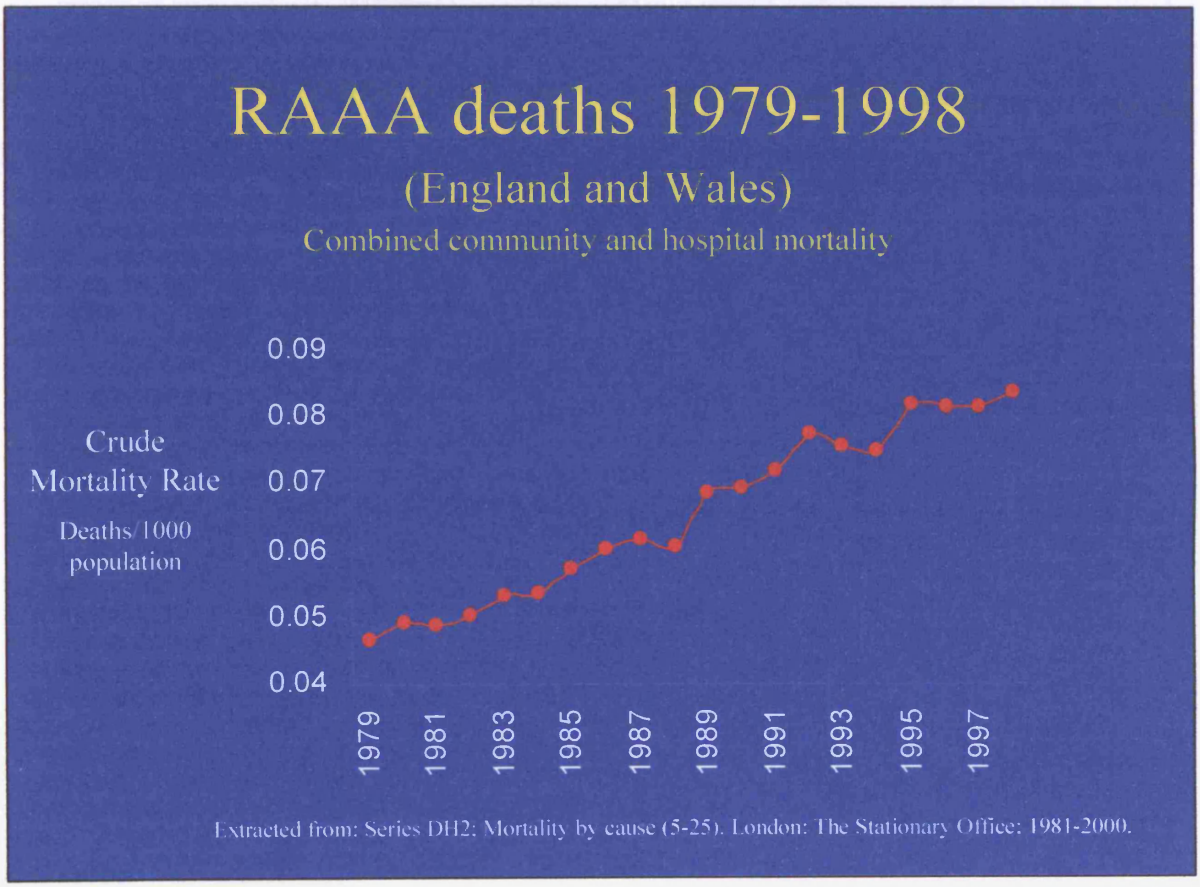


Figure 1.2. Graph showing combined community and hospital mortality from RAAA from 1979 to 1998.

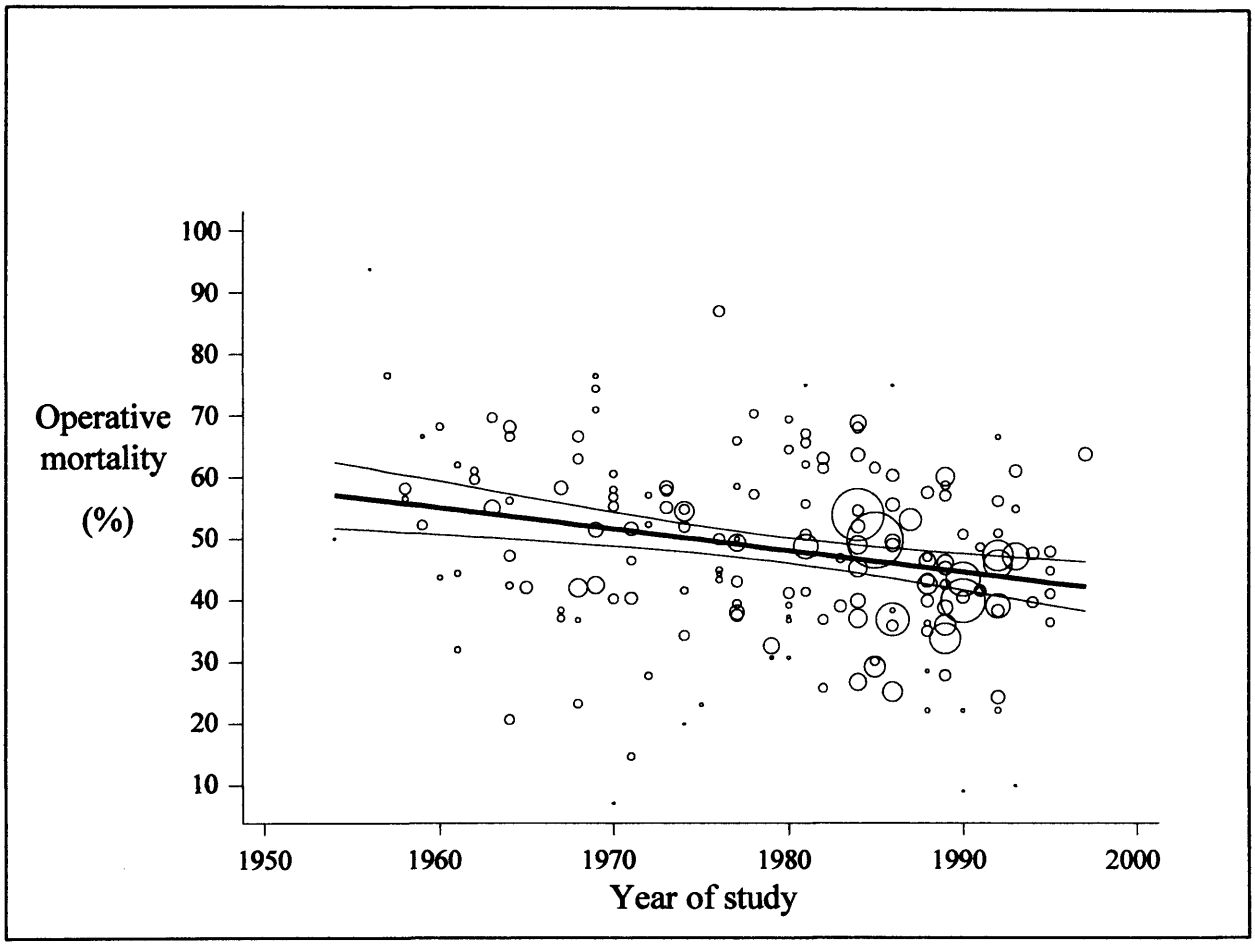


Figure 1.3. Graph showing operative mortality over time for RAAA. Outer lines are 95% confidence interval and circles represent mortality reported in individual studies (Bown MJ 2002).

UK Small Aneurysm Trial and ADAM Study

Two similar prospective randomised controlled trials, the UK Small Aneurysm Trial and the Aneurysm Detection and Management (ADAM) Veterans Affairs Cooperative Study in the USA, have provided level 1 evidence for the management of small AAA (UK Small AAA trial participants 1998) (Lederle FA 2002). Both studies involved over 1000 patients with AAA of between 4 and 5.4 cm in diameter, randomised to undergo elective open AAA repair or 3 or 6 month surveillance with ultrasound or computed tomography scan. In the surveillance group, repair was recommended if the AAA diameter reached 5.5 cm, rate of expansion exceeded 1 cm / year or symptoms developed. The follow up period was 4.6 years in the UK Small

Aneurysm Trial and 4.8 years in the ADAM trial and the primary end point in each trial was death. Both trials demonstrated the same findings for patients with AAA < 5.5 cm; the annual risk of rupture is < 1%, there is no reduction in mortality associated with operative repair, and consequently surveillance is safe. However no similar randomised controlled trials have investigated AAA > 5.5 cm, and therefore the upper AAA diameter for which surveillance is safe is not accurately known

However, 60% of the patients undergoing surveillance eventually underwent repair during the study period. The sequel to the UK Small Aneurysm Trial, the report of 10 year period follow up demonstrated that a total of 74% patients in the surveillance group eventually underwent surgery (UK Small Aneurysm Trial Participants 2002). Interestingly the risk of death was 7.2% lower in the early surgery group than the surveillance group at 8 years, a consequence perhaps of improved lifestyle changes, although perhaps more likely, a reflection of a younger patient group undergoing surgery.

Screening

The aim of AAA screening is to prevent aneurysm rupture by identifying patients with AAA and therefore providing an opportunity to perform elective AAA repair prior to rupture. Ultrasonography is the screening tool of choice due to relatively low cost, lack of radiation, non-invasive nature, accuracy, speed and patient acceptance. Ultrasound scanning can reliably measure the diameter of the aorta in 99% of patients and therefore is more accurate than other screening tests routinely used for other diseases such as mammography and prostate-specific antigen (Lindholt JS 1999) (Thompson RW 2002). Most centres offering AAA screening invite men aged 65

years to undergo an ultrasound scan of the aorta. The demonstration that men with an aortic diameter < 26 mm at the age of 65 do not develop clinically significant AAA over a 12 year period (Crow P 2001) supports this policy.

The Multicentre Aneurysm Screening Study (MASS) in the UK randomised 67 800 men aged 65 to 74 years to undergo an abdominal ultrasound scan of the aorta or no scan (Ashton HA 2002). Patients found to have an AAA (defined as aortic diameter ≥ 3 cm) were followed up for a mean period of 4.1 years with periodic scans. Surgery was recommended if the AAA reached 5.5 cm or expanded at a rate ≥ 1 cm / year. A 53% reduction in AAA related deaths was demonstrated in the group that underwent periodic scanning. This confirmed the findings of previous smaller studies that found a 50% reduction in the incidence in RAAA in screened patients (Scott RA 1994).

It is generally accepted that screening women confers little or no benefit. A randomised controlled trial in Chichester involving 9342 women demonstrated an AAA prevalence of 1.3%, 6 times lower than in men and no reduction in the incidence of rupture in women entered into the study (Scott RA 2002).

Medical management of AAA

The main limitation of AAA screening is that the majority of detected AAA are too small to justify elective repair when discovered and a 'watchful waiting' approach is adopted in the absence of an effective means of preventing AAA growth or rupture. The MASS Study reported that of the 1333 AAA detected through screening, 88% were < 5.5 cm, and therefore elective repair was not indicated when the AAA was discovered (Ashton HA 2002). The increasing utilisation of AAA screening

programmes in the UK will result in the identification of a large number of small AAA, that will require regular surveillance. Unfortunately, at present there is no way of preventing or reducing the rate of AAA expansion or rupture. The potential for a number of pharmacological agents to reduce the rate of AAA expansion has been investigated but at present there is no convincing evidence of benefit.

Beta-blockers

Evidence from animal studies and small human studies has suggested that the beta-blocker, propranolol may inhibit AAA expansion (Steinmetz EF 2003). The proposed mechanism is thought to be through an effect on cardiac contractility and aortic tensile strength (Propanolol Aneurysm Trial Investigators 2002). However, a prospective, randomized, placebo-controlled trial of propranonlol in Canada demonstrated no difference in AAA expansion, the need for AAA repair or mortality in the treatment group (Propanolol Aneurysm Trial Investigators 2002). In addition, 42% of the patients randomised to receive propranolol were unable to continue with treatment because of adverse side effects. A similar study in Denmark demonstrated that 60% of patients using propranolol had to stop treatment and, of concern, patients using propranolol had a statistically higher mortality. This led to the trial being stopped prematurely (Lindholt JS 1999).

Nonsteroidal anti-inflammatory agents

There is some evidence that non-steroidal anti-inflammatory drugs (NSAIDS) may have the potential to reduce the rate of AAA expansion. Indomethacin has been demonstrated to inhibit elastase induced AAAs in rats through inhibition of cyclo-oxygenase 2 (COX 2) leading to a reduction in the inflammatory mediators

interleukin 6 (IL-6) and prostaglandin E_2 (PGE_2), and MMP-9 (Holmes DR 1996) (Miralles M 1999). In human studies indomethacin has been shown to suppress inflammatory mediators and may reduce the rate of AAA expansion (Walton LJ 1999). However, at the moment there is limited evidence of a convincing role.

Matrix metalloproteinase inhibitors

The tetracycline derivative, doxycycline has shown benefit in reducing growth rate in rat models of AAA and tetracycline has been shown to reduce MMP-9 secretion by cultured explants of human AAA tissue (Steinmetz EF 2003). In addition, preoperative treatment with doxycycline in humans has been shown to reduce the production of MMP-9 in aortic tissue fivefold (Curci JA 2000). A small randomised controlled trial of doxycycline versus placebo in 32 patients with small AAA demonstrated reduced rate of aneurysm expansion in patients receiving doxycycline (Mosorin M 2001).

Open AAA repair

The principal aim of elective AAA repair is to prevent aneurysm rupture and therefore prevent aneurysm related death. In each patient with AAA, the decision to operate depends upon the risk of rupture, predicted operative mortality and expected long term survival. Population based studies have demonstrated that the mortality rate following elective open AAA repair is between 4 and 8%, with an average mortality of approximately 5.5 % (Hallin A 2001), which is similar to the 5.8% reported in the UK Small Aneurysm Trial. A number of specialist vascular surgery units report mortality rates < 5%, and some report rates as low as 2% (Ernst CB 1993) (Zarins CK 1997) (Hertzer NR 2002). However, in subgroups of patients with cardiac, respiratory

and renal co-morbidity operative mortality can be as high as 50% (Johnston KW 1989). Despite advances in anaesthesia and critical care, the overall mortality rate for elective AAA repair does not appear to have fallen significantly over the last 20 years (Ernst CB 1993) (Heller JA 2000).

Dubost performed the first successful AAA repair with graft replacement in Paris in 1951 (Dubost C 1951) and the principle of open AAA repair has remained largely unchanged ever since. A transperitoneal approach to the aorta is usually performed, although a retroperitoneal approach can be used. Under general anaesthesia a laparotomy is performed and after mobilisation of the small intestine the peritoneum covering the posterior abdominal wall is divided and the duodenum reflected. The aneurysm neck and iliac arteries are identified and dissected free of the surrounding tissue. Clamps are then applied to the neck and the iliac arteries and the aneurysm sac is incised longitudinally. Lumbar and inferior mesenteric artery back bleeding is controlled with sutures. A synthetic graft (usually Dacron) is inserted into the aneurysm sac and is anastomosed proximally to the neck and distally to the aortic bifurcation if the iliac arteries are disease free. A bifurcated graft is employed if there are associated aneurysms of the common iliac arteries. The cut edges of the aneurysm sac are then approximated to cover the graft before the abdomen is closed.

Although open AAA repair is a well established and durable technique it is associated with a large physiological insult and systemic complications. Laparotomy, aortic cross clamping, tissue trauma, hypothermia and bleeding induce significant physiological stress. Post operative intensive care is almost always mandatory and during the recovery period patients are relatively immobile, cardiorespiratory problems are

common and the recovery can be prolonged, with inpatient stay generally between 7 and 10 days.

Endovascular AAA repair

Endovascular (keyhole) AAA repair was first described by Parodi in 1991 and is the first major advance in the technique of AAA repair in 40 years (Parodi JC 1991). AAA exclusion from the circulation is achieved by placement of a stent-graft inside the aneurysm sac via the femoral arteries under radiological control. The stent-graft consists of a prosthetic vascular graft which is reinforced by metallic struts and has a metallic attachment system which allows fixation to the arterial wall. The stent-graft is usually delivered into the aneurysm inside a sheath, manoeuvred into position under x-ray control and deployed by withdrawal of the sheath. Final fixation at the proximal and distal landing sites is achieved by inflating an angioplasty balloon inside the stent-graft to ensure firm fixation to the arterial wall. The minimally invasive nature of the technique means that it can be performed under general or regional anaesthesia.

Over the last decade many UK vascular units have been performing EVAR and extensive experience of patient selection, intra-operative technique and post-operative problems has been acquired. EVAR avoids the problems associated with laparotomy, resulting in reduced physiological stress and the majority of patients treated can safely return to the surgical ward from theatre, avoiding the need for intensive care. Post operatively, patients mobilise early, and the average length of inpatient stay is less than 4 days (Prinssen M 2004) (Faries PL 2002) (Elkouri S 2004).

However, a number of problems are associated with EVAR. At present not all AAA are morphologically suitable for EVAR and anatomical features such as a wide, tapered or short aneurysm neck, marked neck angulation and narrow or tortuous iliac arteries currently preclude EVAR (Carpenter JP 2001). Careful pre-operative assessment and imaging, usually with contrast CT scanning or angiography is necessary to determine the suitability of an AAA for EVAR and the size of the stent graft required. Currently, in centres using a variety of devices, approximately 60% of patients are suitable for EVAR, and it is envisaged that this number will increase in the future as better stent grafts are introduced (Armon MP 1997) (Wolf YG 2000) (Brewster DC JVS 1998).

Open AAA repair can usually be regarded as a definitive treatment with proven long term durability; in contrast, the relatively recent inception of EVAR means that long term outcome is not known. Endoleak is a phenomenon unique to EVAR, characterised by continued blood flow in the aneurysm sac following stent-graft placement and affects between 10 and 50% of patients (Buth J 2000) (Zarins CK 1999) (Wain RA 1998). The significance of endoleak is unclear and in many cases it is probably a benign phenomenon. However, in some instances endoleak can signify blood entering the sac at systemic blood pressure leading to continued aneurysm expansion and occasionally rupture (Veith FJ 2002) (van Marrewijk C 2002). The relative frequency of complications following EVAR, requiring later secondary intervention necessitates probable lifelong continued radiological surveillance, although the majority of complications can be treated conservatively or with minimally invasive radiological techniques (Elkouri S 2004). This continuing surveillance considerably adds to the cost of EVAR.

A UK multi-centred national randomised trial (EVAR) has been evaluating endovascular aneurysm repair for several years and reported its midterm findings in June 2005. The first arms of the trial, EVAR 1, in which medically fit patients were randomized to either open or endovascular AAA repair, found a 3% higher aneurysm related survival in patients undergoing EVAR at 4 years (4% vs 7%) (EVAR trial participants 2005). However, all cause mortality was similar in the two groups. The second arm of the trial, EVAR 2, randomised patients medically unfit for open AAA repair to EVAR or ultrasound surveillance and best medical therapy (EVAR trial participants 2005). Importantly it was found that EVAR does not improve survival in this patient group.

Role of EVAR in ruptured abdominal aortic aneurysm

The overall operative mortality rate following RAAA repair has not fallen significantly in the last 2 decades and remains between 40 and 50% (Bown MJ 2002). The first report of successful endovascular repair of RAAA was in 1994, and although experience of EVAR in RAAA is still limited, early reports are promising with mortality rates as low as 8% reported (Yusuf SW 1994) (Ohki T 2000) (Lee WA 2004). It is therefore possible that EVAR may provide the first major opportunity to reduce operative mortality in RAAA. The physiological, cardiovascular and metabolic advantages of EVAR over open AAA repair in patients with RAAA, who already have deranged physiology may be even more pronounced than in patients undergoing elective AAA repair.

In the majority of centres a CT scan is required to determine the suitability of the aneurysm for EVAR. One of the major concerns surrounding EVAR for RAAA is the

potential delay in transferring patients with RAAA to theatre via CT. Opponents of EVAR in the treatment of RAAA feel that this delay is of detriment and may result in patients dying before surgery is performed, thereby limiting the benefit of EVAR in RAAA. As a result of this concern, until recently only patients who were haemodynamically stable on arrival at hospital were deemed suitable for EVAR. More recent published series of patients undergoing EVAR for RAAA have included more haemodynamically unstable patients and there is growing interest in EVAR in RAAA. In order to evaluate the viability of EVAR in RAAA two studies were performed at Leicester Royal Infirmary, one to determine the feasibility of obtaining a preoperative CT scan in patients with RAAA and the other to audit the early experience of EVAR in symptomatic and RAAA patients.

Feasibility of preoperative computerised tomography in RAAA

In order to investigate the concern that obtaining a preoperative CT scan to facilitate EVAR results in possibly dangerous delay in transferring patients with RAAA to theatre, I sought to determine the natural history of patients with RAAA who reached hospital but did not undergo surgery. A retrospective case note review was conducted of patients admitted to the Leicester Royal Infirmary with RAAA who did not undergo surgery because of advanced age or associated co-morbidity over an 8 year period between January 1995 and May 2003 to determine the time from admission to death and thus the potential time available for performing a CT scan. Patients were identified from review of all hospital death certificate records for this period. Sixty six patients were identified as having died of RAAA without undergoing surgery, although 6 patients in whom hospital notes could not be located and 2 patients who were in cardiac arrest on arrival at hospital and could not be resuscitated were

excluded from the study. The remaining 56 patients (33 male (59%), 23 female (41%)) had a median age of 85 years (range 71 to 98). Reasons for no operation being performed were; profound shock 9%, cardiac arrest 11%, poor quality of life 29%, malignancy 7%, cardiac disease 15%, respiratory disease 16% and advanced age in 14%. Median systolic blood pressure on admission was 110 mmHg, heart rate was 88 bpm and haemoglobin concentration was 10.5 g/dl. Median interval between onset of symptoms and admission to hospital was 2 hours 30 minutes (range 44 minutes to 36 hours) and the median interval between admission and death was 10 hours 45 minutes (range 1 hour 1 minute to 143 hours 55 minutes). Death within 2 hours of hospital admission occurred in 7 (12.5%) patients and 49 (87.5%) died later than 2 hours after admission. Figure 1.4 displays time from admission to death and Figure 1.5 is a survival curve showing cumulative survival following admission to hospital. Importantly, the majority of the surviving patients after 2 hours remained haemodynamically stable with a median systolic blood pressure of 104 mmHg (range 55 – 170). These data suggest that most patients with RAAA who reach hospital alive are sufficiently stable to undergo a CT scan and the consideration of EVAR.

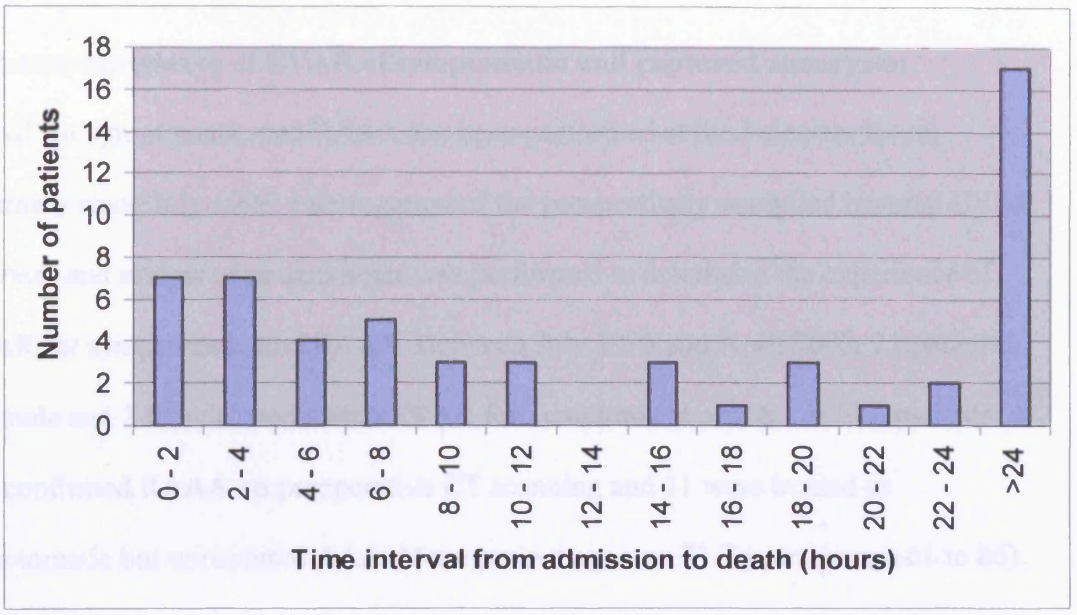


Figure 1.4. Time from admission to death in patients with RAAA not undergoing surgery showing that most patients survive longer than 2 hours.

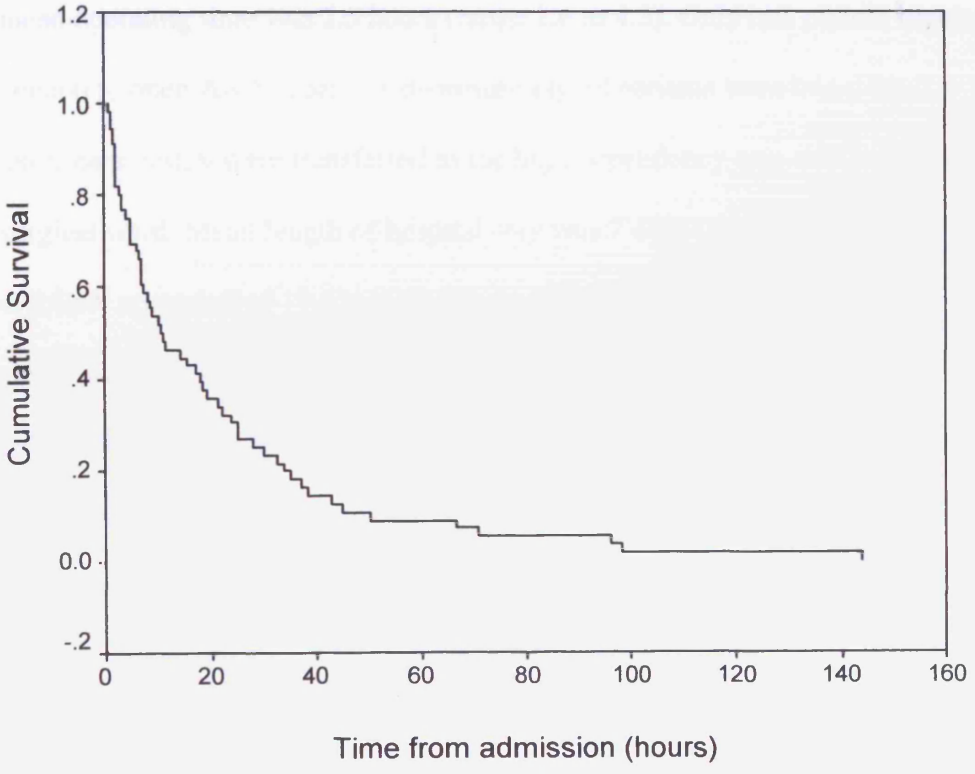


Figure 1.5. Survival curve showing cumulative survival following admission to hospital in patients with RAAA not undergoing surgery.

Leicester experience of EVAR of symptomatic and ruptured aneurysms

EVAR for symptomatic and RAAA has been performed at the Leicester Royal Infirmary since July 1996. Interrogation of the prospectively compiled hospital EVAR database and review of patient notes was performed to determine the experience of EVAR for symptomatic and RAAA. Between July 1996 and April 2003, 21 patients (19 male and 2 female) underwent EVAR for symptomatic or RAAA. Ten patients had confirmed RAAA on preoperative CT scanning and 11 were treated as symptomatic but unruptured AAA. Mean patient age was 73.7 years (range 61 to 86). Three patients had a systolic blood pressure < 100 mmHg on admission. In the patients with confirmed RAAA, the mean time interval between admission and commencement of surgery was 3.3 hours (range 1.5 to 6) and in the total 21 patients, the mean operating time was 2.5 hours (range 1.6 to 4.5). Only one patient required conversion to open AAA repair. Post-operatively 10 patients were transferred to the intensive care unit, 9 were transferred to the high dependency unit and 2 returned to the surgical ward. Mean length of hospital stay was 7 days (range 4 to 20). In the mean follow up period of 19.6 months (range 2 to 86) 4 type 1 and 4 type 2 endoleaks occurred, all the type 1 endoleaks were managed with further endovascular procedures. One peri-operative death, due to a myocardial infarction in the patient who required conversion to open AAA repair and 3 late deaths occurred. Although this is a small series from a single centre the results are very encouraging and compare favourably to the established operative mortality rates for symptomatic and RAAA.

Life expectancy of patients with AAA

Patients with AAA have a reduced life expectancy compared with the rest of the population, and this is largely a consequence of co-existing cardiovascular disease (Brewster J 2003). Approximately 66% of patients with AAA die from a cardiovascular event unconnected with the aneurysm (Powell JT 2001), usually coronary heart disease (CHD), and therefore the presence of AAA is seen as an indicator of high cardiovascular risk (Powell JT 2001). In 1980, Hertzer et al calculated that patients who have undergone AAA repair have an annual mortality rate from CHD of 1.9 to 3.9%, and on the basis of this, AAA is considered to be a CHD risk equivalent, a condition conferring a $\geq$ 20% 10 year risk of CHD (Hertzer NL 1980) (Adult Treatment Panel III 2001). More recent studies have reported similar cardiovascular mortality rates in patients with AAA; the UK Small Aneurysm Trial observed a 28% incidence of mortality due to cardiovascular causes over an 8 year period and 5 year survival rates of between 60 and 74% have been demonstrated in patients with AAA (UK Small Aneurysm Trial Participants 1998). Johnston et al reported a 5 year survival of 67.7% in patients who had undergone AAA repair compared with a 83.1% 5 year survival in a matched population without AAA (Johnston KW 1994). Brady et al in a prospective study utilising data from the UK Small Aneurysm Trial demonstrated that AAA diameter is an independent indicator of cardiovascular and all cause mortality, even after adjustment for cardiovascular risk factors such as smoking, blood pressure and ischaemic ECG changes (Brady AR 2001). In addition to limiting long term survival, cardiovascular disease is the leading cause of death in the perioperative period following AAA repair, accounting for 41% of perioperative deaths in the UK Small Aneurysm Trial (Brady AR 2000) (UK Small Aneurysm Trial Participants 2002).

Summary

AAA is a significant and increasingly important disease. Although a number of risk factors, including a genetic component have been clearly identified, the precise aetiology and mechanism of AAA formation is unknown. The proven benefit of AAA screening will result in the increasing identification of patients with AAA, many of whom will have AAAs too small to justify elective repair, providing a potential opportunity for intervention to control expansion in the future. At present there is no effective means of preventing AAA expansion, although future work may provide a pharmacological or genetic means of intervention. Large randomised controlled trials have provided evidence for which patients should be offered elective AAA repair and the inception of EVAR is an exciting advance in the treatment of both elective and ruptured AAA.

Chapter 2

Pathogenesis of Abdominal Aortic Aneurysms

Contents

Structure of the normal aortic wall

The abdominal aorta is a large fibroelastic conduit that receives, attenuates and distributes blood flow to the lower half of the body by passively distending in systole and recoiling in diastole. In order to perform this function throughout life, the abdominal aorta is a compliant yet extremely resilient structure.

In common with all arteries, the aortic wall is composed of 3 concentric layers; the tunica intima, tunica media and tunica adventitia. The tunica intima, the thin inner layer, is approximately 100μ in diameter and consists of a single layer of endothelial cells that rests on a thin basal lamina. Beneath the basal lamina is a layer of subendothelial supporting tissue containing collagen, elastin, fibroblasts and myointimal cells. The wide intermediate layer, the tunica media, has a diameter of about 500μ and contains a large amount of elastin, conferring high compliance. The elastin fibres are organised in long filaments that are cross-linked by the amino acids desmosine (DES) and isodesmosine (isoDES) (Carmo M 2002). The elastin filaments are arranged in approximately 50 concentric sheets or laminae separated by circumferential smooth muscle cell (SMC) layers, collagenous tissue and extracellular matrix (ECM) components. There is an extensive microfibrillar network surrounding the elastin largely composed of fibrillin. Fenestrations in the elastic lamellae allow diffusion of substances from the aortic lumen through the wall. The tunica adventitia, the outer layer of the aorta, is made up of fibroblasts and connective tissue comprised mostly of collagen arranged in longitudinal spirals although some elastin is also present. The collagen fibres are arranged in stable triple helical structures composed of 3 identical alpha chains, each containing about 340 repeating tripeptide sequences of -Glycine -X-Y-, where frequently X is proline and Y is hydroxyproline (Tromp G

1993). The collagen fibres are stabilised by non-reducible pyridinoline (PYR) and deoxypyridinoline (DPD) cross-links (Carmo M 2002). Collagen is the most abundant fibrous protein in the infrarenal aorta, and although 6 types are present, types I and III account for 90% of the aortic collagen (McGee GS 1991). Type 1 collagen makes up approximately two-thirds of the aortic collagen and confers tensile strength whereas the more extensile type 3 collagen accounts for approximately one-third of the aortic collagen and plays a part in resisting pulse pressure (Powell JT 1993). Small blood vessels, the vasa vasora are present in the adventitia and penetrate the tunica media, providing some, although probably limited blood supply to the adventitia and outer half of the tunica media.

The structural properties of elastin and collagen are complementary. Elastin is an extremely compliant protein that can double its length without deformity (MacSweeney STR 1994) and is essential for relaxation and recoil of the aortic wall during the cardiac cycle, although its tensile strength is limited (Bode MK 2002). At physiological aortic blood pressure the elastin fibres are responsible for bearing most of the haemodynamic load, but at higher pressures, collagen has been shown to be the principal load bearing structure (MacSweeney STR 1994) (Steinmetz EF 2003). Collagen has approximately 20 times the tensile strength of elastin (MacSweeney STR 1994) and it provides the strength of the aortic wall and prevents excessive stretching of the elastin fibres. Elastin is not synthesised in significant amounts in the adult aorta and has a half-life of about 70 years (Powell JT 1992) (Rucker RB 1977); collagen in contrast, is continually synthesised throughout life by the aortic SMC and fibroblasts (McGee GS 2001). The overall aortic collagen content is a product of continuous synthesis and degradation (Bode MK 2002).

SMC are the most abundant cell type in the aortic media, and along with fibroblasts have an important role in the synthesis of the ECM components; collagen, laminin, gelatin, proteoglycan and to a lesser extent in the adult aorta, elastin. Unlike muscular arteries, in which the SMC are innervated and can contract causing arterial constriction, the SMC in the aorta are not innervated to any great extent and therefore contribute little to the biophysical function of the aorta (Dobrin PB 1984).

Pathological changes in AAA

Until recently, AAA were thought to be a direct result of atherosclerotic degeneration, however over the last 20 years it has become evident that a complex interplay between proteolytic, inflammatory, mechanical, genetic, environmental and almost certainly other, as yet unknown processes are involved. The principal pathological features of AAA are a chronic mural inflammatory infiltrate and an imbalance between synthesis and proteolytic digestion of the structural components of the aortic wall, leading to destruction of elastin, collagen and SMC. The end result is a degenerative loss of aortic structural integrity and ability to withstand the expansile force of systolic contraction leading to dilatation and risk of rupture.

Although considerable advances have been made in the understanding of the pathogenesis of AAA, knowledge of the mechanisms involved is incomplete and still evolving. The current inability to satisfactorily reduce the rate of AAA expansion or rupture by pharmacological means is testimony to this. A significant difficulty in the investigation of the pathological changes and causative mechanisms that occur in human AAA is the reliance on animal models or human AAA tissue obtained during surgery. Surgically resected tissue represents end-stage disease and does not give

insight into the pathological changes that occur during the early stages of the disease, a number of years before surgery becomes necessary. Associations can be demonstrated but causative roles and chronology of pathological changes are more difficult to establish.

Fate of elastin and collagen in AAA

Loss of medial elastin is an early and key feature of AAA (White JV 1993) (Sakalihasan N 1993). Various studies have demonstrated a reduction in elastin content of between 63 and 92% (He CM 1994). Campa demonstrated that elastin comprises 35% of the dry weight of the normal aortic media but only 8% of the media in AAA (Campa JS 1987). In addition, the elastin cross-links desmosine and isodesmosine are reduced substantially in AAA, perhaps by as much as 90% (Carmo M 2002). Due to the relative inability of the adult aorta to synthesise mature elastin, this reduction is almost entirely due to elastolysis (Dobrin PB 1989). The amount of elastolytic activity in the aorta of patients with AAA is higher than in those with atherosclerotic occlusive aortic disease or normal aortas and elastolytic activity in the aortic wall is reciprocally related to elastin content (Patel MI 1996) (Campa JS 1987). Elastin damage and breakdown produces elastin-degradation products (EDP), and these peptide have been shown to upregulate secretion of proteases by SMC (Cohen JR 1992) and are chemotactic for the inflammatory cells; macrophages and neutrophils (Tilson MD 1992). Recruitment of these cells into the aortic wall further contributes to elastin loss by the secretion of inflammatory mediators and proteases.

The fate of collagen in AAA is more controversial; its content in AAA has been reported to be diminished (Sumner DS 1970) (Carmo M 2002), unaltered (Dubick

MA 1988) or increased (Menashi S 1987) (Rizzo RJ 1989) (Gandhi RH 1994). This may be related, in part, to the ability of SMC and fibroblasts in the aortic wall to synthesise new collagen as part of a reparative remodelling response to collagen damage or loss (Gandhi RH 1994). Increased collagen turnover is an important feature of AAA and consistently type 1 and 3 procollagen messenger RNA (mRNA) is found to be increased in AAA, implying increased collagen gene expression (Mesh CL 1992) (McGee GS 2001). In addition elevated procollagen peptides, markers of increased collagen production have been demonstrated in the systemic circulation of patients with AAA (Satta J 1995) (Satta J 1997). Carmo et al, in a study that reported less collagen in the walls of AAA than in normal aortas, discovered an increase in the cross-link components: PYR (by 350%) and DPD (by 100%) in AAA, suggesting that while the synthesis of new collagen may have stopped, the existing collagen fibres seemed to have accumulated compensatory cross-links (Carmo M 2002). Interestingly, it has been reported that the amount of type III collagen in the aortic walls of patients with a strong family history of AAA is lower than that in 'sporadic' or non-familial AAA, suggesting a possible collagen gene defect in some patients with AAA (Powell JT 1991).

Insight into the consequences of aortic elastin and collagen loss has been obtained by experimentally digesting elastin and collagen with elastase and collagenase respectively. Dobrin et al discovered that treating segments of artery in vitro with elastase resulted in marked dilatation but not rupture, a phenomenon duplicated in in – vivo animal studies (Dobrin PB 1984). In addition, the aortic compliance decreased, which is thought to be due to the previously unstretched collagen fibres taking a proportion of the wall strain to compensate for the lost elastin. Treating similar

segments of artery with collagenase caused vessel rupture but only slight dilatation (Dobrin PB 1984). Thus, the final event in AAA enlargement and rupture seems to be related to the failure of collagen to resist the pressure in the aorta (Bode MK 2002).

As elastin is destroyed and the tensile stress in the aortic wall is shifted onto collagen (MacSweeney STR 1992) (Campa JS 1987), it appears that increased collagen synthesis is necessary to stabilise the structure and integrity of the aortic wall (Satta J. 1995) (Satta J 1997) (Huffman MD 2000). The cyclic strain on the aortic wall during the cardiac cycle seems to act as a stimulus for collagen synthesis by a number of cells including the SMC (Steinmetz EF 2003). It has been suggested that in small AAAs, collagen synthesis counteracts degradation, but in larger AAA, collagen degradation predominates leading to rapid expansion and rupture (Wassef M 2001). Progressive SMC loss is an established feature of AAA, and it is plausible that as SMC are lost, the remaining cells may not be able to synthesise new collagen at a rate sufficient to replenish the degraded collagen fibres, leading to further expansion and eventual rupture (Dobrin PB 1984) (Dobrin PB 1994). The discrepancies in the reported collagen content in AAA may be a consequence of this. In the later stages of AAA formation, thickening and fibrosis of the adventitia occurs, largely due to the deposition of new collagenous fibres. Significantly, it appears that these new fibres are more soluble than normal collagen and may be more susceptible to degradation (Sakalihasan N 1993) (Freestone T 1995).

Fate of smooth muscle cells in AAA

A further important pathological feature of AAA is a thinning of the tunica media with a concomitant loss of SMC (Annabi B 2002). One study found a 74% reduction

of SMC in AAA compared with normal aortas (Lopez-Chandales A 1997). Apoptosis (Greek: 'falling off') is a process whereby programmed cell death occurs to regulate tissue mass or architecture and apoptosis has been shown to be important in the SMC loss in AAA (Rowe VL 2000). SMC in AAA tissue express the transmembrane molecule Fas (CD 95) that induces apoptosis, providing evidence that aortic SMC are capable of undergoing apoptosis (Henderson EL 1999). The release of cytotoxic mediators, such as proinflammatory cytokines by infiltrating inflammatory cells has been identified as one of the principal mechanisms responsible for the apoptosis of aortic SMC (Thompson RW 1997) (Henderson EL 1999) (Walton LJ 1999). Additionally, there is evidence that SMC from AAA may have reduced replicative potential compared with SMC from normal aortas. Cultured SMC from AAA may exhibit a pattern of accelerated replicative senescence, which does not happen to SMC from normal arteries (Liao S 2000). This suggests that SMC in AAA may age rapidly and exhaust their replicative potential early in the disease process (Thompson RW 2002). The loss of aortic SMC has been proposed as a mechanism by which insufficient synthesis of matrix proteins needed for repair occurs (Henderson EL 1999). However, SMC can also participate in AAA formation through the production of matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9), potent proteolytic enzymes that can degrade elastin and collagen and have been strongly implicated in the pathogenesis of AAA (Crowther M 1996) (Patel MI 1996).

Inflammatory infiltrate

A marked cellular inflammatory reaction characterised by the infiltration of mast cells, monocytes / macrophages (Reilly JM 1996) and B and T – lymphocytes predominantly into the adventitia and outer media of the aortic wall occurs in AAA,

although the inflammation can be transmural (Brophy CM 1991) (MacSweeney ST 1994) (Koch AE 1990). These cells play a key role in the degradation and loss of collagen, elastin, and SMC by the secretion of proteases, protease inhibitors and the important inflammatory mediators, cytokines (Shah PK 1997) (Reilly JM 1996). AAA, unlike normal aortas contain $CD4^+$ and $CD8^+$ T cells that express perforin and Fas, cytotoxic mediators that induce apoptosis. Rabbit studies have demonstrated that the local application of $CaCl_2$ to the aortic adventitia induces a cellular inflammatory response followed by aneurysmal dilatation, implying that inflammatory cells can initiate aneurysm formation (Freestone T 1995). Furthermore, the extent of the adventitial inflammatory infiltrate correlates with both AAA diameter and rate of AAA expansion (Freestone T 1995) (Anidjar S 1992) (Rasmussen TE 2002).

Although the presence and significance of an inflammatory infiltrate in AAA is undisputed, the cause is unclear and a number of theories have been proposed. An infective aetiology has been advocated on the basis of immunohistochemical evidence of Chlamydia pneumoniae infection in as many as 55% of AAA (Juvonen T 1997). Alternatively, it has been suggested that the products of elastin breakdown, such as EDPs or the local production of chemotactic cytokines (chemokines) may be a trigger for the recruitment of infiltrating macrophages (Steinmetz EF 2003). Other theories put forward are that extension of intimal atherosclerosis or the infiltration of oxidised lipid into the aortic media may instigate the inflammatory reaction (Davies MJ 1998) or that an autoimmune reaction may be involved (Gregory AK 1996).

Cytokines

Cytokines are a family of low molecular weight proteins that are important mediators of inflammation and the immune response. Cytokines are synthesised and secreted by SMC in the aortic wall and by infiltrating T cells, B cells and macrophages in the mural inflammatory infiltrate (Newman K 1994). Activated T cells, the main producers of cytokines, are conventionally divided into T helper (Th) Th1 and Th2 subtypes based on the cytokine type they produce. Th1 cells produce inflammatory cytokines such as interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-alpha), and interferon gamma (IFN-gamma), whereas Th2 cells produce anti-inflammatory cytokines such as interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 10 (IL-10).

Increased concentrations of the proinflammatory cytokines; IL-1beta, IL-6 and TNF-alpha have been demonstrated in the aortic wall and systemic circulation of patients with AAA (Newman K 1994) (Juvonen T 1997). Plasma IL-6 concentration has been found to be higher in the iliac artery than the brachial artery in patients with large or inflammatory AAA indicating that the aneurysm itself is a source of circulating IL-6 (Jones K 2001). Elevated circulating IL-6 has been found to correlate with abdominal aortic diameter in patients without AAA, raising the possibility that IL-6 may be involved in early aortic dilatation (Rohde LEP 1999). Serum IL-8 and IFN-gamma concentrations correlate with AAA diameter and rate of AAA expansion respectively (Treska V 2000) (Juvonen T 1997) and serum concentration of macrophage migration inhibitory factor (MIF) has been shown to correlate with both AAA size and rate of expansion (Pan J-H 2003).

Proinflammatory cytokines, such as IL-1beta, IL-6 and TNF-alpha participate in a number of mechanisms implicated in AAA formation. As a group they can inhibit the expression of type I and III collagen and promote the degradation of elastin and collagen by increasing the expression of MMPs, and inhibiting the expression of important inhibitors of MMPs, the tissue inhibitors of matrix metalloproteinases (TIMPS) (Siwik DA 2000) (Newman K 1994) (Juvonen J 1997). In addition cytokines can promote apoptosis and have been shown to kill cultured human SMC (Geng YJ 1996). A subgroup of cytokines, the chemokines have chemotactic properties and play a role in the migration of inflammatory cells into the AAA wall and their subsequent retention. Gene expression of the potent chemokine, interleukin - 8 (IL-8) has been shown to be upregulated in AAA (Armstrong PJ 2002).

The actions of proinflammatory cytokines are balanced by those of their anti-inflammatory counterparts such as IL-4 and IL-10. Antiinflammatory cytokines are also produced by inflammatory cells in AAA, probably in an attempt to restore a physiological balance in response to heightened proinflammatory cytokine activity (Schonbeck U 2002). IL-10 is the primary product of activated Th2 cells and is a potent anti-inflammatory cytokine. IL-10 diminishes the production of proinflammatory cytokines and has been demonstrated to suppress MMP production by human mononuclear phagocytes in vitro (Lacraz S 1995). In addition, IL-10 potently stimulates TIMP-1 biosynthesis at both protein and gene levels, thereby inhibiting MMP action (Lacraz S 1995). It has been shown that the serum IL-10 concentrations are significantly lower in patients with unstable angina compared with patients with stable angina, suggesting that reduced levels of IL-10 favour atherosclerotic plaque instability and rupture (Smith DA 2001).

Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of 25 Zn^{2+} and Ca^{2+} dependent proteinases that are produced by resident aortic wall cells such as SMC (Patel MI 1996) and fibroblasts, and also by infiltrating inflammatory cells, particularly macrophages and lymphocytes. Conventionally the MMPs are divided into 4 categories; collagenases, stromelysins, gelatinases (or type 4 collagenases) and membrane-type MMPs (MT-MMPs) on the basis of substrate specificity, and as a family, are capable of degrading most components of the ECM, including elastin and collagen (Wills A 1996). MMPs have a physiological role in wound healing and tissue repair, and have been implicated in the pathogenesis of a number of conditions including; rheumatoid arthritis, tumour invasion, osteoporosis, rupture of atherosclerotic plaques, as well as AAA. Table 2.1 displays the MMPs important in AAA formation, their primary substrate and cell type of production.

MMP	Primary substrate	Cell type of production
MMP-1	Collagen (type I, III)	Epithelial, inflammatory, mesenchymal
MMP-2	Collagen type IV, elastin	SMCs, fibroblasts
MMP-3	Collagen	
MMP-9	Elastin, collagen	Macrophages, SMCs
MMP-12	Elastin	Macrophages
MMP-13	Collagen type IV	SMCs

Table 2.1. Summary of MMPs implicated in AAA formation along with their primary substrate and cell type of production
(Ailawadi G 2003)

MMPs are regulated at the gene expression, post transcription and protein levels. A number of growth factors and cytokines, particularly IL-1, platelet derived growth factor (PDGF) and TNF-alpha modulate MMP gene transcription (Loftus IM 2002). MMPs are secreted as inactive propeptides, with a shielded catalytic domain (Morgunova E 1999) and enzymatic activation is necessary for MMP activation (Annabi B 2002). An amino-terminal sequence is cleaved by plasmin, the plasmin generating enzymes tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), oxygen free radicals and MT1-MMP (Lehti K 1998). MT1-MMP has been localised to SMC and macrophages (Nollendorfs A 2001) and is an important activator of MMP-2 in vivo (Crowther M 1996). A positive feedback loop exists whereby plasmin activates MMP-1, 3 and 9 directly. MMP-3 then activates further MMP-1, which in turn activates MMP-9 (Dollery CM 1995). Unopposed MMP action leading to uncontrolled degradation of the ECM is prevented by tight regulation and inhibition by tissue inhibitors of metalloproteinases (TIMPS), of which there are 4 types (TIMP1, 2, 3 and 4). TIMPS form non-covalent stoichiometric complexes with MMPs and inhibit their action (Lijnen HR 2003) thereby preventing nonspecific proteolysis (Murphy G 1995) (Loftus IM 2002).

Increased production of MMPs occurs in the media and adventitia of AAA. MMPs 1 (interstitial collagenase), 2 (gelatinase A), 3 (stromelysis-1), 9 (gelatinase B) and 12 (macrophage elastase) are highly effective elastases and collagenases and have been implicated strongly in the pathogenesis of AAA. Their quantity has been shown to be increased at the mRNA and protein level in the wall of AAA compared with non-aneurysmal aortas (Annabi B 2002) (Newman K 1994) (Lijnen HR 2003) (Tung WS 2001) (Patel MI 1996) (Curci JA 1998). Intriguingly, this has not been a consistent

finding, and the expression of MMP-2 has actually been found to be lower in AAA than in normal aorta by Annabi, perhaps reflecting the loss of MMP producing SMC, and a changing MMP profile with AAA size (Annabi B 2002) (McMillan WD 1999). Significantly, Carrell et al demonstrated that MMP-3 (Stromelysin-1) is upregulated in AAA tissue compared with aorta affected by atherosclerotic occlusive disease, a trend not seen in other MMPs in the study (Carrell TW 2002). In addition to degradation of EMC proteins such as elastin and collagen, MMP-3 is a potent posttranscriptional activator of other MMPs, by the activation of the latent proenzyme. In addition to the increased MMP-9 gene expression and protein levels in AAA tissue, elevated plasma levels of MMP-9 have been demonstrated in patients with AAA, the concentration being associated with AAA size and rate of expansion (Lindholt JS 2000) (Armstrong PJ 2002) (McMillan WD 1999). The importance of MMPs in AAA has been highlighted by the following additional observations. Synthetic MMP inhibitors and recombinant TIMP-1 have been shown to prevent AAA formation and growth in rat models (Annabi B 2002) and mice deficient in the gene for MMP-9 are resistant to elastase induced AAA formation (Longo GM 2002).

The precise localisation of MMPs in the aortic wall may be important; MMP-2 is predominantly found in the intimal atherosclerotic plaque and media, whereas MMP-9 is preferentially localised to adventitial macrophages (Newman K 1994) (Freestone T 1995). MMP-2 has much higher activity against elastin than MMP-9 (Freestone T 1995). Freestone et al found higher concentrations of MMP-2 in small AAA, compared with higher levels of MMP-9 in larger AAA (Freestone T 1995) and McMillan found that mRNA expression of AAA is related to size (McMillan WD

1997). This supports the theory that early degradation of elastin is important for AAA formation.

The increased proteolysis in AAA can be a result of increased activity of proteolytic enzymes or a deficiency of antiproteolytic systems (Tilson MD 1993). Recent studies have demonstrated a rise in both MMPs and TIMPS in AAA tissue, suggesting that TIMP upregulation may occur in a physiological attempt to counteract increased MMP activity (Knox JB 1997) (Carrell TW 2002) (Tung WS 2001) (Annabi B 2002). As yet it is unknown whether this increase in TIMP levels is sufficient to properly inhibit the increased MMP activity. However, increased TIMP levels in AAA tissue have not been a consistent finding; Knox et al discovered no difference between MMP and TIMP expression in patients with AAA or occlusive disease (Knox JB 1997) and Brophy et al reported decreased levels of TIMPs in the walls of AAA (Brophy CM 1991). It is unclear whether this difference is a reflection of variable production of TIMPs or a consequence of binding to MMPs (Davies MJ 1998). The balance between MMPs and TIMPs is further complicated by the finding that TIMPs can also induce apoptosis in cells including SMC (Brew K 2000).

Fibrinolytic system

The fibrinolytic system is known to be involved in ECM proteolysis in AAA. The amount of the serine proteases (proteolytic enzymes with serine and histidine in the active centre), plasmin and u-PA and t-PA are known to be higher in AAA than normal aortas, a consequence mainly of macrophage secretion (Jean-Claude J 1994) (Reilly JM 1996) (Saito S 2002). Plasmin is generated from plasminogen, an inactive zymogen, by the action of t-PA or u-PA. In turn, t-PA and u-PA are regulated and

inhibited by the plasminogen activator inhibitors (PAI), PAI-1 and PAI-2 (Rossaak JI 2000). Plasmin activates MMP zymogens and is also capable of limited direct degradation of the ECM (Jean-Claude J 1994) (Shah PK 1997). Reilly et al demonstrated relative fibrinolysis attributable to t-PA was higher in AAA and aortas affected by atherosclerotic occlusive disease compared with normal aortic specimens (Reilly JM 1994). This suggests that the fibrinolytic system may have different roles in AAA and aortic occlusive disease. In AAA its role appears to be the destruction of the ECM and aortic media whereas it causes the accumulation of matrix in occlusive disease. The importance of the fibrinolytic system has been demonstrated in an animal model in which an acellular guinea pig aorta was used as a xenograft in a rat. An aneurysm rapidly developed, however when the graft was seeded with rat SMC overproducing PAI-1, the aorta did not become aneurysmal. This demonstrates that decreased levels of MMPs induced by overexpression of PAI-1 can prevent AAA formation. The apolipoprotein-E (ApoE) deficient mouse model has also been used in the investigation of u-PA in AAA. ApoE deficient mice are hyperlipidaemic and develop AAA (Breslow JL 1996). ApoE deficient mice with targeted deletion of the uPA gene do not develop AAA however (Carmeliet P 1997).

Cysteine proteases

Cathepsin S and cathepsin K are cysteine proteases (proteolytic enzymes with the amino acid cysteine at their active centre) with potent elastinolytic activity that are produced by vascular SMC and macrophages (Reddy VY 1995) and have been shown to be active in atherosclerotic arterial wall remodelling (Wassef M 2001). Cysteine proteases have a number of inhibitors, of which cystatin C is the most abundant and appears most important (Shi GP 1999). Cultured vascular SMC stimulated by the

proinflammatory cytokines, IFN-gamma and IL-1beta, which have been implicated in AAA, secrete cathepsin S (Sukhova GK 1998). An association between cystatin C and AAA has also been demonstrated. Reduced levels of cystatin C have been demonstrated in both patients with atherosclerosis and AAA (Shi GP 1999). Lindholt and colleagues showed that cystatin C is negatively associated with AAA size and also with AAA expansion, a mechanism that is likely to be due to the failure of cysteine protease inhibition (Lindholt JS 2001).

Haemodynamic and aortic wall factors

The abdominal aorta is the commonest site in the body for aneurysm formation, and approximately 90% of AAA arise in the infrarenal section of the aorta. A number of haemodynamic factors and physical properties of the aortic wall may account for this. There is a gradual decrease in the elastin to collagen ratio as the aorta descends from the thorax to the aortic bifurcation, and a large decrease in elastin occurs several centimetres below the renal arteries (Steinmetz EF 2003). The anterior-posterior diameter of the aorta tapers near the bifurcation and this, combined with the reduced compliance caused by the low mural elastin content results in high pulse pressure and increased wall tension (pressure perpendicular to the aortic wall) (McDonald DA 1974). The aortic bifurcation and large branches such as the mesenteric and renal arteries cause turbulent blood flow and reflected pressure waves, further increasing wall tension. The physical stretching of the aortic wall has been implicated in the activation of MMPs (Steinmetz EF 2003) (Moore JE 1992) and the abdominal aorta has been shown to have increased MMP-9 expression and activity compared with the thoracic aorta (Ailawadi G 2003). Clearly however, pressure and flow factors can only act as an adjunct to other mechanisms active in AAA formation as there is little

to suggest that they are in any way different in patients with AAA compared to those who have atherosclerotic aortic disease without aneurysmal dilatation (Powell JT 1989). In addition, as the cylindrical structure of the aorta tends towards a spherical configuration in AAA, the mural pressure is reduced. Laplace's law, which states that in a cylindrical structure such as a normal artery, circumferential wall tension is directly proportional to the diameter and flow pressure, and inversely proportional to the thickness of the wall is frequently used in arterial physiology calculations. In AAA, the transformation of the aorta from an essentially cylindrical structure to one approaching a sphere is important, as this change has been shown to reduce the expected wall tensile stress to less than half that expected in a cylindrical structure of equivalent diameter (Steinmetz EF 2003).

Wall hypoxia has also been proposed as a mechanism contributing to AAA formation. In the thoracic section of the aorta, the vasa vasora are plentiful and are an important blood supply to the thoracic aortic wall. However, in the abdominal aorta discrete vasa vasora end near the renal artery orgins, and distal to this, the vasa vasora are sparse and probably contribute little to wall perfusion. The infrarenal abdominal aorta therefore relies almost entirely on diffusion from the lumen for supply of oxygen and nutrients (Steinmetz EF 2003). Atherosclerosis has a predilection for the abdominal aorta and atherosclerotic thickening of the intima inhibiting diffusion of oxygenated blood from the aortic lumen into the wall has been cited as a possible mechanism by which the aortic wall becomes hypoxic and could be a factor in AAA formation (Steinmetz EF 2003).

In addition to these physical features of the aortic wall, SMC from the abdominal aorta and thoracic aorta appear to have differing replicative potential, which may account for the higher incidence of aneurysms of the abdominal aorta. Powell et al demonstrated that SMC from the thoracic aorta grow well in culture and replicate approximately 20 times whereas those from the abdominal aorta only replicate 5 or 6 times, suggesting that SMC from the thoracic aorta may be better able to repair injury and are less prone to depletion (Powell JT 1989).

Reactive oxygen species and oxidative stress

There is growing interest in the role of oxidative stress caused by the production of reactive oxygen species (ROS) such as superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) in the pathogenesis of AAA. ROS are produced by inflammatory cells and aortic SMC and have a number of pro-aneurysmal activities including activation of MMPs (Rajagopalan S 1996), inhibition of PAI-1 (Satriano JA 1993), upregulation of inflammatory cytokines (Nakahashi TK 2002) and induction of vascular SMC apoptosis (Li PF 1997) (Tan S 1998). ROS have been shown to be higher in AAA tissue than adjacent non-aneurysmal aorta in the same patient (Miller FJ 2002). Insight into the role of oxidative stress in AAA formation has been gained in a rodent model where experimentally induced AAA caused by infusion of porcine elastase results in a 50-fold increase in inducible nitric oxide synthase gene expression, followed by a downregulation in expression of superoxide dismutase, a potent antioxidant (Yajima N 2002).

A number of anti-oxidative and anti-inflammatory agents such as heme oxidase-1 (HO-1) reduce oxidative stress (Dalman RL 2003). HO-1 degrades heme to biliverdin,

releasing free iron and carbon monoxide (CO). Biliverdin is an efficient scavengers of ROS (Yajima N 2002) (Nakahashi K 2002) and CO has an anti-inflammatory function in the arterial wall (Schillinger M 2002). Interestingly, plasma levels of vitamin E, an antioxidant have been found to be reduced in patients with AAA compared to those with coronary artery disease but no AAA, suggesting an imbalance between oxidative and anti-oxidative factors (Sakalihasan N 1996).

The expression of many of the genes involved in oxidative stress are dependent on haemodynamic conditions (Steinmetz EF 2003) (Dalman RL 2003). Increased laminar blood flow and consequently shear stress (pressure parallel to the aortic wall) induces expression of the antioxidants superoxide dismutase (Topper JN 1996) and HO-1 (Tobiasch E 2001). Nakahashi et al demonstrated in an experimental rodent model that increased aortic wall shear stress was associated with a reduction in ROS and slowed aortic dilatation (Nakahashi K 2002). In contrast, turbulent or non-laminar blood flow damages endothelial cells and increases oxidative stress by stimulating the production of ROS (Taylor CA 1999) (De Keulenaer GW 1998).

Nitric oxide (NO) has also been implicated in the pathogenesis of AAA. NO is a gas with a half life of seconds and is formed by the oxidation of L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS), one form of which, inducible NOS (iNOS) can significantly increase the production of NO (Zhang J 2003) (Barbato JE 2004). NO is rapidly oxidised to nitrite and nitrate forms, and nitrite ions have been shown to be involved in elastin damage (Davies MG 1995) (Paik D 1996), oxidative injury, cytokine and MMP upregulation (Lee JK 2001) and apoptosis in SMC (Zhang J 2003). The production of iNOS and nitrite ions are increased in AAA

tissue (Zhang J 2003). There is some evidence from animal studies that inhibition of NOS reduces NO production and AAA expansion, although this is disputed (Lee JK 2001).

Renin-angiotensin-system

Angiotensin II (Ang II) is converted from angiotensin I (Ang I) by the actions of angiotensin converting enzyme (ACE) and chymase-like enzyme. Ang II has been implicated in atherosclerosis and has been shown to be involved in inflammatory processes. In murine studies the infusion of Ang II results in AAA formation in hypercholesterolaemic mice (Daugherty A 2000), possibly by upregulation of u-PA (Wang Y 2001). In addition, Ang II stimulates the production of ROS by SMC and can induce apoptosis, two further pro-aneurysmal processes (Griendling K 1994) (Dimmeler S 1997). The ACE and chymase-like activity in AAA have been demonstrated to be higher than in normal aortas (Tsunemi K 2002). In addition there is some evidence that blocking the production of Ang II with ACE inhibitors inhibits AAA expansion in elastase induced rat AAA models (Liao S 2001). Figure 2.1 summarises the mechanisms known to be important in AAA formation.

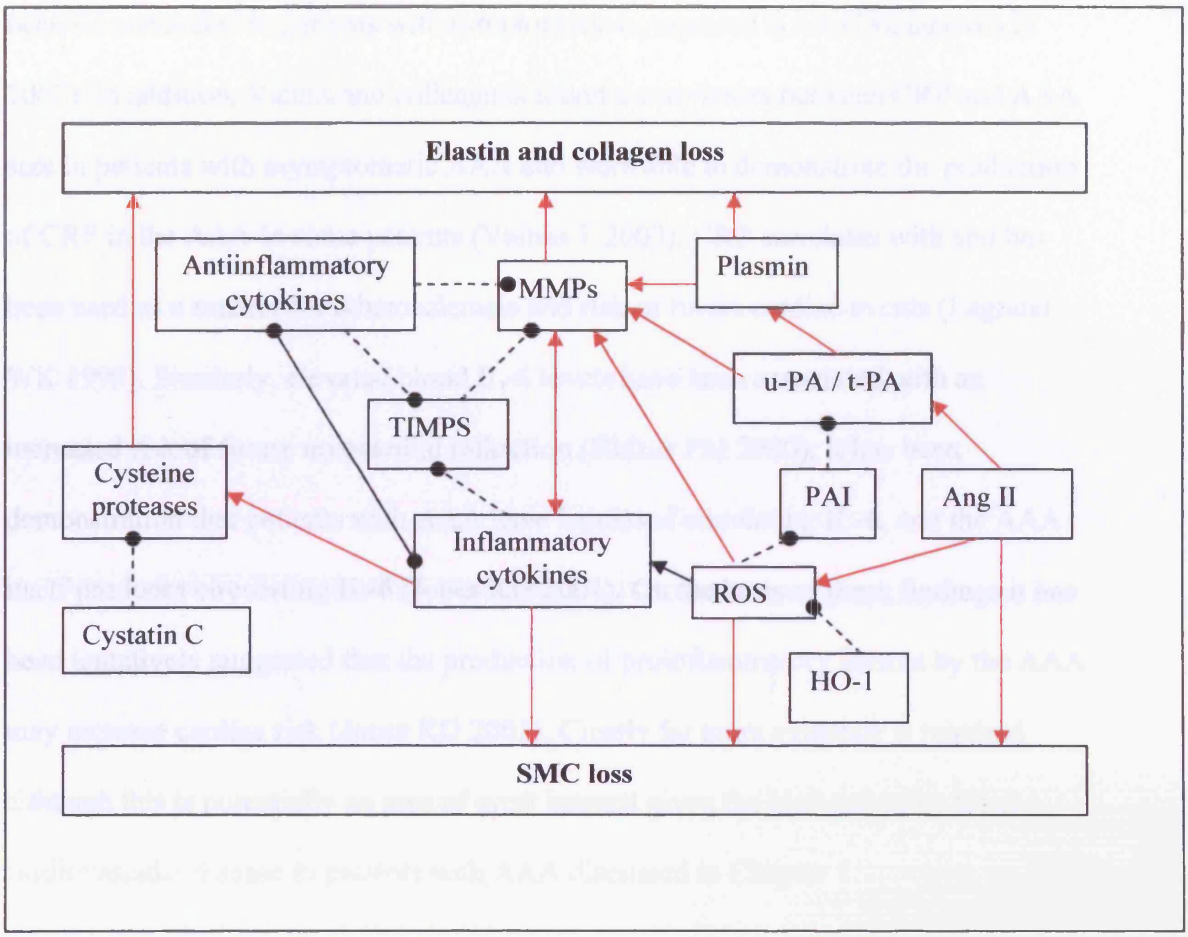


Figure 2.1. Summary of interaction between mechanisms involved in AAA formation leading to elastin, collagen and smooth muscle cell loss.

Key
► activates
● inhibits
MMP: matrix metalloproteinase
TIMP: tissue inhibitor of matrix metalloproteinase
ROS: reactive oxygen species
Ang II: angiotensin II
u-PA: urokinase – type plasminogen activator
t-PA: tissue – type plasminogen activator
PAI: plasminogen activator inhibitor
HO-1: heme oxidase - 1

AAA and cardiac disease

Powell et al, as long ago as 1987 demonstrated that the acute phase protein, C-reactive protein (CRP) is higher in the serum of patients with asymptomatic AAA than those with atherosclerotic occlusive disease (Powell JT 1987) and elevated CRP has since

been demonstrated in patients with symptomatic or ruptured AAA (Domanovits H 2002). In addition, Vainas and colleagues found a correlation between CRP and AAA size in patients with asymptomatic AAA and were able to demonstrate the production of CRP in the AAA in some patients (Vainas T 2003). CRP correlates with and has been used as a marker for atherosclerosis and risk of future cardiac events (Lagrand WK 1999). Similarly, elevated blood IL-6 levels have been associated with an increased risk of future myocardial infarction (Ridker PM 2000). It has been demonstration that patients with AAA have increased circulating IL-6, and the AAA itself produces circulating IL-6 (Jones KG 2001). On the basis of these findings it has been tentatively suggested that the production of proinflammatory factors by the AAA may increase cardiac risk (Jones KG 2001). Clearly far more evidence is required although this is potentially an area of great interest given the high mortality from cardiovascular disease in patients with AAA discussed in Chapter 1.

Evidence for systemic disease

Although elevated levels of circulating proinflammatory cytokines, MMP-9 and CRP have been reported in patients with AAA, most of the mechanisms discussed have only been investigated in the setting of the AAA wall. However, arterial aneurysms are often multiple and up to 7% of patients with AAA have peripheral aneurysms (Sandgren T 2001). Ward discovered that dilated arteries were found at sites not commonly affected by atherosclerosis in patients with AAA and it has been postulated that this may reflect a generalised disorder of connective tissue in patients with AAA (Ward AS 1992). Loftus demonstrated that 40% of infragenicular vein bypass grafts for popliteal aneurysms developed aneurysms compared with only 2% of equivalent grafts performed for atherosclerotic occlusive disease (Loftus IM 1999). This could

reflect a systemic disorder of connective tissue or perhaps a systemic imbalance of proteolytic enzymes in patients with aneurysms.

The genetic component to AAA formation

Although AAA phenotype only becomes evident in late adult life, there appears to be a strong familial and almost certainly genetic component to the aetiology of AAA. Indeed, AAA has been described as one of the most frequent 'familial' diseases (Verloes A 1996). Current understanding of the genetic background to AAA formation is based on the familial clustering of AAA, and the results of candidate gene and linkage analysis studies.

Familial aneurysms

Clifton reported the first familial clustering of AAA in 1977; 3 brothers with ruptured AAA, who were not known to have an underlying familial connective tissue disorder (Clifton MA 1977). This was followed by numerous reports of high familial incidence of AAA. Bengtsson et al performed abdominal ultrasound examinations of siblings of patients with AAA and found that 29% of the brothers and 6% of the sisters had an AAA (Bengtsson H 1989). Webster et al discovered that 13% of siblings of patients with AAA were similarly affected (Webster MW 1991). Johanssen found that 19% of patients with AAA had an affected first degree relative, compared with 2% in a control group without AAA drawn from the same population (Johanssen K 1986). Other studies have consistently demonstrated that as many as 20% of patients with AAA are known to have a first-degree relative with an AAA (Reilly JM 1989) (Norrgard O 1984). This is significantly higher than the recognised autopsy or screening prevalence. Overall, there is approximately a fourfold increased risk of

having an AAA for a brother of a patient with AAA (Baird PA 1995), providing convincing evidence that there is a familial and likely genetic component to AAA formation.

Differences in familial AAA

There is evidence that 'familial' AAA occur at an earlier age and are more likely to rupture than non-familial cases (Baird PA 1995) (Powell JT 1987) (Darling RC 1989). The prevalence of AAA amongst relatives of female patients with AAA is higher than amongst relatives of male patients and it is thought that women with AAA are more extreme deviants from the normal population and the risk to their relatives is correspondingly higher (Powell JT 1987).

Difficulties in genetic analysis

The study of genetic mechanisms involved in AAA formation is complicated by a number of factors. AAA phenotype only becomes apparent in late adult life, and therefore it is unusual to find two or more generations of a family with AAA still alive. This combined with the limited use of autopsies and poor public awareness of AAA makes the construction of genetic pedigrees difficult. The strong association between AAA and risk factors such as smoking and hypertension means that any genetic susceptibility to AAA formation may be modified or confounded by these risk factors. Genes related to AAA with a low penetrance (expression of genetic trait in individual with mutant genotype) further complicate the issue as many patients with AAA related genes may never develop AAA.

Mode of inheritance

A number of modes of inheritance have been proposed in individual families in whom several members have AAA, although no one mode of inheritance has been shown to be universally applicable in different families (Verloes A 1995) (Kuivaniemi H 2003). Powell reported a 7.8% prevalence of AAA amongst first degree relatives of patients with AAA. The proposed genetic explanation was that the disorder is inherited in a multifactoral fashion with an estimated 70% heritability (disease genotype being expressed as disease phenotype), with contributions from several genes combined with environmental factors (Powell JT 1987). Tilson et al found no consistent mode of inheritance in a series of 50 families affected by AAA although autosomal dominant, recessive, multifactoral or X-linked mechanisms were possible in individual families (Tilson MD 1984). In 12 of the families an autosomal dominant pattern could potentially explain the inheritance. However, the fact that 39 male offspring were affected compared with 2 female offspring casts doubt on this mechanism (Tilson MD 1984). It was suggested that there may be both an X-linked and autosomal dominant form of the disease or perhaps a multigene mechanism. Verloes et al collected familial data on 324 probands, affected individuals from whom a pedigree is constructed, with AAA, and found that 81 belonged to multiplex pedigrees, i.e. had an affected relative. Segregation analysis (the comparison of proportion of affected offspring with expected proportion according to a particular genetic hypothesis) suggested that the most likely mode of inheritance was a single gene effect with dominant inheritance, and it was calculated that the frequency of the morbid allele was 1:250 with a penetrance of 0.4 (Verloes A 1995). Kuivaniemi et al in a study of 233 families in whom at least 2 members had an AAA found that in 72% of the families an autosomal recessive inheritance was possible on the grounds that the affected members did not

have an affected parent; in 25% of the families autosomal dominant inheritance was suggested on the basis that the cases had an affected parent (Kuivaniemi H 2003). In the remaining families an autosomal dominant inheritance with incomplete penetrance (expression of genetic trait in individual with mutant genotype) was considered likely. The diversity in mode of inheritance of AAA in these families would support different genetic mutations being involved or a multifactorial pathological process.

Candidate gene approach

The candidate gene approach involves the search for mutations in genes thought to be important in AAA formation. Knowledge of the mechanisms involved in AAA formation allows candidate genes to be characterised as those involved in collagen, elastin or other matrix component production or those involved in the regulation of proteases and their inhibitors (Tilson MD 2002). There are however an enormous number of candidate genes that can be examined. In addition, post-translational modifications of the gene product rather than abnormalities at the gene transcription level, that could lead to AAA formation, are not identified by examining candidate genes. The recent inception of complementary DNA (cDNA) microarrays has made it easier to examine or 'screen' the expression of thousands of genes simultaneously. Combining cDNA microarrays with Northern blotting or quantitative reverse-transcription polymerase chain reaction (RT-PCR) determines whether changes in gene transcription result in change at the mRNA and protein level, and therefore the likelihood of a gene having an important effect (Thompson RW 2002).

Elhers-Danlos syndrome type 4 and Marfan syndrome

Aortic weakening and rupture are seen in two syndromes associated with mutations in genes coding for two of the connective tissue components of the aortic wall: fibrillin (a large glycoprotein that appears to provide a scaffold for elastin deposition in the ECM) in Marfan syndrome and type III collagen in Ehlers-Danlos syndrome type IV (Harpers Biochemistry 2003). These diseases stimulated early interest in genetic defects in connective tissue components of the aortic wall as a possible cause of AAA. Marfans is an autosomal dominant disease caused by mutations in the fibrillin–1 gene, located on chromosome 15 and has a prevalence of at least 6 to 10 per 100,000 (Dietz HC 1994). The mutations result in loss and fragmentation of elastic fibres (Towbin JA 1999). Typical cardiovascular manifestations are ascending aortic aneurysm and dissection, valvular regurgitation, and less frequently AAA (Pyeritz RE 1979). Ehlers-Danlos syndrome type IV is also inherited in an autosomal dominant fashion and is caused by mutation in the type III collagen gene (COL3A1) that prevents the conversion of procollagen to collagen (Superti-Furga A 1989). Ehlers-Danlos is characterised by multiple aneurysms, aortic dissection and death from rupture of the aorta. Although the features of Marfan syndrome and Ehlers-Danlos syndrome type IV are well described, the conditions result from heterogeneous mutations in the fibrillin-1 and type III collagen genes respectively. The same mutation is rarely duplicated in different affected families, indicating that numerous mutations result in the same phenotype.

Connective tissue genes

The first report of a non-syndromal AAA inducing gene mutation was in 1990. Kontusaaru et al isolated a mutation in the type 3 procollagen gene (Col3A1) that

resulted in the conversion of the amino acid glycine to arginine at position alpha 1-619 on the collagen protein in a family in whom multiple members developed AAAs early in life (Kontusaari S 1990). The structural integrity of collagen depends on its tightly wound triple-helical structure and the substitution of arginine for glycine, the smallest amino acid prevents the tight coiling of the collagen helix. The collagen produced was found to be less stable than normal and unfolded at a lower temperature than expected. This defect however, has rarely been subsequently demonstrated (Tromp G 1993) (Powell JT 1991) (Powell JT 1993). Tromp and colleagues sequenced the type 3 procollagen gene in 50 patients with AAA and found that only 2% had a mutation in this gene (Tromp G 1993). Powell et al demonstrated that polymorphic variation detected with the restriction enzyme Ava II in the region 3' to the type 3 collagen gene is significantly more common in patients with AAA than those with aorto-iliac stenotic disease (Powell JT 1993). The polymorphism was also associated with aneurysm diameter and change in the pressure-strain elastic modulus, or stiffness of the aneurysm wall, suggesting that variation in the type III collagen gene may alter the mechanical properties of the aortic wall and predispose to dilatation. The type III procollagen gene is large, having 52 exons, any of which can potentially have a mutation, highlighting the difficulty in identifying individual mutations.

There is no convincing evidence that mutations of the type I collagen or the elastin gene play a role in AAA formation in man. The rare Williams-Beuren syndrome is caused by a mutation on the elastin gene but is not associated with AAA (van Vlijmen–van Keulen CJ 2003). Aortic aneurysms are seen in mice following a targeted deletion of the fibrillin-1 gene and a link has been found between fibrillin-1

genotype and the presence of concomitant abdominal aortic and popliteal artery aneurysms (MacSweeney ST 1996). Powell et al found that in patients with both AAA and a popliteal aneurysm, a particular genotype termed 2-3 fibrillin-1, is significantly over represented. This genotype was found to be associated with increased stiffness of the aortic wall and also increased systolic blood pressure (Powell JT 1996). However, outside Marfan syndrome no associations have been made between familial AAA and fibrillin-1 geneotype.

Connective tissue proteases and their inhibitors

Despite the important role of MMPs in AAA, the investigation of MMP genes has achieved only moderate success. MMP-2 is constitutively expressed and has the characteristics of a housekeeping gene and does not appear to be readily modulated (Crowther M 2000). Polymorphisms have been identified in the human MMP-2 promoter region, with a cytosine (C) to thymine (T) transition at –1306 being associated with differential promoter activity, although none have been associated with AAA formation (Hinterseher I 2006). The MMP-3 gene has an adenine (A) nucleotide insertion / deletion polymorphism at the -1171 position in the promoter sequence that can double the gene transcription activity, however two studies have failed to demonstrate a statistically significant association between this polymorphism and AAA (Yoon S 1999) (Ghilardi G 2003). The MMP-9 gene has a polymorphic site at position -1562 within the promoter region of the gene. A C to T transition exists that results in a 50% increase in promoter activity (Zhang B 1999). The presence of the T allele has been shown to be common in patients with AAA and is a strong independent risk factor for AAA with an odds ratio of 2.41 (Jones GT 2003). This is

potentially an important finding as it could explain the elevated MMP-9 found in the plasma and aortic wall of patients with AAA.

A 4G/5G polymorphism –675 base pairs upstream from the initiation of transcription has been described in the PAI-1 gene (Dawson SJ 1993). The 4G allele binds to an activator, whereas the 5G allele binds to both an activator and a repressor, resulting in reduced transcription of PAI-1 (Rossaak JI 2000). The 5G allele therefore results in less inhibition of the plasminogen activators, leading to increased conversion of plasminogen to plasmin and increased activation of MMPs (Rossaak JI 2000). Rossaak et al discovered that the PAI 4G-allele was more common in 39 patients with familial aortic aneurysms than those with non-familial aneurysms or patients without AAA (Rossaak JI 2000). No difference was found between patients with no family history of AAA and controls, a finding in keeping with the results of two other studies, suggesting that this polymorphism may only have a role in familial AAA (Yoon S 1999) (Jones K 2002). Interestingly, patients with AAA who are homozygous for the 5G allele have a trend towards an increased rate of aneurysm expansion, although statistical significance has not been demonstrated ($p=0.07$) (Jones K 2002).

Eriksson and colleagues found a weak association between cystatin C polymorphisms and AAA growth (Eriksson P 2004). The two polymorphisms investigated were a G to C polymorphism at the -82 position in the promoter region of the gene and a G to A polymorphism at the +148 position in the cystatin C signal peptide. As no control samples were used, the association between these polymorphisms and the presence of an AAA has not been investigated.

The TIMP-1 gene is located on the X chromosome, an interesting finding in view of the male preponderance of aneurysms (Tilson MD 1993). However, Tilson found no evidence of a functional genetic defect in the TIMP-1 gene in 14 patients with AAA (Tilson MD 1993). Although a SNP was identified in codon 101 in two patients, the amino acid sequence of the gene product was conserved. Other studies have also failed to demonstrate an association between TIMP-1 or TIMP-2 gene defects and AAA (Wang X 1999).

Cytokine genes

It has been suggested that the genes coding for cytokines may contribute to AAA formation (Marian AJ 2001). It is feasible that the increased circulating concentrations of proinflammatory cytokines such as IL-1, IL-6 and TNF-alpha in patients with AAA may be a consequence of genetic alterations (Juvonen J 1997) (Marian AJ 2001). Jones investigated an IL-6 guanine / cytosine (G/C) polymorphism at the -174 position in the promoter region of the gene and demonstrated that the C/C genotype was associated with elevated circulating IL-6 and also with increased future cardiovascular mortality in patients with AAA (Jones KG 2001). No association was found between plasma IL-6 or IL-6 genotype and AAA growth and a subsequent study failed to demonstrate an association between IL-6 genotype and the presence of an AAA (Bown MJ 2003).

A small case-control study found that an adenine (A) allele at the polymorphic -1082 position in relation to the start codon in the IL-10 gene is associated with AAA (Bown MJ 2003). The presence of an adenine base at this position results in approximately a 25% reduction in IL-10 production by maximally stimulated lymphocytes (Eskdale J

1998). This makes this polymorphism an interesting and biologically plausible finding as IL-10 has been shown to inhibit proinflammatory cytokine and MMP production and stimulate TIMP production (Lacraz S 1995).

Heme oxidase-1 gene

The HO-1 gene has a promoter polymorphism that results in a variable number of repeating sequences of G and T, termed a (GT)n length polymorphism. The presence of less than 25 GT dinucleotide repeats is associated with increased upregulation of the HO-1 gene, whereas more than 25 repeats is associated with reduced HO-1 inducibility (Yamanda N 2000). Schillinger and colleagues found that patients with AAA were less frequent carriers of the short polymorphism than patients with coronary heart disease, peripheral vascular disease or normal controls (Schillinger M 2002). This implies that a genetic HO-1 deficiency may contribute to AAA disease although not atherosclerosis through an inability to produce CO and bilirubin, protectors against oxidative arterial damage (Willis D 1996).

Angiotensin converting enzyme gene

Polymorphisms in the ACE gene that regulate ACE activity have been identified (Danser AH 1995) (Rigat B 1990). A number of studies have reported an association between ACE gene polymorphisms, particularly an insertion (I) or deletion (D) of a 287 base pair sequence within intron 16 (Rigat B 1992) and cardiovascular risk. The DD genotype is associated with increased tissue and circulating ACE concentration (Danser AH 1995) (Rigat B 1990). Examination of this polymorphism in patients with AAA has produced conflicting results. Pola and colleagues found that the DD and ID genotypes are independent risk factors for AAA in normotensive patients (Pola A

2001), however other investigators have found no association between this polymorphism and AAA or rate of AAA expansion (Hamano K 1999) (Yeung JMC 2002).

Haptoglobin and cholesteryl ester transfer protein genes

Haptoglobin is a haemoglobin transportation protein that has 3 phenotypes dependent on α and β chain subunits present (Powell JT 1990). The Hp 1-1 and Hp 2-1 phenotypes contain α^1 chains, whereas the Hp 2-2 phenotype does not have an α^1 chain. Powell et al found that different alleles in the haptoglobin gene distinguishable with the restriction enzyme AvaII correspond to the different phenotypes, with the presence of the allele for the α^1 gene being associated with AAA (Powell JT 1990). This is in agreement with an earlier finding that a weak association exists between the Hp 2-1 phenotype and AAA (Norrgard O 1984). In addition, it was demonstrated that the Hp 1-1 and Hp 2-1 phenotypes were associated with increased elastinolytic activity. This is a plausible finding as haptoglobin is reported to inhibit the activity of the cysteine protease cathepsin (Snellman O 1967). The haptoglobin gene is on the long arm of chromosome 16, close to 2 genes coding for proteins involved in lipid metabolism: lecitin: cholesterol acetyl transferase (LCAT) and cholesterol ester transfer protein (CETP). The CETP gene is polymorphic and in the same study Powell et al found that a rare polymorphism in this gene was also associated with AAA, although probably unconnected to the haptoglobin polymorphism (Powell JT 1990). On the basis of this finding Ramsbottom et al investigated CETP as a candidate gene for AAA but failed to demonstrate polymorphism frequency differences between patients with AAA and those without (Ramsbottom D 1997).

Autoimmunity

There is some evidence that autoimmunity plays a part in the inflammatory response in AAA (Brophy CM 1991) (Tilson MD 1996). The major histocompatibility complex (MHC) identifies and binds to antigens and present them to host immune cells in autoimmune conditions. Class 1 antigens (HLA-A and B) are recognised by receptors on CD8 positive suppressor T cells and class 2 antigens (HLA-DR and DQ) are recognised by receptors on CD4 positive helper T cells (Sugimoto T 2003). The human leucocyte antigen (HLA) class 2 genes are on chromosome 6 and are highly polymorphic. The HLA-DR B1*02 and HLA-DR B1*04 alleles are strongly associated with AAA in white, black and Japanese populations and the HLA-DR*15 allele and HLA-DR2(15) antigen has also been associated with AAA (Tilson MD 1996) (Hirose H 1998) (Rasmussen TE 2002). The HLA B1*01 allele has been negatively associated with AAA wall inflammation and AAA and the HLA-DQ3 antigen has also been negatively associated with AAA (Rasmussen TE 2002) (Hirose H 1999). Although most work has concentrated on the MHC class 2, an association between the class 1 HLA-A2 and HLA-B61 alleles and AAA has been reported (Sugimoto T 2003).

Linkage analysis

Linkage analysis is another genetic method that has been employed in the pursuit of genetic factors in AAA formation. The technique involves utilising genetic markers that have been mapped to a particular locus on the genome. They test whether or not the marker is co-inherited with AAA at the time of meiotic cross-over. Linkage of a disease phenotype to a marker means that the two are closely related on a piece of DNA. Linkage can be established by analysing large families called index families or

by analysing pairs of siblings, which is of more use in AAA given the late onset and high mortality.

Vaughan et al employed linkage analyses using polymorphic short tandem repeats flanking candidate loci to demonstrate a gene defect for familial aortic aneurysms on chromosome 11q23.3-q24 in three families affected by multiple aneurysms, of both the thoracic and abdominal types (Vaughan CJ 2001). It was demonstrated that the defect was inherited in an autosomal dominant manner. The defect did not appear to be associated with any other abnormalities characteristic of systemic connective tissue disorders. At present few studies have employed this approach in AAA families.

Summary

AAA formation is the end result of a complex multifactoral process leading to a weakening and dilatation of the aortic wall. The principal biochemical mechanisms currently implicated are those involved in the synthesis and degradation of the main wall components; collagen, elastin and SMC. A number of biologically plausible genetic defects have been identified that appear to increase the relative risk of AAA formation. However, no genetic defect has been shown to have a large effect on the absolute risk of AAA formation and therefore genetic defects are not accurate predictors of AAA formation and can not be utilised in AAA screening. Environmental risk factors are important and their interaction with genetic defects has yet to be elucidated.

Chapter 3

Interleukin-10 and its Single Nucleotide Polymorphisms

Contents

Cytokines

Cytokines are soluble proteins or glycoproteins that act as intercellular chemical messengers and are important mediators of inflammation and the immune response. Following their secretion by lymphocytes, macrophages and monocytes, cytokines bind to and activate specific receptors on the surface of target cells, triggering intracellular signal transduction pathways. In this way cytokines regulate cellular processes such as gene transcription and cell differentiation. Conventionally cytokines are divided into T helper (Th) Th1 (cellular immune response) and Th2 (humoral or antibody mediated immune response) groups on the basis of the cell type of production. Th1 lymphocytes principally produce proinflammatory cytokines such as IL-2, IL-12, INF-gamma and TNF-alpha, whereas Th2 lymphocytes largely produce antiinflammatory cytokines such as IL-10 and IL-4. Th2 cytokines inhibit the Th1 cell mediated inflammatory response, thereby creating a Th1/Th2 balance and a regulatory mechanism in inflammation. In physiological as well as pathological states cytokines interact in stimulatory or inhibitory concert in a complex biological pathway.

Interleukin-10

IL-10 is a potent antiinflammatory cytokine that is produced by Th2 lymphocytes, macrophages, dendritic cells, mast cells, B lymphocytes as well as some Th1 lymphocytes. IL-10 was first discovered in 1989 as an inhibitor of murine Th1 cytokine production and was originally termed cytokine synthesis inhibitory factor (Fiorentino DF 1989). Subsequently, human IL-10 was identified as a 160 amino acid protein with a molecular weight of 36 kDa (Moore KW 1993). The secondary structure or peptide backbone conformation of IL-10 comprises 60% helix and the tertiary structure or overall protein shape is a noncovalent V shaped homodimer of

two interpenetrating 65 amino acid polypeptide chains (Moore KW 2001). IL-10 is very similar to the product of the BCRF-1 gene of Epstein-Barr Virus, termed viral IL-10, having 84% homology.

Regulation of IL-10 production

IL-10 production is regulated by a homeostatic mechanism that tends to maintain the pro- / anti-inflammatory cytokine balance. IL-10 production is upregulated by lipopolysaccharide (LPS) or gram negative bacterial endotoxin and proinflammatory cytokines such as IL-1, IFN-beta, IFN-alpha, TGF beta (Frankenberger M 1995) (Porrini AM 1995) (Lalani I 1997) (van der Poll T 1994). The antiinflammatory cytokines; IL-4, 13, TNF-beta inhibit IL-10 production (de Waal Malefyt R 1993) (Chomarat P 1993), as does IL-10 itself via a negative feedback mechanism (de Waal Malefyt R 1991). Administration of exogenous corticosteroids can increase IL-10 production tenfold, suggesting that IL-10 may contribute to the antiinflammatory effect of steroids (Tabardel Y 1996). A number of innate and environmental factors seem to govern an individual's IL-10 production, the most important appearing to be alterations or polymorphisms in the IL-10 gene, although female sex, low body mass index and smoking reportedly reduce IL-10 production (Reuss E 2002) (Takanashi S 1999).

General actions of IL-10

IL-10 is an immunoregulatory cytokine, the principal functions of which are the inhibition of proinflammatory cytokine production by macrophages and the inhibition of accessory functions of macrophages in T cell activation. Following the initial discovery that IL-10 inhibits the synthesis of the proinflammatory cytokines IFN-

gamma, IL-2 and TNF beta by mice Th1 cell clones (Fiorentino DF 1989), IL-10 has subsequently been found to inhibit the production of IL-1, IL-6, IL-8, IL-1beta, TNF alpha and macrophage inflammatory protein (MIP)-1 alpha in a variety of cells including monocytes / macrophages, neutrophils and B and T lymphocytes (Moore KW 1993). As well as limiting Th1 cytokine production, IL-10 is capable of shifting the Th1/Th2 cellular balance in favour of Th2. Th0 cells, the precursors of mature Th1 and Th2 cells, differentiate irreversibly into one or other cell type. IL-10 induces differentiation into Th2 cells and also inhibits IL-12, an important stimulus for Th1 differentiation (Seder RA 1993).

The use of IL-10 deficient ($IL\text{-}10^{-/-}$) mice has elucidated some of the consequences of IL-10 absence. In animal models of sepsis, exogenous LPS induces the release of a multitude of proinflammatory cytokines. The injection of LPS into IL-10 deficient mice results in higher and more prolonged TNF-alpha production than in wild type ($IL\text{-}10^{+/+}$) mice indicating that IL-10 acts to diminish sepsis mediated by TNF-alpha (Berg DJ 1995). In a similar LPS induced sepsis model it has been demonstrated that the release of proinflammatory cytokines precedes that of IL-10. The delayed appearance of IL-10 coincides with down regulation of the proinflammatory cytokines, providing evidence of proinflammatory cytokine stimulation of IL-10 and the inhibitory effect of IL-10 on proinflammatory cytokines (Tedgui A 2001). In humans with septic shock, large amounts of IL-10 are produced, the serum level correlating with the intensity of the inflammatory response, indicating a physiological attempt to restore the normal inflammatory balance (Uboldi de Capei M 2003).

The IL-10 induced inhibition of proinflammatory cytokines appears to be at a number of levels. Inhibition of gene transcription (Wang P 1994) through inhibition of the transcription factor nuclear factor-kB (NF-kB), a protein that binds to the promoter region of the gene and triggers gene transcription, results in reduced IL-10 mRNA and protein (Wang P 1995). IL-10 induced post transcriptional inhibition of proinflammatory cytokines has also been shown to occur (Bogdan C 1992). In contrast to the inhibitory action on T cells, IL-10 is stimulatory in B cells and induces MHC class II expression and promotes cell activation and immunoglobulin IgG, IgA and IgM production (Moore K 1993) (Rousset F 1992) (Uboldi de Capei E 2003).

Actions of IL-10 relevant to AAA pathogenesis

Although IL-10 has not been extensively studied in aneurysmal disease, in-vitro, animal studies and the investigation of IL-10 in other disease states has elucidated some of its functions and allowed insight into its possible role in AAA.

As discussed in the previous chapter, cytokines are key regulators of a number of mechanisms implicated in AAA formation. The pro- /anti-inflammatory cytokine balance regulates MMP and TIMP expression, type I and III collagen expression, SMC apoptosis and cathepsin S secretion (Siwik DA 2000) (Newman KM 1994) (Juvonen J 1997) (Geng YJ 1996) (Cohen SB 1997) (Sukhova GK 1998). By direct inhibition of proinflammatory cytokines, it is likely that IL-10 has the potential to interfere with a number of these pro-aneurysmal processes, and potentially protect against aneurysm formation.

The pro - / anti-inflammatory cytokine balance is an important controller of MMP activity. Proinflammatory cytokines stimulate MMP production whereas IL-10 downregulates MMP production. IL-10 achieves this by directly inhibiting MMP gene transcription and indirectly via the inhibition of proinflammatory cytokines (Lacraz S 1995). In addition, IL-10 potently stimulates TIMP-1 biosynthesis at both the protein and gene levels, thereby providing a further inhibitory mechanism (Lacraz S 1995). Additional evidence for the role IL-10 plays in MMP production comes from the work of Silvestre et al who demonstrated that IL-$10^{-/-}$ mice have exaggerated MMP-9 production in response to hindlimb ischaemia, a stimulus for MMP production (Silvestre JS 2001).

IL-10 has been found to decrease urokinase type plasminogen activator (uPA) and the urokinase plasminogen activator receptor (uPAR) in monocytes in vitro (Paysant J 1998). By inhibiting uPA and its receptor it would be reasonable to expect that the conversion of plasminogen to plasmin is impaired, thereby reducing MMP activation and direct plasmin induced ECM degradation. However, it must be borne in mind that at present, there is no evidence that IL-10 inhibits tissue plasminogen activator (tPA), which has similar activity and function to uPA. Therefore, the extent to which the plasminogen to plasmin conversion is impaired by IL-10 induced uPA and uPAR inhibition is unclear.

Inducible nitric oxide synthase (iNOS) catalyzes the formation of NO, which is oxidised to nitrite ions. Nitrite ions have been implicated in AAA formation through their ability to damage elastin, cause aortic wall oxidative injury, upregulate proinflammatory cytokines and MMPs and stimulate SMC apoptosis (Zhang J 2003)

(Davies MG 1995) (Paik D 1996) (Lee JK 2001). There is some evidence from animal studies that reduction of NO through inhibition of NOS reduces the rate of AAA expansion (Lee JK 2001). Mallat et al investigated IL-10 expression in relation to iNOS in advanced atherosclerotic plaques, represented by 18 atherosclerotic carotid arteries and 3 AAA tissue samples. IL-10 expression was associated with a three-fold reduction in the probability of iNOS expression and reduced apoptosis (Mallat Z 1999). Unfortunately the AAA samples represented a small percentage of the tissue examined and no separate analysis was made. Nonetheless, this provides further evidence of the ability of IL-10 to interrupt a mechanism implicated in AAA pathogenesis. Table 3.1 lists the actions of IL-10 that may be important in inhibiting AAA formation.

Functions of IL-10 relevant to AAA pathogenesis
Proinflammatory cytokine inhibition
MMP inhibition
TIMP activation
iNOS inhibition
NO production inhibition
Apoptosis inhibition
uPA inhibition

Table 3.1. Functions of IL-10 that may protect against AAA formation.

Abbreviations: MMP, matrix metalloproteinase. TIMP, tissue inhibitor of metalloproteinase. iNOS, inducible nitric oxide synthase. NO, nitric oxide. uPA, urokinase type plasminogen activator.

IL-10 in vascular disease

Much of the investigation into the role of IL-10 in vascular disease has focused on atherosclerosis. Although uncertainty surrounds the exact nature of the relationship between atherosclerosis and AAA, there is some evidence to support the theory that atherosclerosis is important in the initiation of aneurysmal dilatation. Irrespective of whether a true causal relationship exists, atherosclerosis is potentially a useful analogous pathology in which to contemplate the possible role of IL-10 in AAA formation in the absence of detailed investigation in AAA tissue. The two conditions have a number of shared pathological mechanisms; both are macrophage and T-lymphocyte mediated inflammatory processes in which the proinflammatory / antiinflammatory cytokine and the MMP / TIMP balances are of critical importance. Despite these similarities in mechanisms, atherosclerosis and AAA have some fundamental differences and therefore caution must be exercised in drawing definitive conclusions from this model.

Over the last decade it has become clear that the initiation, progression and acute clinical manifestations of atherosclerosis are dependent on inflammatory processes (Ross R 1999). Early in the progression of atherosclerosis, endothelial dysfunction stimulates monocyte, leukocyte and platelets adherence to the endothelium via the adhesion molecules intercellular adhesion molecule – 1 (ICAM-1) and vascular cell adhesion molecule – 1 (VCAM-1) (Ross R 2001). Monocytes then infiltrate the endothelium to reach the subendothelial space in a process aided by the ECM degrading properties of uPA, plasminogen and MMPs and are transformed into macrophages (Paysant J 1998). SMC migrate to the lesion and then proliferate, resulting in thickening of the arterial wall (Ross R 1999). In advanced plaques, a

fibrous cap forms over the lesion. MMPs, which are present in atherosclerotic but not normal arteries, induce ECM and fibrous cap degradation leading to plaque rupture, a frequent cause of acute clinical manifestations of atherosclerosis such as unstable angina, MI or distal embolization (Smith DA 2001) (Tziakas DN 2003) (Brown DL 1995) (Vine N 1991).

Throughout the development of atherosclerosis, macrophages and T lymphocytes secrete proinflammatory cytokines such as IL-6, IFN-gamma and TNF-alpha. These cytokines orchestrate atherogenic cellular processes including inflammatory cell migration, SMC migration and proliferation, MMP production, TIMP inhibition, cellular apoptosis and reduced collagen synthesis (Ross R 1999) (Hansson GK 2001) (Libby P 1995) (Whitman SC 2000) (Liuzzo G 2000). IFN-gamma in particular has been shown to enhance plaque progression and stimulate clinical manifestations of coronary artery disease (Gupta S 1997) (Whitman SC 2000) (Liuzzo G 2000). Patients with acute coronary syndromes, in addition to having a localised inflammatory response have been shown to have elevated systemic proinflammatory factors such as CRP and IL-6 (Smith DA 2001) (Liuzzo G 1994) (Biasucci LM 1996), both of which are predictive of future cardiovascular events and poor outcome (Lindahl B 2000) (Heeschen C 2003).

Protective effect of IL-10 in atherosclerosis

The production of the antiinflammatory agents IL-10, TGF-beta and interleukin-1 receptor antagonist (the production of which is stimulated by IL-10) creates a Th1/Th2 counteregulatory balance that tends to maintain the inflammatory homeostasis of the arterial wall and prevents unopposed proinflammatory

atherosclerotic progression (Tedgui A 2001). IL-10 appears to be one of the most important antiinflammatory factors involved in the inhibition of atherosclerosis and has been identified at the mRNA and protein level in atherosclerotic although not in normal arteries (Mallat Z 1999). Plaque macrophages and lymphocytes appear to be the principal cell types responsible for IL-10 production, although there is some evidence that SMC may contribute (Uyemura K 1996) (Mallat Z 1999). In contrast to the considerable degree of overlap in the actions of many proinflammatory cytokines, IL-10 seems to have a number of individual or unique functions and is therefore a key moderator of atherosclerosis (Girndt M 2002). IL-10 inhibits atherosclerotic change at a number of levels. Early in the disease process, IL-10 inhibits ICAM-1 and VCAM-1 thereby reducing monocyte / endothelium adherence and monocyte proliferation and differentiation (Krakauer T 1995) (Pinderski Oslund LJ 1999). The IL-10 induced reduction in cell bound and monocyte intracellular uPA and UPAR appears to further limit monocyte migration and adhesion (Paysant J 1998). Throughout all stages of atherosclerotic progression, the most important action of IL-10 is probably the deactivation of macrophages and the inhibition of proinflammatory cytokine production (Mallat Z 1999). The potent atherogenic, proinflammatory cytokine IL-12 is inhibited by IL-10 and the two cytokines appear to have cross-regulatory roles in atherosclerosis (Uyemura K 1996).

There is evidence that the proinflammatory / antiinflammatory cytokine balance is a determinant of outcome in acute coronary syndromes (Heeschen C 2003). It has been demonstrated that patients with unstable angina have reduced circulating IL-10 compared with those with no angina or stable angina, suggesting IL-10 has a protective role (Smith DA 2001) (Tziakas DN 2003). High circulating IL-10 is

associated with improved prognosis in patients with acute coronary syndromes, a phenomenon that seems to result from a plaque stabilising effect (Heeschen C 2003).

The use of IL-$10^{-/-}$ mice has elucidated some of the vascular consequences of IL-10 deficiency. IL-$10^{-/-}$ mice develop severe hypercholesterolaemia induced atherosclerosis (Pinderski Oslund LJ 1999) (Mallat Z 1999). Transgenic expression of IL-10 reverses the atherosclerotic change, underlying the important protective effect of IL-10 (Mallat Z 1999). Apolipoprotein E (Apo $E^{-/-}$) deficient mice are used as a model of atherosclerosis, and the use of combined IL-$10^{-/-}$ and Apo $E^{-/-}$ mice allows the consequences of IL-10 deficiency in atherosclerosis to be determined. These mice have increased low density lipoprotein levels, Th1 responses defined by IL-12 and IFN-gamma plasma concentration, and increased early atherosclerotic lesion size compared with only Apo E-/- deficient mice (Caligiuri G 2003). This early increase in lesion size can be explained by the key role played by macrophages in the early stages of atherosclerosis and their exquisite sensitivity to the Th1/Th2 balance. Furthermore, plaque MMP-9 expression is increased in IL-$10^{-/-}$ deficient mice, indicating the inhibitory role of IL-10 on plaque MMP production. Atherosclerotic plaques in IL-10 deficient mice have numerous inflammatory cells, high IFN-gamma expression and low collagen content in comparison with lesions from wild type mice, features that predispose to plaque instability (Mallat Z 1999).

Overall, IL-10 appears to have a decisive role in the prevention of atherosclerosis and appears to stabilise vulnerable atherosclerotic plaques. The similarities in the inflammatory mechanisms of aneurysmal disease (discussed more fully in Chapter 2) and atherosclerosis mean that it is likely that some of the roles played by IL-10 in

atherosclerosis are mirrored in AAA. This would suggest that IL-10 may be an important inhibitory factor in AAA.

Therapeutic roles

Attempts have been made to utilise the antiinflammatory and immunosuppressive properties of IL-10 in a number of diseases regulated by a Th1 cytokine response such as inflammatory bowel disease, rheumatoid arthritis, psoriasis and transplant rejection. Early trials of recombinant IL-10 have shown promise and safety. Therapeutic intervention to increase endogenous IL-10 or to administer exogenous IL-10 in the treatment of atherosclerosis has been proposed, but at present little work has focused on this promising possibility (Heeschen C 2003) (Terkeltaub 1999).

IL-10 and its single nucleotide polymorphisms

Deoxyribonucleic acid (DNA) is a double stranded helical macromolecule, famously described by Watson and Crick in 1953 (Watson J. Nature 1953). The helix comprises 2 single strands of DNA running antiparallel to each other, held together by noncovalent hydrogen bonds. These bonds form between the complementary nucleotide bases adenine (A), thymine (T), guanine (G) and cytosine (C). The bases are attached to a sugar molecule, deoxyribose and the adjacent sugar molecules are joined via a phosphate group. DNA is present in every nucleated cell of the body and contains an organism's entire genetic code. The genetic information is arranged in nucleotide triplets termed codons, with each codon coding for an amino acid in the polypeptide product. Degeneracy exists, meaning that a number of different codons code for the same amino acids.

The human genome consists of approximately 3 billion nucleotide bases. There is less than 1% variation in the human genome, with approximately 1 in every 1000-2000 nucleotides differing between individuals. Much of this genetic variation is caused by gene polymorphisms which may be microsatellite polymorphisms (repeating sequences of nucleotides), the insertion or deletion of one nucleotide, or more commonly, the replacement of one nucleotide for another (for example G being replaced with A) termed single nucleotide polymorphisms (SNPs) (Reynard MP 2000). There are an estimated 3 million SNPs in the human genome, although 95-98% occur in sections of genes that do not code for proteins, so-called conservative polymorphisms, and therefore do not result in amino acid alteration in the gene product. However, even some conservative polymorphisms can affect gene transcription and therefore protein production by altering transcription factor binding sites (Keen LJ 2002). It appears that, in this way, cytokine gene SNPs may cause individual variation in cytokine production and cumulative cytokine exposure (Marian AJ 2001).

Although most diseases are not caused by individual gene defects, recently much investigation has focused on SNPs as possible determinants of susceptibility to developing specific diseases. A number of association studies comparing SNP patterns in patients with a particular disease to patients without the disease have demonstrated that SNPs causing high production of proinflammatory cytokines predispose to inflammatory diseases whereas high producers of antiinflammatory cytokines may be protected. Polymorphisms of the IL-10 gene have been associated with numerous conditions in which the pro- / anti- inflammatory balance is disturbed and therefore appear to be of clinical importance. However, it must be borne in mind

that many SNP studies are small candidate gene studies and the possibility that many of the reported positive associations are the result of chance, or type 1 error, must be considered. Publication bias towards publishing small studies with positive results rather than large studies with negative results may account for the apparent numerous SNP associations with disease. Indeed, many reported SNP - disease associations have not been confirmed when retested (Marian AJ 2001).

The study of SNPs is not straightforward, and even the demonstration of an association between a SNP and a disease state must be interpreted with caution. A demonstrated SNP - disease association may be true and the SNP may influence disease susceptibility; alternatively the polymorphism may be closely linked to the true allele causing the association, the phenomenon of linkage disequilibrium. A further explanation could be that patients with a particular disease are genetically different from the rest of the population, and coincidentally also differ in the polymorphism (population stratification) (Andreotti F 2002) (Strachan T 1997).

The huge number of SNPs in the human genome means that on each gene, multiple SNPs exist, which, in combination may have additive or deleterious overall effects on gene expression. It is hoped that eventually, use of whole genome linkage disequilibrium studies of up to one million SNPs will elucidate the effect multiple SNPs may have. It has been argued that even a polymorphism that influences the production of a protein important in a disease may contribute only a small amount to the heritable component of protein production (Lane DA 2000) (Andreotti F 2002). Nonetheless the study of isolated SNPs in candidate gene studies has demonstrated many strongly significant associations that are biologically plausible and therefore the

study of SNPs seems to be an area where progress in the understanding of the mechanisms and possible genetic basis of disease can be made.

IL-10 gene

The IL-10 gene is located on chromosome 1 (1q31-q32) and contains 5 exons, areas of the gene that are expressed. Gene expression is tightly controlled at the transcriptional level, although some constitutive expression occurs (Turner DM 1997) (Moore KW 1993) (Burdin N 1997) (Bienvenu J 1995). In common with other cytokine genes, the IL-10 gene is highly polymorphic, with 28 SNPs having been described. None of these SNPs have been found to result in amino acid substitution in the IL-10 protein product, but a number influence transcription factor binding and therefore gene transcription and IL-10 production (Lazarus R 2002). There is marked inter individual variation in IL-10 production and gene polymorphisms are reported to contribute to between 50 and 75% of the variation (Westendorp RG 1997) (Reuss E 2002). The 3 most intensively investigated and apparently important SNPs lie in the gene promoter region and are a G to A substitution at the -1082 position upstream from the transcription start site (-1082 (G/A)), a C to T substitution at the -819 position (-819 (C/T)) and a C to A substitution at the -592 position (-592 (C/A)). The polymorphisms at the -819 and -592 positions are linked with only the -819/-592 CC or TA haplotypes (linked DNA markers that are inherited as a single unit) existing. Two $(AC)^n$ microsatellite repeats, termed IL10.G and IL10.R, 1.2 kb and 4 kb upstream of the transcription start site have been identified and may also influence gene transcription (Eskdale J 1995) (Eskdale J 1996). Investigation of the effect SNPs have on IL-10 production has yielded seemingly conflicting results. The presence of an A allele at the -1082 position is generally regarded as a 'low producer' genotype,

with a 25% reduction in IL-10 production (Eskdale J 1998). This is based on Turner et al reporting that the presence of an A allele is associated with lower IL-10 production than in A allele negative Conclavin A (Con A) stimulated lymphocytes, an effect independent of the polymorphisms at the -592 and -819 positions. In this study, the alleles at the -819 and -592 positions had no influence on IL-10 production (Turner DM 1997). However, a study of IL-10 in human plasma during and following cardiopulmonary bypass found lower IL-10 production is associated with the -1082 GG genotype (Galley HF 2003) and AA homozygotes at the -592 position have been associated with low IL-10 production in IFN-alpha stimulated peripheral blood mononuclear cells (PBMC) (Rosenwasser LJ 1997). The -592 A allele has also been associated with low IL-10 production in critically ill patients (Lowe PR 2003).

The 3 polymorphisms are closely linked with only 3 out of the 8 possible haplotypes existing in the Caucasian population; G/C/C, A/C/C and A/T/A at positions -1082/ -819/ -592 respectively (Turner DM 1997). The GCC, ACC and ATA haplotypes are associated with high, intermediate and low IL-10 production in Con A stimulated PMBC (Edwards-Smith CJ 1999). The G/C/C homozygous haplotype is associated with a 1.3 times higher level of IL-10 production in Con A stimulated cells (Turner DM 1997). Reuss et al provided supporting evidence by demonstrating that the G/C/C haplotype is associated with a 20% higher transcriptional activity compared with the 2 other haplotypes, which were, however, associated with a comparable IL-10 production (Reuss E 2002). The apparent inconsistencies between effects of these SNPs may be a consequence of different cells and stimuli used in different studies and little is known about the effect these SNPs have on non stimulated cells. (Lio D 2002) (Keen LJ 2002). Further evidence of a significant role of polymorphisms comes from

the observation that patients with the supposed low producer genotypes; the -1082AA and the ACC haplotype are more likely to have inflammatory conditions such as rheumatoid arthritis or inflammatory bowel disease (Lio D 2002) (Tagore A 1999).

IL-10 genotype studies

A difficulty in SNP studies is the high variation in SNP frequency between different populations, as demonstrated in Tables 3.2 and 3.3. For example the IL-10 -1082 G allele is present in between 40 and 60% of the Caucasian population, whereas it occurs in only 0 to 5% of some Asian populations (Keen L 2002). However, even amongst Caucasian populations large differences in allele frequencies occur as demonstrated in Tables 3.2 and 3.3. This may partly explain the differing effects SNPs have been found to have, and therefore the control population in SNP studies is vitally important. The GA heterozygous genotype is most common, perhaps suggesting an evolutionary survival advantage (Uboldi de Capei M 2003). The fact that this genotype is associated with intermediate IL-10 production may reflect potential disadvantage associated with high or low IL-10 production.

Author	Country	N	Condition	-1082AA	-1082GA	-1082GG	A	G
Girndt M (2002)	Germany	300	CRF	36	42	22	57	43
		-	Control	-	-	-		
George S (2004)	UK	66	RAS	27	61	12	57.5	42.5
		100	Control	19	44	37	41	59
Bown MJ (2003)	UK	100	AAA	34	49	17	58.5	41.5
		100	Control	23	48	29	47	53
Koch W (2001)	German	998	CAD	30	49	20.8	54.5	45.5
		793	MI	30.4	48.3	21.3	54.6	45.5
		340	Control	30.9	47.3	21.8	55	45
Galley HF (2003)	UK	150	CPB	32.7	43.3	24	54.4	45.7
		130	Control	32.0	43.8	24.2	53.9	46.1
Karjalainen J (2003)	Finland	242	Asthma	30	50	20	55	45
		395	Control	31	51	19	56.5	44.5
Lio D (2002)	Italy	190	Centarians	45.8	42.6	11.6	67.1	32.9
		260	Control	37	52.6	10	63.3	36.7
Costeas PA (2003)	Greek Cypriot	100	Control	40	44	16	62	38
Uboldi de Capei M (2003)	Italy	140	Control	34	54	12	61	39
Lowe PR (2003)	Aberdeen	128	Control	32	43.8	24.2	53.9	46.1
		67	Critically ill	19.4	53.7	26.9	46.3	53.8
Donger C (2001)	Belfast	424	Control	26.7	49.0	24.2	51.2	48.8
		422	MI	26.5	46.4	27.0	49.7	50.3
	Glasgow	352	Control	22.7	50.3	27.0	47.9	52.2
		373	MI	21.4	50.4	28.1	46.6	53.4
	Strasbourg	176	Control	27.3	52.3	20.4	53.5	46.6
		189	MI	26.5	54.0	19.6	53.5	46.5
Meenagh A (2002)	N Ireland	93	Control	19.4	46.2	34.4	42.5	57.5
	Zulu	84	Control	33.4	57.1	9.5	62	38
	Omani	79	Control	44.3	41.8	13.9	65.2	34.8
	Singapore Chinese	81	Control	95.1	4.9	0	97.6	2.5
	Mexican Mestizos	39	Control	46.2	35.9	17.9	64.2	35.9

Table 3.2 Studies comparing IL-10 -1082 genotype and allele frequencies in patients with and without disease states. Abbreviations: (MI, myocardial infarction), (CPB, cardiopulmonary bypass), (MS, multiple sclerosis) (RAS, renal artery stenosis), (CAD, coronary artery disease).

Author	Country	N	Condition	-592CC	-592CA	-592AA	C	A
Girndt M (2002)	Germany	300	CRF	55	37	8	73.5	26.5
		-	Control	-	-	-		
George S (2004)	UK	66	RAS	47	38	15	66	34
		100	Control	65	34	1	82	18
Bown MJ (2003)	UK	100	AAA	55	36	9	73	27
		100	Control	62	33	5	78.5	21.5
Koch W (2001)	Germany	998	CAD	54.5	39.1	6.4	74	26
		793	MI	56.6	37.1	6.3	75.2	24.9
		340	Control	51.5	40.6	7.9	71.8	28.2
Costeas PA (2003)	Greek Cypriot	100	Control	61	31	8	76.5	23.5
Uboldi de Capei M (2003)	Italian	140	Control	50	44	6	76.5	23.5
Lowe RP (2002)	Aberdeen	67	Critically ill	65	33.5	1.5	81.8	18.3
		132	Control	67	29	4	81.5	18.5
Donger C (2001)	Belfast	423	MI	62.2	32.9	5.0	78.7	21.4
		427	Control	63.5	33.1	3.7	80	20
	Glasgow	374	MI	65.2	32.9	1.9	81.7	18.4
		352	Control	58.8	36.6	4.5	77.1	22.9
	Strasbourg	190	MI	51.6	42.1	2.6	72.7	27.4
		176	Control	55.7	38.6	5.7	75	25
Meenagh A (2002)	N Ireland	93	Control	64.5	34.4	1.1	81.7	18.3
	Zulu	84	Control	38	56.0	6.0	66	34
	Omani	79	Control	57.0	36.7	6.3	75.4	24.7
	Singapore Chinese	81	Control	11.1	43.2	45.7	32.7	67.3
	Mexican Mestizos	39	Control	35.9	46.2	17.9	59	41
Karjalainen (2003)	Finland	242	Asthma	59	37	4	77.5	22.5
		395	Control	57	39	4	76.5	23.5

Table 3.3 Studies comparing IL-10 -592 genotype and allele frequencies in patients with and without disease states. Abbreviations: (MI, myocardial infarction), (RAS, renal artery stenosis), (CAD, coronary artery disease).

It is possible that IL-10 genotype influences all cause survival and therefore genotype frequency may change with population age. Lio et al found that the -1082 G allele frequency in Italian centenarian men is significantly higher than in randomly selected men of all ages from the same population group (Lio D 2002). Although this is an isolated finding, it is potentially of interest as ageing appears to involve increased inflammatory activity. It also highlights the importance of properly age matched case and control patients in SNP association studies.

A number of studies have investigated IL-10 polymorphisms in cardiovascular disease. Girndt et al investigated patients with chronic renal failure undergoing dialysis, a patient group subject to aggressive atherosclerosis. The -1082 A allele, which is associated with reduced IL-10 production ability and impaired inflammation down regulation, was found to be associated with elevated C-reactive protein (CRP) and fibrinogen and lower albumin levels. The -1082 AA homozygote group were also at significantly higher risk of an adverse cardiovascular event and cardiovascular mortality compared with patients with the G allele (Girndt M 2002).

A large case control study of the IL-10 gene polymorphisms in 998 patients with asymptomatic coronary artery stenosis, 793 with MI, and 340 without CAD, failed to demonstrate differences in IL-10 or TNF-alpha allele or genotype frequencies either singly or in combination (Koch W 2001). A further large study investigating IL-10 gene polymorphisms in patients with MI failed to demonstrate a difference in either IL-10 genotype alone or in combination with TNF-alpha genotype (Donger C 2001).

A case control study of 100 patients with AAA and 100 age and sex matched control patients without AAA undertaken by Bown et al in Leicester demonstrated that the presence of the -1082 A allele is significantly associated with the presence of an AAA ($p=0.03$) with an odds ratio of 1.8 for this allele being a risk factor for AAA. In addition, the -1082/-819/-592 GCC/GCC genotype which is associated with high IL-10 production, was found to be more common in the control group, although statistical significance was not reached ($p= 0.06$) (Bown MJ 2003). These are interesting and biologically plausible findings given the possible protective role of IL-10 in AAA (Turner DM 1997). Indeed, SNPs may, in part, account for the genetic component to AAA formation. However, the relatively small power of this study means that further investigation is required to reach definitive conclusions on the role of IL-10 polymorphisms in AAA.

Summary

Existing work has demonstrated that IL-10 inhibits a number of mechanisms important in AAA formation and has a protective effect in atherosclerosis. At present there has been limited direct work on IL-10 in AAA. Cytokine gene SNPs are strongly implicated in a number of disease states in which the pro- / anti-inflammatory balance is disturbed. The finding that a 'low producer' IL-10 polymorphism is associated with AAA in a small case control study suggests that the investigation of IL-10 and its polymorphisms in AAA is a potentially fruitful area for further research. Initially this will result in better understanding of the mechanism involved in AAA pathogenesis and perhaps eventually result in a therapeutic option to prevent AAA formation or expansion.

Chapter 4

Aims and Scope of Thesis

AAA represents a common and frequently fatal vascular pathology. The incidence appears to be increasing and AAAs are currently the 8th commonest cause of death in the UK, accounting for approximately 9500 deaths annually (Multicentre Aneurysm Screening Study Group 2002) (Office for national statistics 2001). The recent inception of AAA screening programmes has led to the identification of large numbers of patients with small AAA, for which, presently there is no treatment. Attempts to pharmacologically curtail AAA expansion have proved fruitless so far and operative intervention in patients with large or ruptured AAA is still the only available treatment. Greater understanding of the pathogenesis of AAA is required if progress is to be made in developing a medical treatment for AAA.

The principal pathological features of AAA are well described; elastin loss, increased collagen turnover, smooth muscle cell apoptosis and the infiltration of chronic inflammatory cells lead to weakening of the aortic wall, progressive aneurysmal dilatation and risk of rupture. However, the causative mechanisms underlying these pathological changes are less well understood and there is evidence to support the involvement of a number of proteolytic, inflammatory, mechanical, genetic and environmental processes.

Proinflammatory cytokines have been implicated in AAA formation through diverse actions which include MMP activation, TIMP inhibition, inhibition of collagen expression, induction of SMC apoptosis, and the chemotaxis of inflammatory cells into the aortic wall (Siwik DA 2000) (Newman KM 1994) (Juvonen J 1997) (Geng YJ 1996) (Cohen SB 1997) (Sukhova GK 1998). Increased concentrations of proinflammatory cytokines have been demonstrated in the aortic wall and systemic

circulation of patients with AAA and the concentrations of specific cytokines have been shown to correlate with AAA size and rate of expansion (Newman KM 1994) (Juvonen ATB 1997) (Treska V 2000) (Juvonen 1997) (Pan JH 2003).

The actions of proinflammatory cytokines are balanced by those of their antiinflammatory counterparts such as the potent IL-10. Although IL-10 has not been extensively studied in aneurysmal disease; in-vitro and animal studies, and the investigation of IL-10 in other disease states, has elucidated some of its functions and allowed insight into a potentially protective role in AAA. IL-10 diminishes the production of proinflammatory cytokines, and consequently it is reasonable to speculate that it will attenuate some of their aneurysm forming activity. In addition, IL-10 has been demonstrated to directly suppress MMP production by human mononuclear phagocytes in vitro (Lacraz S 1995) and to potently stimulate TIMP-1 biosynthesis at both protein and gene levels (Lacraz S 1995). IL-10 also has an inhibitory role in the fibrinolytic system through inhibition of urokinase type plasminogen activator (uPA) and the urokinase plasminogen activator receptor (uPAR) in monocytes in vitro (Paysant J 1998). This inhibitory function in the conversion of plasminogen to plasmin may contribute to reduced MMP activation and also direct plasmin induced ECM degradation.

Recently attention has focused on single nucleotide polymorphisms (SNPs) as determinants of disease susceptibility. Cytokine gene SNPs have been implicated in a number of disease states in which the pro- / anti-inflammatory balance is disturbed. The 3 most intensively investigated and apparently functionally important SNPs in the IL-10 gene lie in the gene promoter region and are a G to A substitution at the -1082

position upstream from the transcription start site (-1082 (G/A)), a C to T substitution at the -819 position (-819 (C/T)) and a C to A substitution at the -592 position (-592 (C/A)). The polymorphisms at the -819 and -592 positions are linked with only the -819/-592 CC or TA haplotypes (linked DNA markers that are inherited as a single unit) existing. This results in 3 possible haplotypes occurring in the Caucasian population; G/C/C, A/C/C and A/T/A at positions -1082/ -819/ -592 respectively (Turner DM 1997).

There is marked inter individual variation in IL-10 production and gene polymorphisms are reported to contribute to between 50 and 75% of the variation (Westendorp RG 1997) (Reuss E 2002). The presence of an A allele at the -1082 position is regarded as the 'low producer' genotype, with a 25% reduction in IL-10 production (Eskdale J 1998). The GCC, ACC and ATA haplotypes are associated with high, intermediate and low IL-10 production (Edwards-Smith CJ 1999).

A pilot case control study of 100 patients with AAA and 100 age and sex matched control patients undertaken by Bown et al in Leicester in 2003 demonstrated that the presence of the -1082 A allele is significantly associated with the presence of an AAA (P=.03) with an odds ratio of 1.8 for the presence of this allele being a risk factor for AAA. In addition, the -1082/-819/-592 GCC/GCC genotype which is associated with high IL-10 production, was found to be more common in the control group, although statistical significance was not reached (p= 0.06) (Bown MJ 2003). The finding of associations between low IL-10 producing genotypes and AAA are interesting and biologically plausible given the possibly protective role of IL-10 in AAA (Turner DM. Eur J Immunogenet 1997). The relatively small power of the study means that

further investigation is required to reach definitive conclusions on the role of IL-10 polymorphisms in AAA but suggests that the investigation of IL-10 and its polymorphisms in AAA is a potentially fruitful area for further research.

In this thesis the role of IL-10 and its functional gene polymorphisms -1082 (G/A), -819 (C/T) and -592 (C/A) in the pathogenesis of AAA was investigated. A case control study was performed to investigate the IL-10 allele, genotype and haplotype frequencies in patients with AAA and matched control patients with normal diameter aortas. The plasma IL-10 concentrations were measured in patients with AAA and those with normal diameter aortas to investigate whether there is an association between the presence of AAA and circulating IL-10. In addition, the effect of the IL-10 gene polymorphisms on circulating IL-10 in both groups was investigated.

In the patients with AAA in whom serial measurements of AAA diameter over time had been taken, thereby allowing determination of aneurysm expansion rate, the effect of IL-10 gene polymorphisms on rate of expansion was investigated.

The presence of IL-10 in the aortic wall of patients undergoing AAA repair was sought and the concentration measured.

The null hypotheses for the thesis are as follows:

1. IL-10 allele and genotype frequencies at positions -1082, -819 and -592 are not associated with the presence of AAA.
2. Plasma IL-10 concentration is not associated with the presence of AAA.
3. Plasma IL-10 concentration is not influenced by the IL-10 genotype.
4. IL-10 polymorphisms on the IL-10 gene at positions -1082, -819 and -592 are not associated with AAA expansion rate.
5. IL-10 is not present in the aortic wall.

Chapter 5

Methods

Contents

Study design and patient recruitment

The principal aims of the thesis as discussed in the previous chapter were to:

1. Investigate the IL-10 gene allele and genotype frequencies at the -1082, -819 and -592 positions in patients with AAA and those with normal diameter aortas.
2. Investigate the circulating IL-10 concentration in patients with AAA and those with normal diameter aortas.
3. Investigate the effect of the IL-10 genotype on circulating IL-10 concentration.
4. Investigate the relationship between IL-10 genotype and rate of AAA expansion.
5. Investigate IL-10 concentration in the AAA wall.

The relative rarity and long latent period of AAA would make a longitudinal or prospective study to investigate the role of the IL-10 gene polymorphisms and circulating IL-10 concentration in AAA extremely complicated, expensive and time consuming. In order to address the first two aims of the thesis a case control study design was chosen. If carefully designed and conducted, a case-control study can rapidly and economically yield reliable answers to the questions posed. Patients with a condition, the cases, are identified and their exposure to a suspected risk factor is then compared to that of control patients without the disease. In this way the association between the IL-10 gene polymorphisms and AAA can be calculated along with an odds ratio for a specific polymorphism being a risk factor for AAA.

The main problem associated with case-control studies is susceptibility to bias and a number of study design points need to be addressed in order to conduct a robust study. The starting point in the case-control study was the definition and identification of case and control patients. Clearly, the definition of cases and controls is crucial and as

discussed in Chapter 1, a number of definitions have been applied to AAA. I chose to define a normal infrarenal aortic diameter as being less than 2.5 cm, the most commonly applied definition. Any patients with an aortic diameter greater than or equal to 2.5 cm were initially recruited as cases.

In order to correctly categorise patients as cases or controls, accurate measurement of aortic diameter is required. Ultrasound scanning affords economical and accurate measurement of aortic diameter and I chose to recruit patients in whom abdominal aortic diameter had been formally measured with ultrasound scanning. It is known that aortic measurement on computed tomography scanning differs slightly from that measured on ultrasound so all measurements used in the study were from ultrasound scanning.

Patient selection is an important cause of bias in case-control studies, and to avoid this, selection of cases and controls is vitally important. Theoretically, the cases should comprise all the patients with the disease in a defined study population. However, in practice this is clearly impossible and selection of cases was based upon recruiting a representative sample of patients with AAA into the study. The control group provides the background exposure to the risk factor expected in the population, and the control group should be representative of patients with normal diameter aortas who would be selected as cases if they had AAA, thereby representing the population at risk of becoming cases.

In order to minimise patient selection bias both cases and controls were recruited from the same sources; vascular surgical out patient clinics, admissions to hospital for

elective AAA repair and from the Leicestershire AAA screening programme. Since the inception of the AAA screening programme in 1996, approximately 1300 men aged 65 years are selected annually from the patient lists of general practitioners who wish to participate in the programme, and undergo an abdominal ultrasound scan to measure the diameter of the abdominal aorta. During the study period, from September 2002 until June 2004, 2 experienced ultrasonographers, based at the Leicester Royal Infirmary visited 65 general practitioner surgeries in Leicestershire and performed abdominal ultrasound examinations using a portable ultrasound machine (*Aloka echo camera SSD-500*). If a patient was found to have an abdominal aortic diameter of 2.5 cm or less the patient was reassured and discharged. If the aortic diameter was greater than 2.5 cm but less than 5 cm the patient underwent a further scan 6 months later. Patients with AAA larger than 5 cm were referred to the vascular surgery out-patient department and underwent scans every 3 or 6 months until operative intervention was indicated. Approximately 4% of the screened patients were found to have an AAA. The screening programme therefore provided a source of control patients as well as incident and prevalent cases.

Patients with AAA recruited from the vascular surgery out-patient clinic or during hospital admission for AAA repair had been identified as having an AAA in the screening programme or had incidentally been found to have an AAA during previous clinical or radiological abdominal examination. A further 63 patients aged over 70 years without AAA following abdominal imaging were recruited from the vascular surgery ward.

I attended AAA screening sessions in general practice surgeries in Leicestershire, hospital outpatient clinics and visited patients due to undergo elective AAA repair on the ward to recruit patients. During the period of subject recruitment I attempted to recruit all patients I encountered. There were no selection criteria other than having aortic diameter measured on ultrasound scanning. The subjects were not consecutive as recruiting all patients from these sources during the period of study was impossible.

Data collection

In a case-control study, matching of cases and controls needs to be performed, so that as far as possible, similar patients are being compared. Patients can be matched individually, by pairing cases and controls, or by having case and control groups with, for example, similar age and sex distribution. Potential confounding factors must be identified and allowance must be made in the analysis. In order to standardise the cases and controls, after obtaining informed consent, risk factors for AAA and past medical history were ascertained by directly interviewing patients and from review of hospital notes. As it was impossible for me to be blinded as to whether the patient was a case or control at the time of recruitment a standardised proforma was used for data collection (Figure 5.1).

Patient ID				**Date of collection**				
Sex				**Age**				
Time sample taken				**Time frozen**				
Aortic diameter								
Previous aortic measurements								
Date								
Diameter								
How diagnosed								
Started smoking				**Stopped smoking**				
Cigarettes / day				**Change in smoking**				
Hypertension treatment				**BP**				
MI				**CABG**				
Angina				**Diabetes**				
CVA / TIA				**CEA**				
PVD				**Claudication distance**				
Cholesterol				**Family history**				
COPD								
Medication								

Figure 5.1. Copy of proforma used for data collection

Abbreviations: MI myocardial infarction, BP blood pressure, CABG coronary artery bypass graft, CVA cerebrovascular accident (stroke), TIA transient ischaemic attack, CEA carotid endarterectomy, PVD peripheral vascular disease, COPD chronic obstructive pulmonary disease

Smoking history was recorded, including age of commencing, the average number of cigarettes smoked per day and if appropriate, the age of stopping smoking. If there had been a change in the smoking pattern, for example a reduction in cigarettes smoked, this was recorded. Evidence of ischaemic heart disease and history of

myocardial infarction, angina or previous coronary artery bypass graft or coronary stent was recorded. Treatment for hypertension was recorded. A past history of cerebrovascular disease was defined by a history of stroke, transient ischaemic attack (TIA) or carotid endarterectomy. Peripheral vascular disease was classified by claudication distance, previous lower limb arterial reconstruction or amputation as a result of peripheral vascular disease. In addition, data was recorded on diabetes, cholesterol and chronic obstructive pulmonary disease. The presence of a known AAA in blood relatives was recorded.

Wherever possible, data that was subject to patient recall bias was not used. Information supplied by the patient was confirmed, wherever possible, by review of medical records. It is established that patients with a particular disease are more likely to remember exposure to a risk factor for the disease than patients without the disease, the phenomenon of recall bias. Self reported smoking habit is likely to be subject to recall bias, and is known to be unreliable and patient knowledge of a relative with an AAA is almost certainly subject to recall bias. In addition, there is evidence that researchers gather data differently in cases and controls. The study design aimed to reduce this risk by using a standard proforma and asking the exact same questions of cases and controls.

In patients with AAA in whom serial ultrasound scans had been performed, the dates of previous scans and the AAA diameter at each scan was recorded in order to calculate the rate of expansion. At the end of the patient recruitment period the AAA screening database was interrogated and hospital notes were reviewed to determine

subsequent AAA measurements. This allowed the effect the IL-10 gene polymorphisms had on rate of AAA expansion to be investigated.

Laboratory methods

The materials and techniques used to determine the plasma IL-10 concentration and IL-10 gene polymorphisms are discussed.

Blood sampling

After obtaining informed, written consent, blood samples were obtained by direct venous puncture of the median cubital vein using a standard 21 gauge needle and 10 ml syringe after cleaning the skin with an antiseptic swab. Care was taken to avoid touching the venepuncture site following cleaning. The blood was immediately transferred into a sterile 7 ml glass bottle containing EDTA (ethylenediaminetetraacetic acid), as an anticoagulant (*BD Vacutainer*) as there is some evidence that plastic bottles and endotoxin contamination can induce ex-vivo cytokine production.

Frequently up to 15 patients were recruited into the study in a session at a general practice surgery or hospital out-patient clinic. It is known that individual cytokine levels can decay ex-vivo at room temperature and to prevent this, following procurement, the samples were kept on melting ice in a closed polystyrene ice box until transfer to the laboratory. The length of time prior to transporting the samples was minimised so that samples were processed no more than 4 hours following venepuncture.

The plasma fraction of blood was used to quantify the IL-10 concentration and the white cells were used for DNA extraction prior to polymorphism identification. In order to separate the blood components, samples were centrifuged on immediate return to the laboratory at 770g (2000 RCF) for 10 minutes at 4°C as recommended by *Biosource*, the manufacturers of the Enzyme Linked-Immunosorbent Assay (ELISA) kit used to quantify the plasma IL-10 concentration. The centrifuge was allowed to cool to 4°C prior to sample insertion. The plasma fraction was then pipetted off, carefully ensuring that there was no white blood cell contamination. The plasma was then snap frozen in liquid nitrogen and stored at -80°C in a cryovile (*Sarstedt*). In order to allow DNA extraction the white cell layer (including some plasma above and red cells below) was pipetted off using a sterile Pasteur pipette, snap frozen in liquid nitrogen and similarly stored at -80°C. This method of plasma and white blood cell storage allowed later processing and analysis in batches.

IL-10 Enzyme Linked-Immunosorbent Assay (ELISA)

The plasma IL-10 concentration was quantified using a commercially available ELISA kit with a minimum detectable IL-10 concentration of 0.2 pg/ml (*Biosource Human IL-10 Ultrasensitive*). An ELISA is an immunoassay that utilises an in-vitro antigen antibody reaction to determine the concentration of antigen in a sample. A monoclonal capture antibody specific for IL-10 is coated to a 96 well ELISA plate and when this capture antibody comes into contact with a sample containing IL-10, it binds to the IL-10 and holds it against the surface of the well. In accordance with the manufacturer's instructions, 100 µl of a standard diluent buffer was added to all the wells except the ones designated as chromagen blanks. Following this, 8 serially diluted samples of 100µl of a standard solution of known IL-10 concentrations were

pipetted into individual wells. In this way samples with IL-10 concentrations of 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 pg/ml were analysed. Plasma samples of unknown IL-10 concentration were pipetted into the remaining wells, after the addition of a further 50 µl of standard diluent buffer. To reduce inter-assay variability each standard, sample and the chromagen blank was analysed in duplicate to allow calculation of a mean value.

During an incubation period of 2 hours at 37°C, binding occurred between the IL-10 in the samples and the immobilised capture antibody, the amount of IL-10 binding being proportional to the IL-10 concentration of the solution. The plate was then washed to remove unbound free IL-10 and 100 µl of a second antibody to IL-10, the detection antibody, which is preconjugated to a biotin molecule was added to all the wells except the chromagen blank. During a second incubation period of 1 hour at room temperature binding occurred between the detection antibody and a different site on the IL-10 molecule, and again, the amount of antibody binding was proportional to the amount of IL-10 present. Binding of the detection antibody to IL-10 thus completes the 'sandwich'. A further washing step was performed to remove unbound detection antibody leaving the IL-10 molecule bound to the plate via the capture antibody and to the detection antibody conjugated to the biotin molecule.

Next, 100 µl of a solution containing streptavidin conjugated to the enzyme horseradish peroxidase which binds strongly to the biotin conjugated to the detection antibody was added and a third incubation step of 30 minutes at room temperature was performed. Again, the amount of binding was proportional to the original IL-10 concentration. 100 µl of chromagen, which is the substrate for the horseradish

peroxidase enzyme was then added. Chromagen is colourless, but on binding with horseradish peroxidase it breaks down to its blue coloured product. The intensity of the colour is proportional to the amount of IL-10 present. An incubation of 30 minutes was performed at room temperature in the dark before 100 µl of a stop solution was added to each well. At this stage the contents of the wells changed from blue to yellow.

The yellow colour intensity was quantified by spectrophotometry (optical density scanning) of the plate at 450 nm. The absorbance level of the chromagen blank was subtracted from the absorbance of the wells to adjust for the absorbance of the reagents used.

The standard samples of known IL-10 concentration allowed the construction of a graph of IL-10 concentration against measured optical density and a curve of best fit to be applied. The equation of the curve was derived and the IL-10 concentration of the patient plasma samples was then calculated from the measured optical density. Microsoft Excel was used to construct a spreadsheet of optical density values and to construct the standard curve and calculate the IL-10 concentration of the plasma samples. Figure 5.2 displays a typical standard curve of optical density and sample concentration.

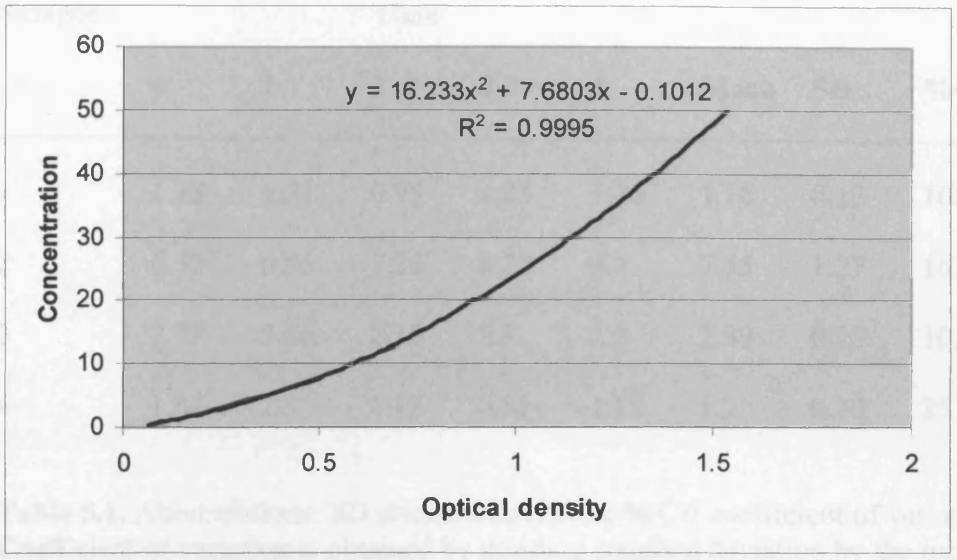


Figure 5.2. Example of a standard curve of optical density against IL-10 concentration
The equation for the curve is $y=16.233x^2 + 7.6803x - 0.1012$
$R^2 = 0.9995$ is the correlation coefficient for the curve (a summary of the strength of linear association between the variables)

Effect of delay in processing samples on IL-10 concentration

Cytokine levels decay in ex-vivo blood. Many of the blood samples in the study were taken from patients undergoing AAA screening in general practitioner surgeries and had to be transported to the laboratory on ice. Therefore the change in cytokine levels over time was of concern. In order to determine the effect of delay in processing the samples, a small study was conducted to determine the effect of delay prior to freezing on measured IL-10 concentration. Blood was taken from 4 patients, transferred into 4 glass bottles containing EDTA and kept on ice for 1, 2, 3 and 4 hours respectively prior to being centrifuged and snap frozen. No samples in the study remained on ice longer than 4 hours prior to processing. The IL-10 concentration was then quantified by ELISA to determine the change in measurable IL-10 concentration over time. Table 5.1 and Figure 5.3 display the change in IL-10 concentration in pg/ml over time.

Sample	Time							
	0	1	2	3	4	Mean	SD	%CV
1	1.18	1.21	0.95	1.25	1.25	1.16	0.12	10.3
2	6.32	6.56	7.26	8.22	9.4	7.55	1.27	16.8
3	2.73	2.56	2.15	2.3	2.2	2.39	0.25	10.5
4	1.64	1.3	1.12	0.81	1.15	1.2	0.30	25

Table 5.1. Abbreviations: SD standard deviation, % CV coefficient of variation Coefficient of variation is obtained by dividing standard deviation by the mean and is a statistical measure of the deviation of a variable from its mean.

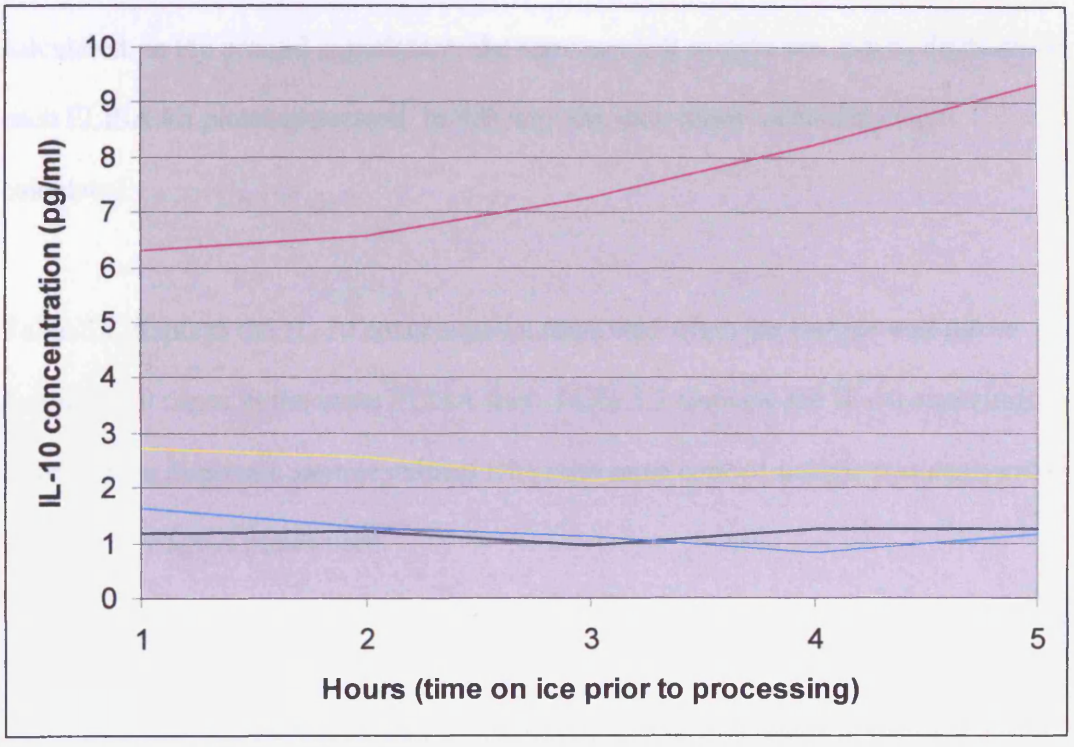


Figure 5.3. Graph of measured IL-10 concentration in samples from 4 patients which were kept on ice for 0 to 4 hours prior to processing.

Conclusion

The coefficient of variation for each sample was low (10.3, 16.8, 10.5, 25). This suggests that there is no significant change in IL-10 concentration in samples stored

on ice for up to 4 hours prior to processing. This provides justification for processing samples up to 4 hours following collection.

Assay variability

As with any experimental study, ELISA is subject to a degree of error. Factors such as temperature, concentration of reagents and pipetting error may affect the efficacy of binding between antibody and antigen and therefore introduce inaccuracy. In order to test the reproducibility of the results obtained, 2 experiments were conducted. In the first, a control sample made up from the standard provided in the kit was analysed in 20 separate wells in the same assay. This allowed the intra-assay variability to be calculated. In the second experiment, the same control sample was run in duplicate in each ELISA kit plates processed. In this way the inter-assay variability was calculated.

Table 5.2 displays the IL-10 concentration measured when the sample was run in duplicate 20 times in the same ELISA tray. Table 5.3 displays the IL-10 concentration (mean of the duplicate sample values) when the same control sample was analysed in each of the ELISA plates used.

Sample	IL-10 conc (pg/ml)
1	2.02
2	2.33
3	1.93
4	2.10
5	1.98
6	2.02
7	2.13
8	1.97
9	1.77
10	2.19
11	1.63
12	2.22
13	2.35
14	1.99
15	2.50
16	2.13
17	1.78
18	2.15
19	2.19
20	2.29
Mean	2.08
SD	0.21
%CV	10.1%

Table 5.2. IL-10 concentration values obtained when a control sample was analysed in 20 wells of a single ELISA plate, (SD, standard deviation), (CV, coefficient of variation)

Sample	IL-10 conc (pg/ml)
1	1.88
2	1.99
3	2.31
4	1.73
5	1.78
6	1.86
7	2.22
8	1.75
9	1.94
Mean	1.94
SD	0.20
%CV	10.3%

Table 5.3. IL-10 concentration values obtained when the same control sample was analysed in each ELISA plate. (SD, standard deviation), (CV, coefficient of variation)

The experiments demonstrate acceptably low intra-assay and inter-assay variability (10.1% and 10.3% respectively).

DNA extraction from blood

Due to the large number of blood samples processed a commercially available DNA extraction kit (*Puregene genomic DNA isolation kit*) was used to extract white blood cell DNA. The principle is based on the salting out method described by Miller SA et al (Miller SA 1988).

As DNA is contained in the nucleated white blood cells, the red blood cells, which are lacking in genomic DNA are lysed to facilitate their separation from the white blood cells. The DNA is extracted from the white blood cells by cell lysis with an anionic detergent in the presence of a DNA stabiliser. The DNA stabiliser inhibits the activity

of DNases, enzymes that degrade DNA. Contaminants in the solution such as proteins are removed by salt precipitation. The DNA is then recovered by precipitation with alcohol and dissolved in a buffered solution containing a DNA stabilizer.

The precise protocol used was as follows:

1. **Cell lysis** – 300 µl of white cell rich blood was added to 900 µl of cell lysis solution containing EDTA, ammonium chloride and sodium bicarbonate and incubated at room temperature. The mixture was then centrifuged and the supernatant is removed with a pipette leaving behind a pellet and 10-20 µl of residual liquid. The pellet was then vortexed to resuspend the white cells before 300 µl of cell lysis solution was added.
2. **Protein precipitation** – 100 µl of protein precipitation solution containing ammonium acetate was added to the cell lysate before a further vortex step followed by centrifuge. This resulted in the precipitated protein forming a dark brown pellet at the bottom of the tube.
3. **DNA precipitation** – The supernatant containing the DNA was poured into a clean microcentrifuge tube containing 300 µl of 100% isopropanol (2 – propanol). The sample was mixed and then centrifuged to produce a small pellet of DNA at the bottom of the tube. The supernatant was poured off and the DNA pellet was washed with 300 µl of 70% ethanol. After a further centrifuge step the ethanol was poured off.
4. **DNA hydration** – 100 µl of DNA hydration solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM tris(hydroxymethyl)aminomethane at pH 7-8 was added to the DNA and

vortexed to mix. The mixture was then incubated at 65°C to facilitate rehydration. The mixture was then stored at – 20°C until used.

DNA was extracted from samples in batches of 12, as this was the number of wells in the microcentrifuge machine used. Great care was taken to avoid potential contamination of samples with foreign DNA as any contaminating DNA would be amplified in the same way as the patients' DNA in the subsequent polymerase chain reaction (PCR). All reagents used were guaranteed by the manufacturer to be free from DNA contamination and the reaction tubes, pipettes and pipette tips were kept free from possible contaminants and used only for DNA extraction or the setting up PCR. All procedural steps in which reaction tubes were opened were performed in a Class 2 microbiological air flow cabinet that had been first cleaned with a 70% solution of industrial methylated spirit, and gloves were worn at all times. In each run of 12 samples, 11 patient samples were processed along with a 'water blank' containing all the reagents but with double distilled water instead of blood. This 'water blank' was amplified in a PCR reaction in the same way as the patient samples to ensure contamination had not occurred. In total 80 batches of 11 samples with 1 water blank were run. None of the 80 water blanks when amplified in a PCR reaction contained DNA.

Genotyping IL-10 single nucleotide polymorphisms

Polymerase chain reaction (PCR)

PCR is an extremely powerful technique used for the amplification of a defined DNA target sequence or template to produce numerous copies of the target sequence. The PCR reaction is based upon the in vivo cellular DNA replicative process occurring in

nature and relies upon the action of a DNA polymerase (an enzyme that creates new DNA strands using a DNA template), 2 oligonucleotide primers (short polynucleotide chains to which new deoxyribonucleotides (dNTPs) can be added), dNTPs (DNA precursors) and magnesium ions. The template DNA to be amplified is added to the reaction mix and undergoes amplification in a process involving 3 main cycles:

1. Denaturation of target DNA at temperature 93-95°C
2. Primer annealing at temperature approximately 50-70°C
3. DNA synthesis / extension at temperature approximately 70-75°C

The first step in PCR is denaturation, in which the template DNA is heated to approximately 95°C to break the hydrogen bonds between the complementary bases on the two strands of DNA. This produces two single strands of DNA (ssDNA). The DNA is then flash-cooled to prevent the strands re-annealing and to allow oligonucleotide primers to bind or anneal to the ssDNA. Primers are sequences of usually 15 to 25 nucleotides that are complementary to specific areas upstream and downstream of the area of target DNA to be amplified. In this way, the section of DNA to be amplified is very specific. In the presence of a heat stable DNA polymerase (a DNA extension enzyme), Mg^{2+} and a mix of the DNA precursors, the 4 deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), the synthesis of new DNA strands takes place. In each cycle the number of DNA strands doubles. The new DNA strands are complementary to the individual DNA strands of the original target DNA sequence. The cycles are then repeated, often around 30 times and the new strands of DNA produced in previous cycles are amplified resulting in an exponential increase in the number of DNA strands. Theoretically the number of DNA strands

produced is 2^n where n is the number of cycles. This means that after 20 cycles of amplification there will be approximately 1 x 10^6 copies of the template (Figure 5.4).

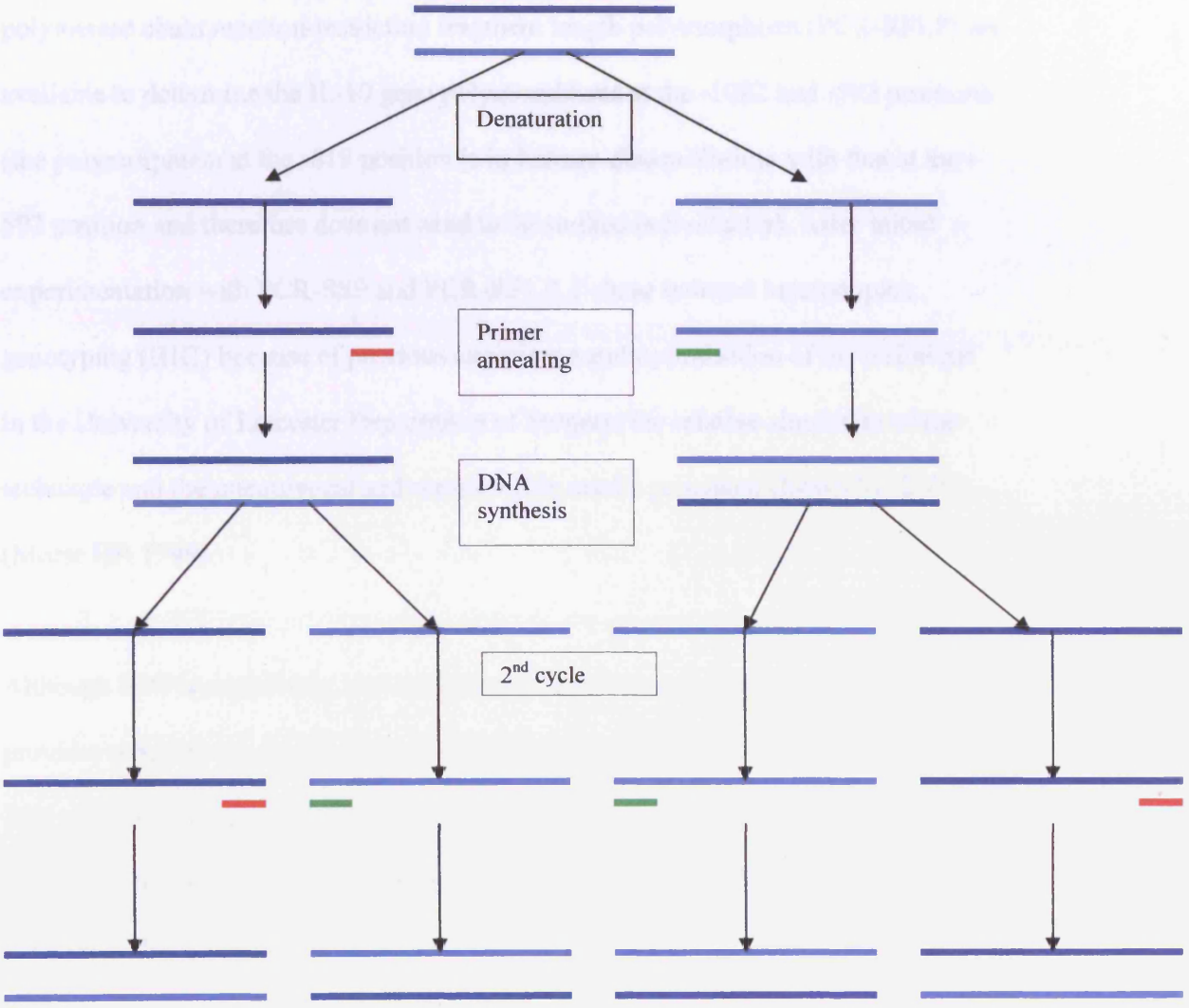


Figure 5.4. Diagramatic representation of two cycles of PCR process.

DNA strands

Forward and reverse primers

Induced heteroduplex genotyping

A number of PCR based methods including polymerase chain reaction-sequence-specific primers (PCR-SSP or amplification refractory mutation system PCR) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) are available to determine the IL-10 gene polymorphisms at the -1082 and -592 positions (the polymorphism at the -819 position is in linkage disequilibrium with that at the -592 position and therefore does not need to be studied individually). After initial experimentation with PCR-SSP and PCR-RFLP, I chose induced heteroduplex genotyping (IHG) because of previous experience and optimisation of the technique in the University of Leicester Department of Surgery, the relative simplicity of the technique and the unequivocal and reproducible results generated (Bown MJ 2005) (Morse HR 1999).

Although IHG is a relatively new technique, it has found widespread acceptance and provides reliable and simple means of genotyping large numbers of samples.
IHG utilises two separate PCR reactions. In reaction one, the region of DNA containing the polymorphic site, the target sequence, is amplified in a standard PCR reaction as described above. In reaction two, an artificially constructed heteroduplex generator molecule which is almost identical to the DNA target sequence is amplified. The heteroduplex generator molecule has been deliberately modified immediately adjacent to the polymorphic loci with insertion, deletion or substitution of specific nucleotide bases. In the case of the IHG used to identify the IL-10 gene polymorphisms at the -1082 and -592 positions, there are 4 and 5 adenine base insertions (polyA) downstream of the polymorphic locations.

Following amplification of the target sequence and the heteroduplex generator in separate reactions, the two amplicons (PCR products) are mixed, heated to 95°C to break the inter-strand hydrogen bonds and then cooled. During the cooling phase the single strands of amplified DNA and heteroduplex generators bind to one another. This results in 3 different products; double stranded template DNA, double stranded heteroduplex generators and one strand of template DNA binding to one strand of the heteroduplex generator due to the almost complementarity between the 2 sequences.

In order for the latter combination to bind, a conformational change must occur and the heteroduplex generator bends to allow bases on either side of the insertion to bind to complementary bases on the DNA sequence. This causes a loop in the heteroduplex generator and the double stranded structure takes on a rigid shape that is specific to the SNP present. Figure 5.5 is a schematic representation of the process of IHG. On binding to the heteroduplex generator the hybrid double strand undergoes a 3-dimentional conformation change dependent on the alleles present. This is shown by the different angles on the 'arms'.

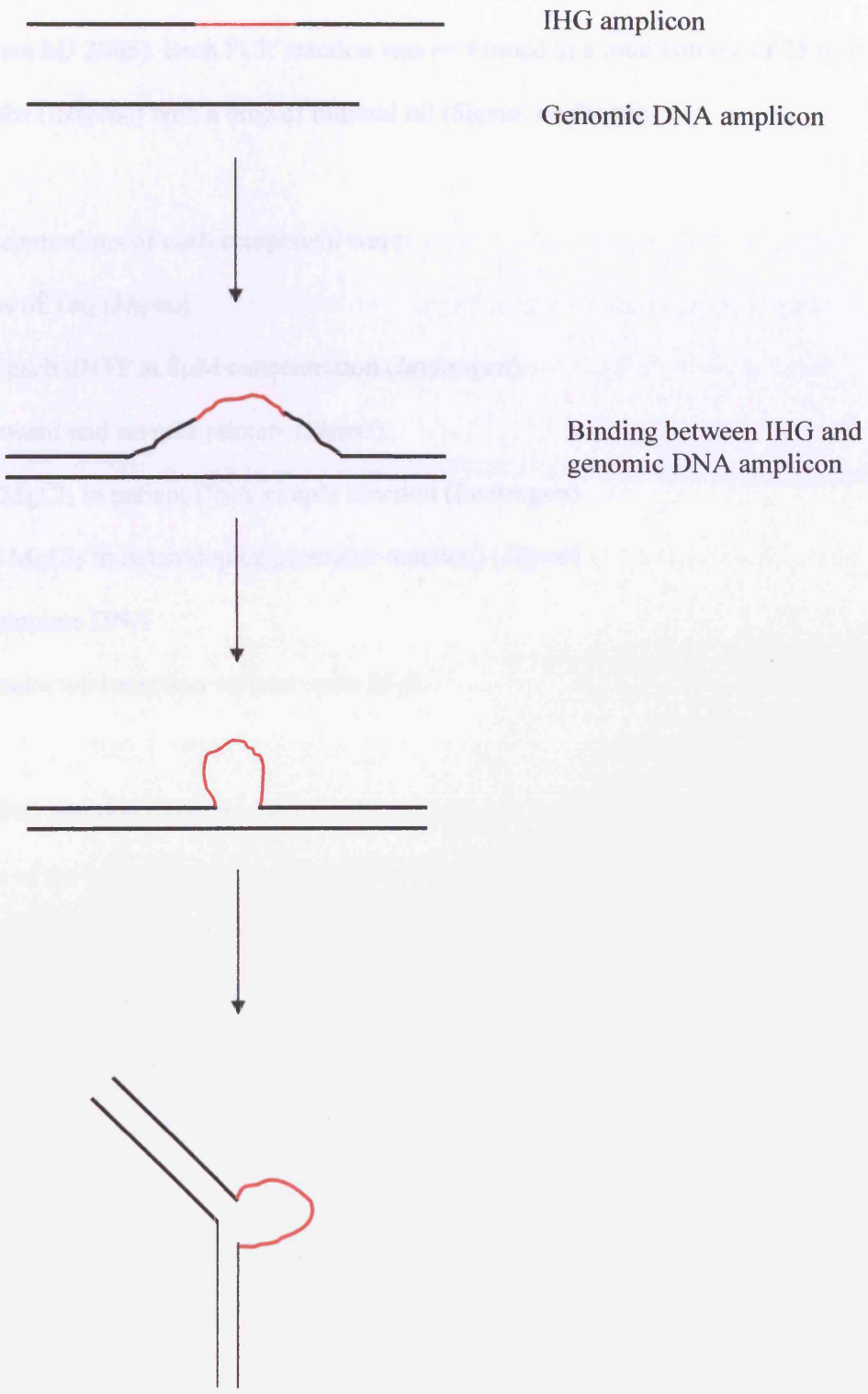


Figure 5.5. Diagram demonstrating conformation change in heteroduplex.

The exact protocol followed was based on the method previously described by Bown et al (Bown MJ 2005). Each PCR reaction was performed in a total volume of 25 µl in a PCR tube (*ABgene*) with a drop of mineral oil (*Sigma*) on the top.

The concentrations of each component were:

0.25 units of Taq (*Sigma*)

2.5 µl of each dNTP at 8µM concentration (*Invitrogen*)

5 µM forward and reverse primers (*Sigma*)

1.5 mM $MgCl_2$ in patient DNA sample reaction (*Invitrogen*)

(2.5 mM $MgCl_2$ in heteroduplex generator reaction) (*Sigma*)

2.5 µL template DNA

H_2O to make total reaction volume up to 25µL.

For the IHG reaction to determine the alleles present at the -1082 position the sequence of the heteroduplex generator (*Sigma*) was:

AATCCAAGACAACACTACTAAGGCTTCTTTGGGAGAAAAAGGGGAAGTA

GGGATAGGTAAGAGGAAAGTAAGGGACCTCCTATCCAG

Forward primer sequence: 5'-AATCCAAGACAACACTACTAAGGC-3'

Reverse primer sequence: 5'-CTGGATAGGAGGTCCCTTA-3'

For the IHG reaction to determine the alleles present at the -592 position the sequence of the heteroduplex generator (*Sigma*) was:

GAAATCGGGGTAAAGGAGCCTGGAACACATCCTGTGACCCCGCCTGTAAA

AACTGTAGGAAGCCAGTCTCTGGAAAGTAAAATGGAAGGGCTGCTTGGG

AACT

Forward primer sequence: 5'-GAAATCGGGGTAAAGGAGCC-3'

Reverse primer sequence: 5'-AGTTCCCAAGCAGCCCTTCC-3'

Each reagent was diluted to the concentration described and stored as a stock solution. A mastermix was prepared to allow numerous reactions to be prepared simultaneously and improve pipetting accuracy. In practice this meant that 40 samples and 1 water blank could be amplified simultaneously. Figure 5.6 is a photograph of the thermal cycling machine used (*Perkin Elmer Thermal Cycler 480*).

The following PCR reaction conditions were used (all conditions had previously been optimised):

-1082 reaction conditions

Initial denaturation:	5 minutes at 95°C	
Denaturation:	1 minute at 95°C	30 cycles
Anneal:	1 minute at 57°C	
Elongation:	1 minute at 72°C	
Final extension:	5 minutes at 72°C	

-592 reaction conditions

Initial denaturation:	5 minutes at 95°C	
Denaturation:	1 minute at 95°C	30 cycles
Anneal:	1 minute at 62°C	
Elongation:	1 minute at 72°C	
Final extension:	5 minutes at 72°C	

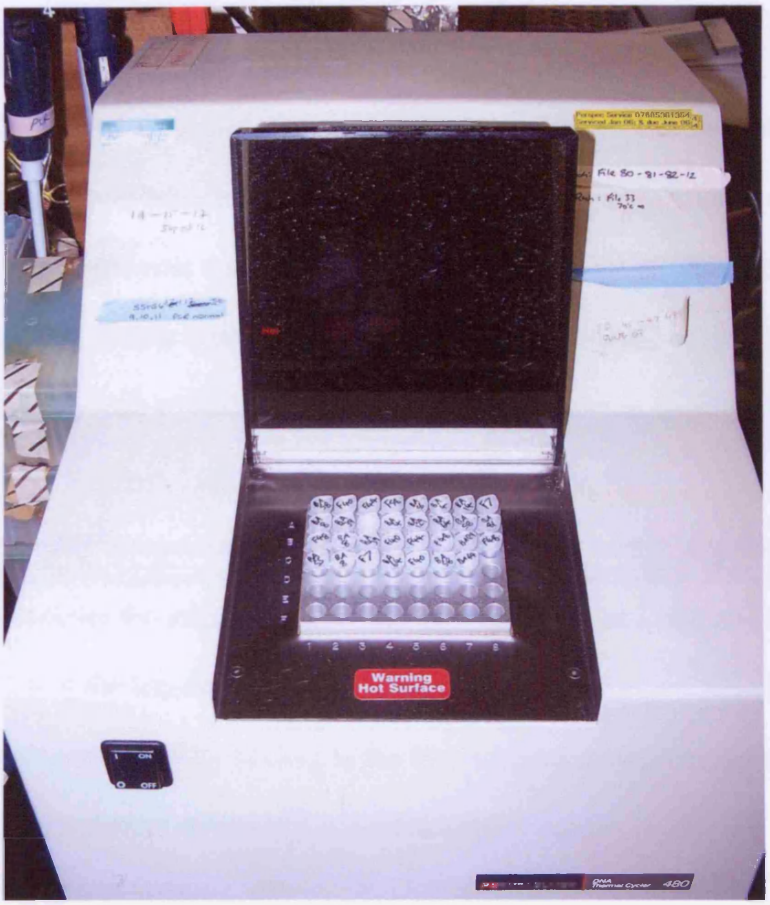


Figure 5.6. Photograph of PCR tubes containing sample and mastermix about to undergo PCR in thermal cycler machine.

Following the final extension phase the PCR reaction tubes containing the amplicons were stored at 4°C in preparation for the next step; mixing of the sample amplicons with heteroduplex generator amplicons.

In this step 12.5µl of sample amplicons was mixed with a similar volume of heteroduplex generator amplicons, transferred to the PCR thermal cycler machine and heated to 95°C and cooled in a stepwise manner by 1°C every 30 seconds down to 48°C. This enabled breaking of the inter-strand hydrogen bonds and some binding of DNA and heteroduplex generator amplicons.

Polyacrylamide gel electrophoresis

The different heteroduplexes were then differentiated by electrophoresis on a non-denaturing polyacrylamide gel in a vertical electrophoresis tank. Each product has different electrophoretic mobility and this allowed the three products (double stranded template DNA, double stranded heteroduplex generators and one strand of template DNA binding to one strand of the heteroduplex generator) to clearly separate.

Polyacrylamide forms cross-linked chains resulting in a matrix structure. This matrix structure impedes the migration of DNA, which migrates at a rate inversely proportional to the $\log_{10}$ of their size. In addition, the conformational change in the DNA molecules caused by binding to the heteroduplex generators depending on the polymorphism present results in a unique migration pattern on the gel.

A 15% acrylamide gel was used as this has resolution sufficient to separate 25-43 base pairs on DNA product. After initial experimentation with commercially available gels it was found that improved images were obtained with hand cast gels. A mixture was made to allow 4 gels to be cast simultaneously (Figure 5.7). This was composed of:

20ml of 30% acrylamide / bisacrylamide (*Geneflow*)

11.7ml of double distilled H_20

8ml of 5x Tris-Borate-EDTA (TBE) (*Invitrogen*)

0.28ml of 10 % ammonium persulphate (*Geneflow*)

7µl of Tetramethylethylenediamine (TEMED) (*Invitrogen*)

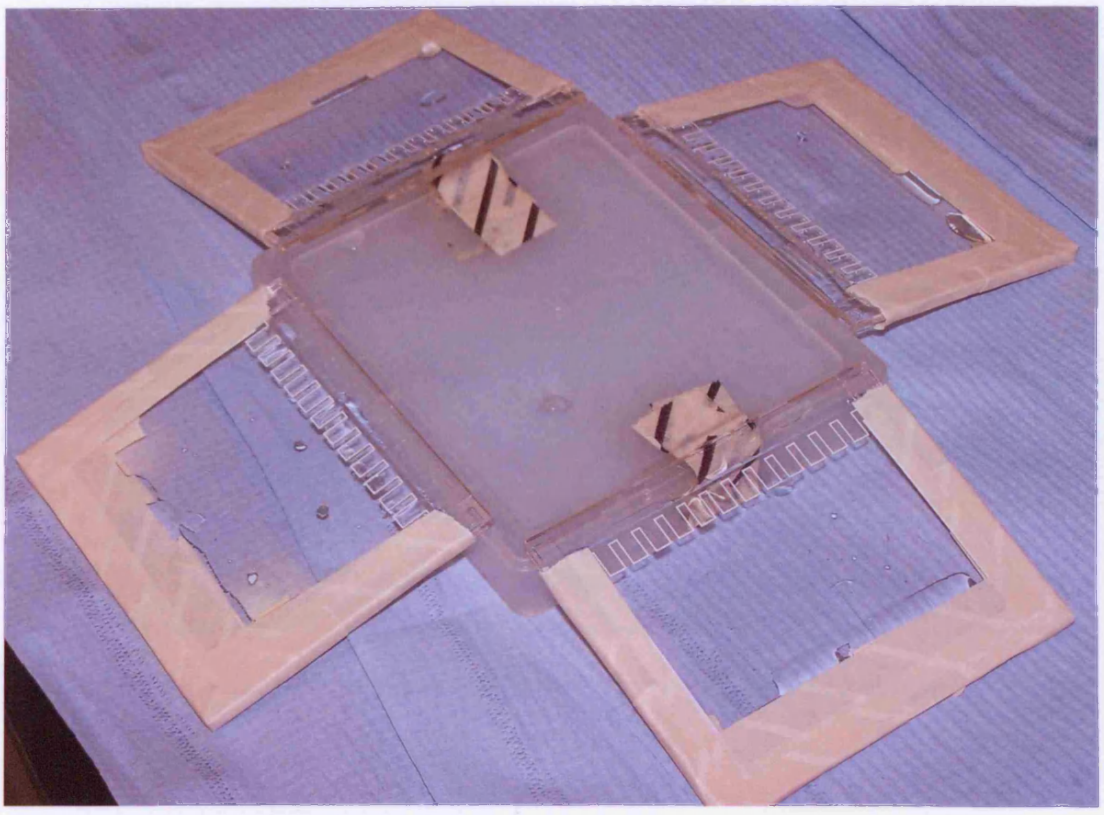


Figure 5.7. Hand cast gels being allowed to set. Autocave tape was used to seal sides and base of gel casting plates.

The electrophoresis buffer used was 1xTris-borate (TBE). This was prepared in a 5xstock solution made with 54g Tris base, 27.5g boric acid and 20 ml 0.5M EDTA (pH 8.0) and made up to a volume of 1 litre with double distilled water. A 5x dilution was performed prior to use.

2.5 µl of a gel loading buffer (blue juice) (*Invitrogen*) was mixed with each sample and 20 µl of the mixture was loaded into each well on the gel (Figure 5.8). A loading buffer is important as it increases the density of the sample which allows easier loading into the well and allows visualisation of sample in the well.

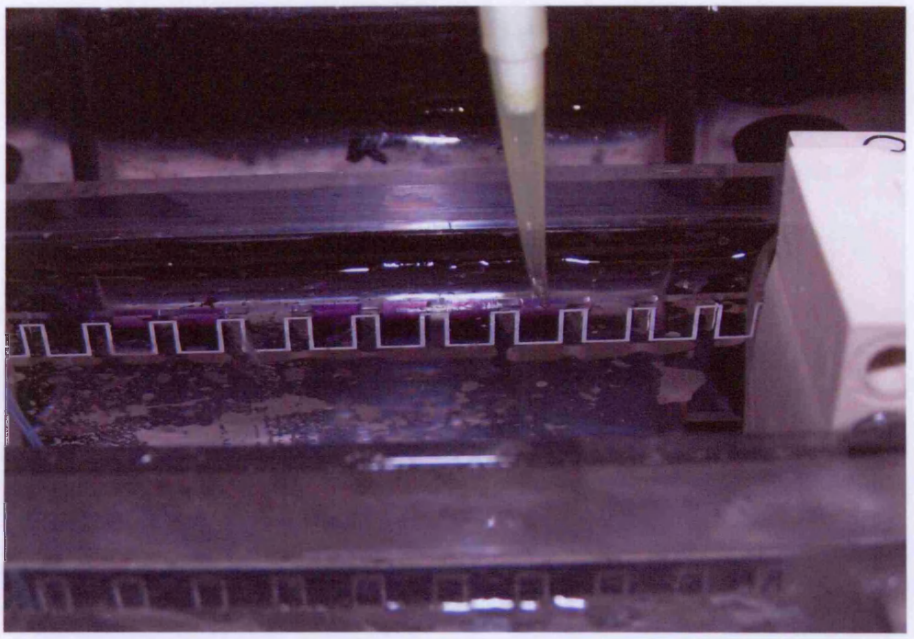


Figure 5.8. Samples containing amplicon and loading buffer being loaded into wells in acrylamide gel.

The samples then underwent electrophoresis at 100 volts for 2 hours and thirty minutes (Figure 5.9).

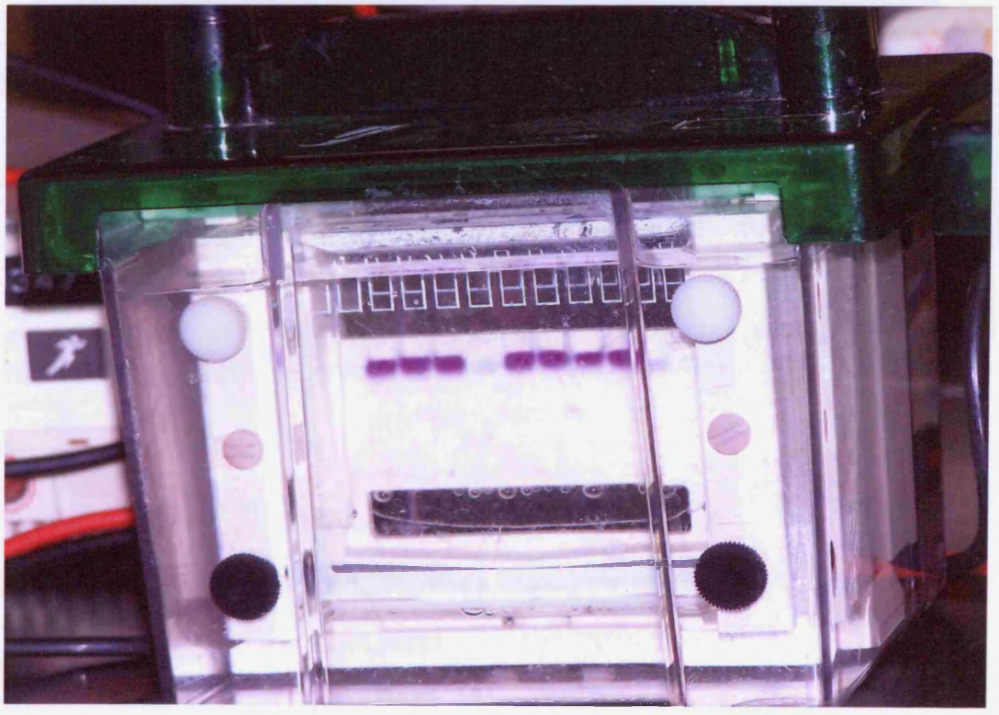


Figure 5.9. Samples undergoing electrophoresis. The loading dye allows migration of samples in gel to be monitored.

Ethidium bromide, which intercalates between the nucleotide bases was used to stain the DNA, and therefore allow visualisation of the DNA products under ultraviolet light. Unlike agarose gels, acrylamide gels cannot be cast in the presence of ethidium bromide because it inhibits acrylamide polymerisation. Therefore following electrophoresis the gels were soaked in ethidium bromide (*Invitrogen*) solution (0.5 μg / ml) diluted with 1xTBE buffer for 35 minutes prior to ultra violet visualisation. Figure 5.10 shows a typical gel image viewed under ultraviolet light.

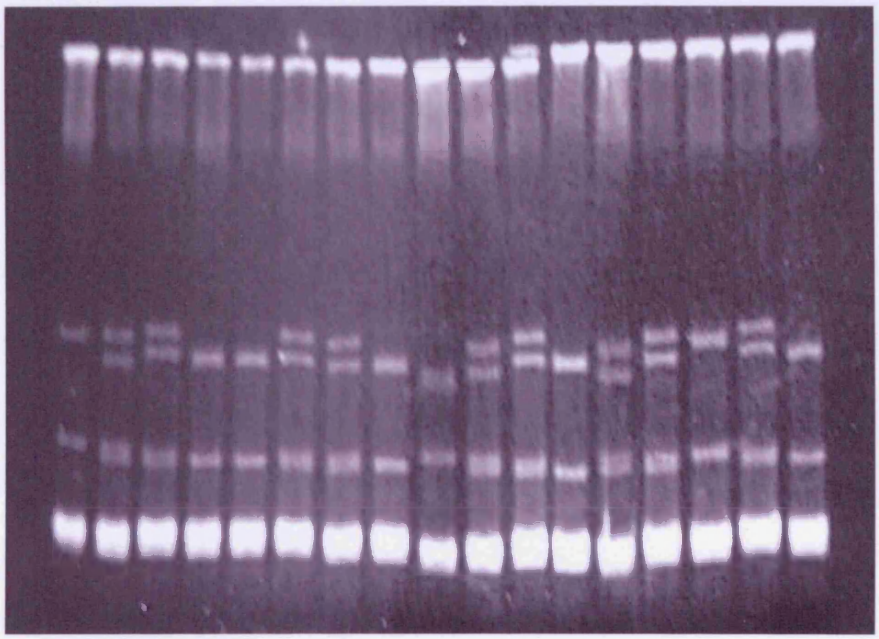


Figure 5.10. Image of a polyacrylamide gel following electrophoresis and staining with ethidium bromide. Typical pattern of bands for GG, GA and AA genotypes are displayed.

Prevention of contamination

With all PCR reactions a number of points must be considered. PCR is an extremely powerful technique and because of this great care must be taken to ensure contamination of the samples does not occur. If foreign DNA is present in the reaction, this will be amplified in the same way as the template DNA. All reagents

used were guaranteed by the manufacturer to be free from contamination. The reaction tubes and pipette tips were kept free from possible contaminants and used specifically for PCR. The PCR reaction mixture was prepared in a Class 2 microbiological air flow cabinet and in an area in the laboratory separate from that used for DNA preparation or PCR product analysis, as contamination from previously amplified DNA is the commonest source of problems. At all stages from blood sampling to gel electrophoresis gloves were worn. A negative control consisting of all reagents and primers but without DNA was included with every 11 blood samples from which DNA was extracted and with each PCR run to ensure that contamination was not present.

Validation of IHG

In order to validate the induced heteroduplex genotyping technique for IL-10 gene polymorphisms 100 samples of known genotype were genotyped for polymorphisms at the -1082 and -592 positions. There was a 100% concordance in genotype.

In order to test the reproducibility of the results a number of random samples were repeated. Table 5.4 shows the number of reactions repeated for the polymorphisms at the -1082 and -592 positions. All samples showed 100% concordance.

Position	Repeated x2	Repeated x3	Repeated x4	Repeated x5
-1082	177	24	15	2
-592	71	10	0	1

Table 5.4. Table shows the number of reactions repeated when determining the polymorphisms at the -1082 and -592 positions.

Measurement of IL-10 in the aneurysm wall

In order to investigate the presence of IL-10 in the AAA wall I obtained 1 x 1 cm pieces of infrarenal aorta from 22 patients undergoing elective AAA repair. During the operation, following clamp application and sac opening, the operating surgeon removed the tissue specimen from the anterior section of the sac.

Immediately after procurement the tissue was placed into a cryovial (Sarsted) and snap frozen in liquid nitrogen and stored at -80°C to allow future analysis in batches. A standard protocol was followed to homogenise the samples. The frozen samples were cut into 100 mg pieces (wet weight) and washed for 20 seconds at 4°C and agitated in PBS (phosptate buffered saline composed of 0.14 M NaCl, 0.01 M Po4 buffer, 0.0003 M KCl at a pH of 7.45) (*Invitrogen*) to remove blood. The tissue was then diced into small pieces and placed in 1 ml of ice cold homogenisation buffer (50 mM Tris – HCl, 2 M urea, 20 mM NaCL, 0.1% w/v EDTA, 0.1% v/v Bioj 35, 1 x complete mini protease inhibitor cocktail (*Roche Diagnostics*). The samples were then homogenised using an Ultra – Turrax T-25 homogeniser (*Iha Labortechnik*). The homogenates were then sonicated for 3 x 10 seconds at an amplitude of 12 μm amplitude in a Soniprep 150 sonicator (*Sanyo*) on ice. Cellular debris was removed by centrifuging for 1 hour at 16,000g at 4°C. The supernatant was extracted and a 100 μl aliquot was used to determine the total protein concentration of the homogenised samples using the BCA protein assay method (*Pierce*).

Once the total protein content of each sample was known all the samples were manipulated to 1mg/ml and stored at -80°C until ELISA analysis to determine the IL-10 concentration.

Chapter 6

Adequacy of Cardiovascular Risk Reduction in Patients with Abdominal Aortic Aneurysm

Contents

Background

Manifestations of occlusive arterial disease are prevalent in patients with AAA and coronary heart disease is the leading cause of death. Recently evidence of the magnitude of the cardiovascular risk reduction afforded by pharmacological therapies has become available. Antiplatelet agents, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), angiotensin-converting enzyme (ACE) inhibitors and beta adrenergic receptor blockers (beta-blockers) reduce cardiovascular risk in patients with specific manifestations of atherosclerotic occlusive arterial disease (Antithrombotic Trialists' Collaboration 2002) (Heart Protection Study Collaborative Group 2002) (The Heart Outcomes Prevention Evaluation Study Investigators 2000) (Gottleib SS 1998). Used individually the relative - risk of fatal and non fatal cardiovascular events is reduced by approximately 25%, and when used in combination the risk reduction may be as great as 75% (Yusuf S 2002). Atherosclerotic occlusive arterial disease, and in particular coronary heart disease (CHD), is prevalent in patients with abdominal aortic aneurysm (AAA) resulting in reduced life expectancy compared with the rest of the population (Brown OW 1981) (Batt M 1999).

Methods

Despite the strong association between AAA and cardiovascular risk, the potential role of pharmacological risk reduction in patients with AAA has not been thoroughly investigated and remains unclear. There is sparse mention of AAA in recommendations aimed at targeting patients for cardiovascular risk reduction and little is known about the adequacy of current risk reduction in patients with AAA. A study was therefore conducted to determine the prevalence of cardiovascular disease

and risk factors in patients with AAA, and to ascertain the number of patients in whom a statin, anti-platelet agent, beta-blocker and ACE inhibitor are indicated and the number of patients who are currently receiving adequate evidence based cardiovascular risk reduction therapy.

In patients with no history of occlusive arterial disease, prediction of their CHD risk over 10 years was estimated using a CHD risk assessment calculator based on the Framingham Heart Study data (Wilson PWF 1998). This risk assessment calculation is based upon attaching weighting to patient age, sex, systolic and diastolic blood pressure, total and high density lipoprotein cholesterol (HDL-C), diabetes and smoking habit. In the UK, it is generally accepted as a minimum standard of care that a statin is indicated in the primary prevention of cardiovascular disease in patients without arterial occlusive disease with a $\geq$ 30% predicted risk of CHD in the next 10 years (Department of Health 2000). An antiplatelet agent is recommended in the primary prevention of cardiovascular disease in patients with a 10 year CHD risk $\geq$ 15%, and I used this as an indication for an antiplatelet agent in patients with no evidence of occlusive arterial disease (Sanmuganathan PS 2001) (British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Society, British Diabetic Association 1998).

Patients were considered suitable for an antiplatelet agent, statin, beta-blocker or ACE inhibitor in accordance with evidence and recommendations from the Antithrombotic Trialists' Collaboration, the Heart Protection Study, the Heart Outcomes Prevention Evaluation (HOPE) Study, the National Service Framework for Coronary Heart Disease, the Joint British Recommendations on Prevention of Coronary Heart Disease

in Clinical Practice and the Antiplatelet Therapy in Peripheral Arterial Disease Consensus Statement summarised in Table 6.1 (Antithrombotic Trialists' Collaboration 2002) (Heart Protection Study Collaborative Group 2002) (The Heart Outcomes Prevention Evaluation Study Investigators 2000) (Gottleib SS 1998) (Sanmuganathan PS 2001) (British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Society, British Diabetic Association 1998) (Department of Health 2000). Figure 6.1 is an algorithm for the treatment of cardiovascular risk.

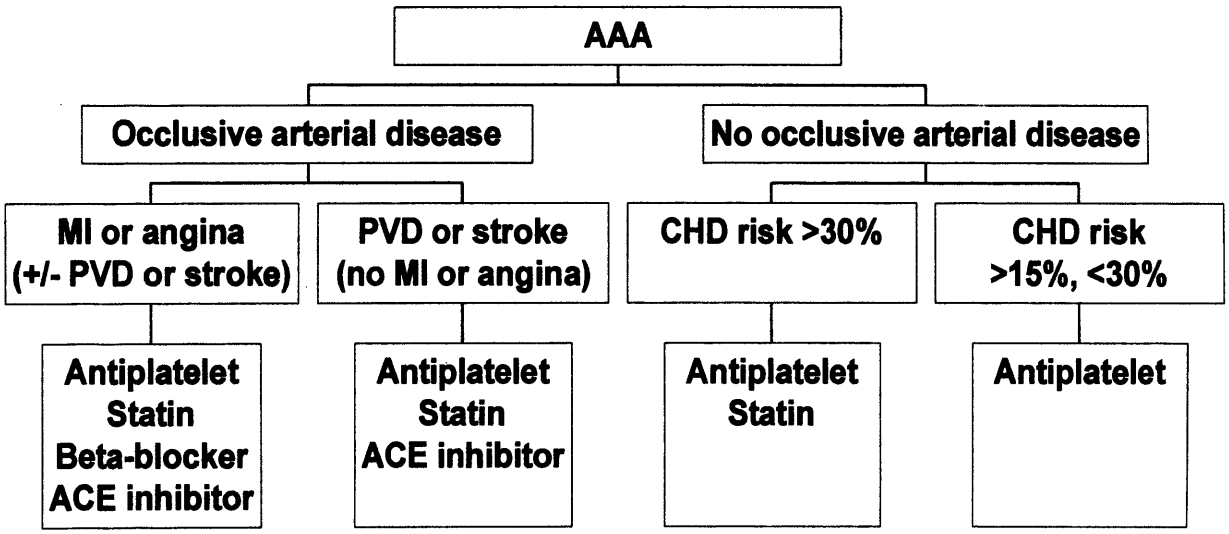


Figure 6.1. Algorithm for pharmacological cardiovascular risk reduction in patients with AAA. Occlusive arterial disease is defined as MI, angina, PVD or ischaemic stroke. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction. PVD, peripheral vascular disease. ACE, angiotensin converting enzyme.

1. An antiplatelet agent is indicated in all patients with evidence of occlusive arterial disease (MI, angina, ischaemic stroke or PVD).
2. An antiplatelet agent is indicated in primary prevention in patients without occlusive arterial disease in whom the estimated 10 year CHD risk $\geq$ 15%.
3. An antiplatelet agent should be strongly considered in patients with AAA without occlusive arterial disease.
4. A statin is indicated in primary prevention in patients in whom the estimated 10 year CHD risk $\geq$ 30%.
5. A statin is indicated in all patients with CHD or evidence of occlusive vascular disease (MI, angina, ischaemic stroke or PVD).
6. An ACE inhibitor is indicated in all patients with MI, PVD, ischaemic stroke or DM who have at least 1 other risk factor (eg. hypertension, smoking, high total cholesterol, low HDL-C or documented microalbuminuria).
7. Beta-blockers should be given to all patients with a history of MI, and their use in patients with angina reduces the number of ischaemic episodes encountered.

Table 6.1. Summary of current evidence and recommendations for antiplatelet, statin, beta-blocker and ACE inhibitor use in cardiovascular risk reduction. Abbreviations: MI, myocardial infarction. PVD, peripheral vascular disease. CHD, coronary heart disease. HDL-C, high density lipoprotein cholesterol. DM, diabetes. ACE, angiotensin converting enzyme.

Results

The first 313 patients with AAA recruited into the case control study were analysed. Two hundred and eighty (89%) of the patients were men and 33 (11%) were women. The median age was 71 years (range 53 – 91). Seventy patients (22%) had previously

undergone AAA repair and 243 (78%) were undergoing AAA surveillance or consideration of elective AAA repair at the time of the study. Mean AAA diameter when recruited into the study or at the time of AAA repair was 5.2 cm (range 3 to 10). Table 6.2 displays the prevalence of associated cardiovascular disease in the patients with AAA.

Cardiovascular pathology	Number of patients	%
Hypertension	228	73
CHD (MI or angina)	107	34
MI	69	22
Angina only	38	12
Ischaemic stroke	46	15
PVD	80	26

Table 6.2. Prevalence of cardiovascular pathology in patents with AAA. If patients had more than 1 vascular pathology they are included in more than 1 category. N=313. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction. PVD, peripheral vascular disease.

Of the total 313 patients, 202 (65%) had a blood lipid measurement in the previous 5 years, the mean total cholesterol was 5.0 mmol/l (range 2.9 to 8.5). Two hundred and twenty eight patients (73%) were undergoing treatment for hypertension. Mean systolic blood pressure was 146 mmHg and the mean diastolic blood pressure was 84 mmHg. Two hundred and eight patients (66%) had a systolic pressure $\geq$ 140 mmHg or a diastolic pressure $\geq$ 90 mmHg. Twenty one patients (7%) were known to have diabetes. Twenty seven (9%) patients denied ever having smoked cigarettes, 75 (24%)

were current smokers and 211 (67%) were former smokers. The patients with a history of cigarette smoking had a mean pack year history of 38.2 years.

One hundred and seventy four patients (56%) had evidence of occlusive arterial disease (MI, angina, PVD or stroke), thereby having indication for an antiplatelet agent, statin and ACE inhibitor (Table 6.1). One hundred and seven patients of the 174 (61%) had a history of MI or angina and therefore, in addition, had an indication for a beta-blocker (Table 6.1 and Figure 6.1). Table 6.3 displays the numbers and percentages of patients with occlusive arterial disease with indication for specific therapy and the numbers and percentages appropriately treated.

Therapy	**Patients with Indication (%)**	**Patients on Treatment (%)**
Antiplatelet	174 (100)	129 (74)
Statin	174 (100)	79 (45)
Beta-blocker	107 (61)	41 (38)
ACE inhibitor	174 (100)	67 (39)

Table 6.3. Numbers and percentages of patients with occlusive arterial disease with indication for specific therapy and numbers and percentages appropriately treated. N=174. Abbreviation: ACE, angiotensin converting enzyme.

One hundred and thirty nine patients (44%) with AAA were not known to have occlusive arterial disease and therefore an indication for a statin was based upon the demonstration of a 10 year CHD risk $\geq$ 30% (Table 6.1 and figure 6.1). Fifty one of the 139 patients (37%) had not had a recent blood lipid measurement. Eighty eight (63%) had a total cholesterol and HDL-C measurement thereby allowing 10 year

CHD risk calculation. The mean 10 year CHD risk in the 88 patients was 22% (range 5 to 53). Twenty two of the 88 patients (25%) had a 10 year CHD risk ≥ 30%, thereby having an indication for a statin.

Of the 88 patients with a determinable 10 year CHD risk, 68 (77%) had a 10 year risk ≥ 15%, thereby having an indication for antiplatelet treatment. Figure 6.2 illustrates statin use depending on lipid measurement and indication. Four patients without occlusive arterial disease had diabetes and an additional cardiovascular risk factor, and in accordance with the results of the HOPE study should use an ACE inhibitor. Table 6.4 displays the numbers of and percentages of patients without occlusive arterial disease with indication for specific therapy and the numbers and percentages appropriately treated.

Therapy	**Patients with indication (%)**	**Patients treated (%)**
Antiplatelet (N=88)	68 (77)	17 (25)
Statin (N=88)	22 (25)	2 (9)
ACE inhibitor (N=139)	4 (3)	2 (50)

Table 6.4. Numbers and percentages of patients with no evidence arterial occlusive disease with indication for specific therapy and numbers and percentages appropriately treated. Indication for an antiplatelet agent or statin could only be determined in 88 patients.
Indication for a statin based upon ≥ 30% 10 year CHD risk and indication for antiplatelet based upon ≥ 15% risk

Table 6.5 displays the total numbers and percentages of the 313 patients with AAA with indication for specific therapy and those undergoing appropriate pharmacological risk reduction.

Therapy	Patients with indication for therapy (%)	Patients using treatment when indicated (%)
Antiplatelet (N=262)	242 (92)	146 (60)
Statin (N=262)	196 (75)	81 (41)
Beta-blocker (N=313)	107 (34)	41 (38)
ACE inhibitor (N=313)	178 (57)	69 (39)

Table 6.5. Overall numbers and percentages of patients with AAA who have indication for specific therapy and numbers and percentages of patients being appropriately treated. In the consideration of patients in whom an antiplatelet agent or statin are indicated the 51 patients in whom 10 year CHD risk could not be established have been excluded.

Table 6.6 gives a breakdown of vascular pathology in the 313 patients with AAA and the total number and percentage of patients using an antiplatelet, statin, ACE inhibitor and beta-blocker.

Combination of cardiovascular Pathologies	Number of patients (%)	Patients (%) using Statin	Patients (%) using Antiplatelet	Patients (%) using Beta-blocker	Patients (%) using ACE inhibitor
AAA only	139 (44)	24 (17)	35 (25)	33 (24)	35 (25)
AAA and PVD	38 (12)	8 (21)	18 (47)	12 (32)	11 (29)
AAA and CHD	63 (20)	36 (57)	53 (84)	30 (48)	23 (37)
AAA and stroke	23 (7)	8 (35)	16 (70)	8 (35)	6 (26)
AAA, PVD and CHD	27 (9)	18 (67)	23 (85)	5 (19)	16 (59)
AAA, PVD and stroke	6 (2)	2 (33)	3 (50)	1 (17)	2 (33)
AAA, CHD and stroke	8 (3)	3 (38)	8 (100)	3 (38)	2 (25)
AAA, CHD, Stroke and PVD	9 (3)	4 (44)	8 (89)	3 (33)	7 (78)

Table 6.6. Breakdown of associated manifestations of occlusive arterial disease in patients with AAA and the numbers and percentage receiving an antiplatelet agent, statin, beta-blocker and ACE inhibitor. N=313.
Abbreviations: CHD, coronary heart disease. MI, myocardial infarction. PVD, peripheral vascular disease.

Discussion

The high prevalence of cardiovascular pathology and risk factors in patients with AAA mean that most have an indication for an antiplatelet agent, statin, ACE inhibitor or beta-blocker. Seventy five percent of the 174 patients with occlusive arterial disease and 88 patients without evidence of occlusive arterial disease who had a lipid measurement had a clear indication for a statin by virtue of having a manifestation of occlusive arterial disease or a predicted 10 year CHD risk of $\geq$ 30%. If the 51 patients without occlusive arterial disease who did not have a lipid

measurement are included in the analysis and it is assumed that the proportion of the 51 patients with indication for a statin is the same as that in the patients without occlusive arterial disease who had a lipid measurement (25%), then 67% would have an indication for a statin. An antiplatelet agent was indicated in 92% of the patients with AAA (excluding the 51 patients who had not had a lipid measurement). Again, if it assumed that the proportion of the 51 patients with indication for a antiplatelet agent is the same as that in the patients without occlusive arterial disease with a lipid measurement (77%), then 90% would have an indication for an antiplatelet agent. Thirty four percent of patients had an indication for a beta-blocker because of a history of MI or angina and 57% had an indication for an ACE inhibitor in accordance with the findings of the HOPE study (The Heart Outcomes Prevention Evaluation Study Investigators 2000) (Gottleib SS 1998).

However, suboptimal cardiovascular risk management is demonstrated in patients with clear indication for specific therapy; only 60% were using an antiplatelet agent, 41% a statin, 38% a beta-blocker and 39% an ACE inhibitor when indicated. Additionally, 35% of patients had not had a blood lipid measurement in the previous 5 years and the finding that 66% of the patients had a BP $\geq$ 140 / 90 suggests hypertensive control could be improved. It is established that smoking cessation can reduce the risk of MI by half and the finding that one quarter of the patients were current smokers highlights a further opportunity for intervention to reduce cardiovascular risk (Yusuf S 2002).

The presence of AAA is an indicator of high cardiovascular risk and coronary artery lesions demonstrable on angiography have been reported in approximately 30% of

patients with AAA (Hertzer NR 1984) (Young JR 1986). In 1980, Hertzer et al demonstrated that patients who have undergone AAA repair have an annual CHD mortality rate of 1.9 – 3.9%, and on the basis of this, the American 2001 Third Report of the National Cholesterol Education Program Adult Treatment Panel (ATP III) document considers AAA to be a CHD risk equivalent, a condition conferring a ≥ 20% 10 year risk of CHD (Hertzer NR 1980) (Adult Treatment Panel III 2001)

More recent studies have reported similar mortality rates; the UK Small Aneurysm Trial observed a 28% incidence of mortality due to cardiovascular causes over an 8 year period and 5 year survival rates of between 60 and 74% have been demonstrated (The United Kingdom Small Aneurysm Trial Participants 2002) (Batt M 1999) (Reigel MM 1987) (Roger VL 1989). In addition to limiting long term survival, cardiovascular disease is the leading cause of death in the perioperative period following AAA repair, accounting for 41% of perioperative deaths in The UK Small Aneurysm Trial (Brady AR 2000).

Although the primary action of statins is reduction in blood lipids, there is evidence that they improve endothelial function and reduce inflammation, resulting in atherosclerotic plaque stabilisation and reduction in risk of acute arterial events (Poldermans D 2003) (Kertai MD 2003). The 2003 Heart Protection Study, a randomised controlled trial of 20 536 patients aged 40 to 80 with occlusive arterial disease (defined as CHD, ischaemic stroke or PVD) receiving 40 mg of simvastatin daily or placebo, demonstrated a reduction of approximately 25% in cardiovascular events and death in patients receiving simvastatin, irrespective of baseline cholesterol level (Heart Protection Study Collaborative Group 2002). There is growing evidence

that statins may also reduce perioperative mortality in patients undergoing major vascular surgical procedures (Poldermans D 2003). A recent retrospective case-controlled study of 2816 patients found that statin use reduces perioperative mortality fourfold in patients undergoing major noncardiac vascular surgery, a finding that was supported by a randomised controlled trial of perioperative atorvastatin versus placebo in 100 patients undergoing vascular surgical procedures (Poldermans D 2003) (Durzzo AES 2004). Kertai et al, in a retrospective observational study of 570 patients reported that long term statin use following AAA repair is associated with reduced all cause and cardiovascular mortality, a finding in spite of the patients on statins having a higher prevalence of CHD (Kertai MD 2004). Although ATP III recommends aggressive LDLC lowering therapy in patients with AAA, at present the evidence for the routine use of statins in patients with AAA is limited (Adult Treatment Panel III 2001). This study demonstrates that most patients with AAA have associated cardiovascular pathology for which statins confer survival benefit, strengthening the case for routine statin therapy in the majority of patients with AAA.

Due to potentially severe bleeding complications, the indication for an antiplatelet agent in cardiovascular risk reduction is dependent upon the demonstration that benefit exceeds the risk (He J 1998). Risk of bleeding in patients using aspirin is constant, irrespective of the severity of cardiovascular disease, and the cardiovascular benefit appears linearly dependent upon absolute cardiovascular risk (He J 1998). Reduction in cardiovascular events and mortality when used in the secondary prevention of cardiovascular disease is well established (Antithrombotic Trialists' Collaboration 2002). The Antithrombotic Trialists' Collaboration in their meta-analyses of 287 randomised trials of antiplatelet therapy versus control or one

antiplatelet regimen versus another demonstrated that an antiplatelet agent reduces vascular events by 25% and vascular mortality by one sixth in patients at increased risk of occlusive vascular events (defined by a history of MI, angina, stroke or cerebral ischaemia, PVD or atrial fibrillation) (Antithrombotic Trialists' Collaboration 2002). In primary prevention, aspirin use cannot be justified without formal CHD risk estimation. The precise level of CHD risk necessary to justify antiplatelet treatment is controversial. In the USA, treatment has been recommended in patients with 10 year CHD risk levels of 6% and 10%, whereas in the UK treating patients with risk ≥15% is generally recommended (US Preventive Services Task Force 2002) (Sanmuganathan PS 2001) (Pearson TA 2000). The estimation that the presence of AAA confers a ≥ 20% 10 year CHD risk provides the rationale for the Antiplatelet in Vascular Surgery Consensus Statement recommending that an antiplatelet agent should be strongly considered in patients with AAA without evidence of occlusive arterial disease (Peripheral Arterial Diseases Antiplatelet Consensus Group 2003). This study's demonstration that 92% of patients with AAA overall, and 77% without evidence of occlusive arterial disease have an indication for an antiplatelet agent supports this, but at present no studies have determined the actual risk reduction associated with antiplatelet use in patients with AAA.

In patients who have suffered MI, a beta-blocker is known to reduce mortality by as much as 40% and although there is no evidence that their use in patients with stable angina improves survival, their use reduces the frequency of ischaemic episodes (Gottleib SS 1998) (Pepine CJ 1994). Perioperative use of beta-blockers in patients at high risk of cardiovascular disease or undergoing major vascular surgery significantly lowers risk of MI and cardiac death in the perioperative period and up to 2 years

postoperatively (Poldermans D 1999) (Mangano DT 1996). It has been proposed that the likely mechanism may be a reduction in myocardial oxygen demand or an atherosclerotic plaque stabilising effect (Poldermans D 1999).

ACE inhibitors confer a proven survival benefit in patients with left ventricular systolic dysfunction, and improve outcome in patients post MI (Pfeffer MA 1992). The HOPE Study of 9297 patients at increased risk of vascular disease randomised to receive either the ACE inhibitor ramipril or placebo, expanded the indication for treatment by demonstrating that ramipril lowers the risk of MI, stroke or cardiovascular death by 22% in patients with coronary artery disease, stroke, PVD or diabetes plus one other cardiovascular risk factor (hypertension, high total cholesterol, low HDLC, smoking or documented microalbuminuria) (The Heart Outcomes Prevention Evaluation Study Investigators 2000). The precise mechanism of action is unclear, and probably not entirely dependent on blood pressure reduction (The Heart Outcomes Prevention Evaluation Study Investigators 2000). ACE inhibitors appear to mitigate endothelial dysfunction in atherosclerosis, have a role in plaque stabilisation and may possess antithrombotic properties (Halkin A 2002). This study demonstrates that by virtue of meeting the criteria laid out in the HOPE study, over half of patients with AAA should be using an ACE inhibitor (The Heart Outcomes Prevention Evaluation Study Investigators 2000).

Strategies for reducing cardiovascular risk are evolving and focus has shifted away from treating individual risk factors such as hypertension or high lipid levels to concentrating on an individual's overall absolute cardiovascular risk. In patients without occlusive arterial disease, risk calculation based on the Framingham Heart

Study data allows an easy assessment of absolute 10 year CHD risk and the need for primary prevention (Wilson PWF 1998). Secondary prevention has been simplified and indications expanded by the demonstration that all patients with evidence of occlusive arterial disease benefit from a statin, antiplatelet agent and ACE inhibitor (Antithrombotic Trialists' Collaboration 2002) (Heart Protection Study Collaborative Group 2002) (The Heart Outcomes Prevention Evaluation Study Investigators 2000). The effects of each agent appear to be independent and additive and it has been estimated that used in combination, two thirds to three quarters of future vascular events could be prevented (Yusuf S 2002). This, combined with the relative safety of treatment, has led to advocates of a 'polypill', suggesting that all patients over 55 or those with manifestations of occlusive vascular disease should take a combined pill containing a statin, aspirin, 3 antihypertensive agents and folic acid (Wald NJ 2003). However, at the moment this is no more than a theoretical argument and cardiovascular risk reduction remains dependent upon identifying patients with indication for specific therapy. The lack of studies investigating the potential benefit of similar therapeutic measures in patients with AAA means that little direct evidence exists for the benefit of pharmacological cardiovascular risk reduction in patients with AAA. Currently, the identification of patients with AAA who will benefit from using an antiplatelet agent, statin, beta-blocker or ACE inhibitor depends upon demonstrating associated cardiovascular disease for which there is evidence for their use.

Side effects of the pharmacological agents discussed are less common than previously thought and more therapeutic options within each drug class are becoming available. Patients who are intolerant of aspirin can often tolerate alternative antiplatelet agents

such as dipyridamole or clopidogrel (Peripheral Arterial Diseases Antiplatelet Consensus Group 2003) (CAPRIE Steering Committee 1996). Symptoms sufficiently severe to stop beta-blocker treatment occur in only 0.8% of patients and although cited as a relative contraindication, most patients with PVD satisfactorily tolerate a beta-blocker (Law MR 2003). Statins are very safe, with liver failure and rhabdomyolysis being the only serious adverse effects, occurring at a rate of only one case per million person years (Law MR 2003). The only absolute contraindication to statin use is active or chronic liver disease (Law MR 2003). ACE inhibitors are well tolerated, with unwanted symptoms sufficiently severe to stop treatment occurring in 0.1% of patients, however they can cause a decline in renal function or hypotension and therefore their use requires monitoring (Law MR 2003) (The Heart Outcomes Prevention Evaluation Study Investigators 2000). The large proven benefit of the therapies discussed means that unless an absolute contraindication to treatment exists, treatment should be initiated and patient tolerance established.

The main limitation of the study is that the identification of previous MI, ischaemic stroke, PVD and angina is dependent on the accuracy of a prior diagnosis. An attempt to accurately diagnose cardiovascular pathology is beyond the scope of the study. Only a small number of patients have an assessment of their left ventricular function available, so I have made no mention of this in the analysis of patients who should receive an ACE inhibitor. Although this approach will tend to underestimate the true prevalence of cardiovascular pathology and the number of patients with an indication for cardioprotective therapy, it allows an accurate evaluation of adequacy of current cardiovascular risk factor reduction in patients with known indications. I have used a 10 year CHD risk $\geq$ 30% as the indication for statin therapy in patients with no

occlusive arterial disease to avoid overestimating the number of patients with indication for treatment. Generally 10 year CHD risk ≥ 30% is considered the minimum accepted standard of care and although initiating treatment at predicted risk levels as low as 15% has been advocated, local recommendations depend, in part, on available resources (British Cardiac Society, British Hyperlipidaemia Association, British Hypertension Society, British Diabetic Association 2000).

Summary

Despite the strong association between AAA and cardiovascular risk, and the demonstration that most patients with AAA have an indication for some form of pharmacological intervention, the identification and treatment of cardiovascular risk in patients with AAA is suboptimal. A likely contributing factor is the lack of direct evidence for pharmacological cardiovascular risk reduction in patients with AAA and the consequent lack of reference to AAA in many recommendations aimed at reducing cardiovascular risk. The growing popularity of AAA screening programmes will result in the increasing identification of patients with AAA, and therefore patients at high cardiovascular risk. Frequently, patients with AAA are not under the care of a cardiovascular physician and it is important that all physicians and surgeons encountering patients with AAA associate the presence of an AAA with high cardiovascular risk and ensure rational and appropriate evidence based therapies are used. This approach will undoubtedly increase the life expectancy of patients with AAA.

Association between genotype, cardiovascular pathology and IL-10 levels

All 313 patients recruited into the study had a detailed cardiovascular history taken. They were also genotyped for the polymorphisms at the IL-10 -1082 and -592 positions using Induced Heteroduplex Genotyping (described fully in Chapter 5). In 114 patients the plasma IL-10 level was measured using a commercially available ELISA kit (*Biosource Human IL-10 Ultrasensitive*). The other 199 patients did not have a measurement of plasma IL-10 because they were in the post-operative period, which may raise cytokine concentrations, or the length of time elapsed between venepuncture and the processing of the samples exceeded 4 hours. In Chapter 5 it was found that a delay of 4 hours or less between procurement of the sample and processing did not significantly affect the IL-10 level but it was felt that longer delays may create inaccuracy in the measurement of IL-10.

The data collected allowed me to investigate the associations between the IL-10 -1082 and -592 genotypes and the presence of hypertension, MI, stroke, IHD and diabetes adjusting for cardiovascular risk factors. It also enabled me to investigate the associations between the genotypes and IL-10 level, again adjusting for potential confounding factors.

Methods

Logistic regression analysis was used to investigate the associations between the genotypes and hypertension, MI, stroke, IHD and diabetes. Odds Ratios and 95% Confidence Intervals are presented for the association between genotype and account; independently, adjusted for all covariates, and adjusted for statistically significant covariates only.

A generalised linear model was used to compare the mean IL-10 level between the genotypes, adjusting for cardiovascular risk factors. IL-10 level was found to be non-normally distributed and therefore the data was log-transformed for analysis purposes. Statistical analysis was performed using SPSS Version 11.0.

Results

Genotype and presence of cardiovascular pathology

Table 6.7 displays the 3 genotypes at the -1082 position in patients with and without a history of hypertension, MI, stroke, IHD and diabetes. Table 6.8 displays the equivalent information for the genotypes at the -592 position.

	Number of subjects (%) Genotype at -1082 position		
Outcome	**GG**	**GA**	**AA**
Hypertension			
No	20 (28.6%)	45 (28.1%)	20 (24.1%)
Yes	50 (71.4%)	115 (71.9%)	63 (75.9%)
MI			
No	58 (82.9%)	119 (74.4%)	67 (80.7%)
Yes	12 (17.1%)	41 (25.6%)	16 (19.3%)
Stroke			
No	59 (84.3%)	138 (86.3%)	70 (84.3%)
Yes	11 (15.7%)	22 (13.8%)	13 (15.7%)
IHD			
No	50 (71.4%)	98 (61.3%)	57 (68.7%)
Yes	20 (28.6%)	62 (38.8%)	26 (31.3%)
Diabetes			
No	66 (94.3%)	147 (91.9%)	79 (95.2%)
Yes	4 (5.7%)	13 (8.1%)	4 (4.8%)

Table 6.7. -1082 genotypes for the 313 patients depending on the presence or absence of hypertension, MI, CVA, IHD and diabetes. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction.

Outcome	Number of subjects (%) Genotype at -592 position CC	CA	AA
Hypertension			
No	51 (27.6%)	29 (25.2%)	5 (38.5%)
Yes	134 (72.4%)	86 (74.8%)	8 (61.5%)
MI			
No	142 (76.8%)	91 (79.1%)	11 (84.6%)
Yes	43 (23.2%)	24 (20.9%)	2 (15.4%)
Stroke			
No	154 (83.2%)	100 (87.0%)	13 (100%)
Yes	31 (16.8%)	15 (13.0%)	0 (0%)
IHD			
No	118 (63.8%)	77 (67.0%)	10 (76.9%)
Yes	67 (36.2%)	38 (33.0%)	3 (23.1%)
Diabetes			
No	171 (92.4%)	108 (93.9%)	13 (100%)
Yes	14 (7.6%)	7 (6.1%)	0 (0%)

Table 6.8. -1082 genotypes for the 313 patients depending on the presence or absence of hypertension, MI, CVA, IHD and diabetes. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction.

Logistic regression analysis

Logistic regression analysis was used to investigate the association between the genotypes and hypertension, MI, stroke, IHD and diabetes making adjustment for the potential risk factors or covariates: age, sex, hypertension, history of smoking and diabetes. Table 6.9 displays the association between -1082 genotypes GG, GA and AA and history of MI, stroke, hypertension, IHD and diabetes, with genotype as an independent factor, then adjusted for all covariates and finally adjusted for statistically significant covariates only. Table 6.10 displays the equivalent information for the -592 genotypes CC, CA and AA.

Outcome	Type HI Test of Gene Effect	Pairwise Comparison	Odds Ratio	95% CI	P-value
MI					
Independent	P=0.28	AA vs GG	1.15	0.51 to 2.64	0.74
		GA vs GG	1.67	0.81 to 3.41	0.12
Adjusted for all	P=0.33	AA vs GG	1.17	0.50 to 2.73	0.78
Covariates		GA vs GG	1.65	0.79 to 3.47	0.14
Adjusted for age and	P=0.22	AA vs GG	1.16	0.50 to 2.71	0.69
hypertension		GA vs GG	1.77	0.85 to 3.68	0.08
Stroke					
Independent	P=0.89	AA vs GG	1.00	0.42 to 2.39	0.84
		GA vs GG	0.86	0.39 to 1.88	0.63
Adjusted for all	P=0.89	AA vs GG	0.95	0.39 to 2.31	0.91
Covariates*		GA vs GG	0.84	0.38 to 1.85	0.64
Hypertension					
Independent	P=0.76	AA vs GG	1.26	0.61 to 2.60	0.47
		GA vs GG	1.02	0.55 to 1.91	0.71
Adjusted for all	P=0.72	AA vs GG	1.23	0.59 to 2.56	0.45
covariates*		GA vs GG	0.96	0.51 to 1.81	0.57
IHD					
Independent	P=0.26	AA vs GG	1.14	0.57 to 2.29	0.73
		GA vs GG	1.58	0.86 to 2.91	0.10
Adjusted for all	P=0.32	AA vs GG	1.12	0.54 to 2.29	0.71
Covariates		GA vs GG	1.54	0.82 to 2.92	0.13
Adjusted For hypertension	P=0.20	AA vs GG	1.09	0.53 to 2.23	0.58
only		GA vs GG	1.63	0.87 to 3.06	0.07
Diabetes					
Independent	P=0.58	AA vs GG	0.84	0.20 to 3.47	0.53
		GA vs GG	1.46	0.46 to 4.64	0.31
Adjusted for all	P=0.45	AA vs GG	0.86	0.20 to 3.61	0.49
covariates*		GA vs GG	1.66	0.51 to 5.38	0.22

* No covariates were statistically significant

Table 6.9. Association between the -1082 genotype as an independent factor and history of MI, stroke, hypertension, IHD and diabetes, then adjusted for all covariates and adjusted for statistically significant covariates only. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction.

Outcome	Type III Test of Gene Effect	Pairwise Comparison	Odds Ratio	95% CI	P-value
MI					
Independent	P=0.75	AA vs CC	0.60	0.13 to 2.81	0.57
		CA vs CC	0.87	0.50 to 1.53	0.80
Adjusted for all covariates	P=0.77	AA vs CC	0.66	0.13 to 3.28	0.69
		CA vs CC	0.84	0.47 to 1.51	0.95
Adjusted for age and hypertension	P=0.79	AA vs CC	0.69	0.14 to 3.36	0.73
		CA vs CC	0.84	0.47 to 1.50	0.98
Stroke**					
Independent	P=0.39	CA vs CC	0.75	0.38 to 1.45	0.39
Adjusted for all covariates*	P=0.33	CA vs CC	0.71	0.36 to 1.40	0.33
Hypertension					
Independent	P=0.59	AA vs CC	0.61	0.19 to 1.95	0.34
		CA vs CC	1.13	0.66 to 1.92	0.31
Adjusted for all covariates*	P=0.61	AA vs CC	0.60	0.19 to 1.93	0.34
		CA vs CC	1.10	0.64 to 1.89	0.34
IHD					
Independent	P=0.58	AA vs CC	0.53	0.14 to 1.99	0.40
		CA vs CC	0.87	0.53 to 1.42	0.65
Adjusted for all covariates	P=0.68	AA vs CC	0.62	0.16 to 2.44	0.57
		CA vs CC	0.85	0.51 to 1.42	0.85
Adjusted for hypertension only	P=0.62	AA vs CC	0.58	0.15 to 2.29	0.52
		CA vs CC	0.83	0.50 to 1.39	0.83
Diabetes**					
Independent	P=0.63	CA vs CC	0.79	0.31 to 2.02	0.63
Adjusted for all covariates*	P=0.58	CA vs CC	0.77	0.30 to 1.98	0.58

* No covariates were statistically significant
** Insufficient numbers to compare AA

Table 6.10. Association between the -592 genotype as independent factor and history of MI, stroke, hypertension, IHD and diabetes as independent factors, then adjusted for all covariates and adjusted for statistically significant covariates only. Abbreviations: CHD, coronary heart disease. MI, myocardial infarction.

Determinants of plasma IL-10 Level

Table 6.11 displays the plasma IL-10 level in the 114 patients in whom it was measured along with genotype at the -1082 position. Table 6.12 displays the same information for the -592 genotype. The IL-10 levels were not normally distributed so the levels were then log-transformed.

	Genotype at -1082 position		
IL-10 level	**GG**	**GA**	**AA**
Raw data			
N	24	55	35
Mean	1.50	1.83	3.40
Median	1.22	1.17	0.93
Standard Deviation	1.36	2.23	13.71
Minimum	0	0	0
Maximum	5.68	11.65	82.02
Log-transformed Data			
N	24	55	35
Mean	-0.09	-0.04	-0.21
Median	0.20	0.16	-0.07
Standard Deviation	1.26	1.36	1.40
Minimum	-3.91	-3.91	-3.91
Maximum	1.74	2.46	4.41

Table 6.11. IL-10 level for patients with the -1082 GG, GA and AA genotypes along with log-transformed data.

	Genotype at -592 position		
IL-10 level	**CC**	**CA**	**AA**
Raw data			
N	73	37	4
Mean	1.72	3.31	1.80
Median	1.07	1.02	1.59
Standard Deviation	2.01	13.34	1.70
Minimum	0.02	0.02	0.22
Maximum	11.65	82.02	3.82
Log-transformed Data			
N	73	37	4
Mean	0.01	-0.34	0.06
Median	0.07	0.02	0.22
Standard Deviation	1.18	1.62	1.32
Minimum	-3.91	-3.91	-1.52
Maximum	2.46	4.41	1.34

Table 6.12. IL-10 level for patients with the -592 CC, CA and AA genotypes along with log-transformed data.

Effect of IL-10 -1082 genotype on IL-10 concentration

Initially an unadjusted analysis was undertaken to determine the effect of the -1082 genotype on the IL-10 level. Applying the -1082 genotype as the only covariate did not reveal a significant effect (P =0.85). Table 6.14 shows a pair-wise comparison of genotype against back transformed mean IL-10 level, again no significant difference was demonstrated.

Pair-Wise Comparison	Ratio	95% Confidence Interval	P-value
AA vs GA	0.85	0.47 to 1.51	0.57
AA vs GG	0.89	0.44 to 1.81	0.75
GA vs GG	1.05	0.54 to 2.02	0.88

Table 6.14. Pair-wise comparison of -1082 genotype against back transformed IL-10 level.

Adjusting for covariates

A generalised linear model was then employed to determine the effects of cardiovascular risk factors (age, sex, smoking history, hypertension and diabetes) along with genotype at the -1082 position on the IL-10 level (Table 6.15). None of the covariates were found to be associated with IL-10 level.

Covariate	P-value
Position -1082	0.87
Age	0.92
Sex	0.71
Ever Smoked	0.27
Hypertension	0.39
Diabetes	0.71

Table 6.15. Results of generalised linear model to determine effects of covariates on IL-10 level.

Comparing the least square mean IL-10 levels for each genotype at the -1082 position when adjusted for the other covariates in a pair-wise analysis did not reveal any effect (Table 6.16).

Pair-Wise Comparison	Ratio	95% Confidence Interval	P-value
AA vs GA	0.86	0.47 to 1.55	0.60
AA vs GG	0.90	0.43 to 1.86	0.77
GA vs GG	1.05	0.53 to 2.08	0.90

Table 6.16. Difference between least square means (adjusted) in pair-wise comparison of genotype at -1082 position.

Effect of IL-10 -592 genotype on IL-10 concentration

Initially an unadjusted analysis was undertaken to determine the effect of the -592 genotype on the IL-10 level. Applying the -592 genotype as the only covariate did not reveal a significant effect (P =0.43). Table 6.17 shows a pair-wise comparison of genotype against back transformed mean IL-10 level, again no significant difference was demonstrated.

Pair-Wise Comparison	Ratio	95% Confidence Interval	P-value
AA vs CA	1.50	0.37 to 6.12	0.57
AA vs CC	1.06	0.27 to 4.17	0.93
CA vs CC	0.70	0.41 to 1.21	0.20

Table 6.17. Pair-wise comparison of -592 genotype against back transformed IL-10 level.

Adjusting for covariate

A generalised linear model was then employed to determine the effects of cardiovascular risk factors (age, sex, smoking history, hypertension and diabetes) along with genotype at the -1082 position on the IL-10 level (Table 6.18). None of the covariates were found to be associated with IL-10 level.

Covariate	P-value
Position -592	0.32
Age	0.86
Sex	0.65
Ever Smoked	0.19
Hypertension	0.38
Diabetes	0.64

Table 6.18. Results of generalised linear model to determine effects of covariates on IL-10 level.

Comparing the least square mean IL-10 levels for each genotype at the -1082 position when adjusted for the other covariates in a pair-wise analysis did not reveal any effect (Table 6.19).

Pair-Wise Comparison	Ratio	95% Confidence Interval	P-value
AA vs CA	1.65	0.38 to 7.04	0.50
AA vs CC	1.09	0.26 to 4.55	0.90
CA vs CC	0.66	0.38 to 1.15	0.14

Table 6.19. Pair-wise comparison of -592 genotype against back transformed IL-10 level.

Effect of medication on IL-10 level

The effect of medication on IL-10 level was investigated and the results summarised in Table 6.20. Patients taking a statin, ACE inhibitor, aspirin or a beta blocker did not have a significantly different IL-10 level compared to those not on medication.

	On medication		Not on medication		
Medication	Number	Mean IL-10	Number	Mean IL-10	P-value
Statin	33	3.44	81	1.75	0.23
ACE inhibitor	34	3.86	80	1.55	0.42
Aspirin	53	2.06	61	2.91	0.09
Beta blocker	34	1.44	80	2.58	0.28

Table 6.20. IL-10 level in patients using or not using a statin, ACE inhibitor, aspirin or beta blocker along with P-values (Mann Whitney Test was used). Abbreviation: ACE, angiotensin converting enzyme.

Summary

No association was found between IL-10 genotype at the -1082 or -592 positions and history of hypertension, MI, CVA or diabetes when considered as independent variables or when age, sex, smoking history, hypertension and diabetes were adjusted

for. Similarly, the -1082 and -592 genotypes were not associated with IL-10 level when considered as independent variables or when age, sex, smoking history, hypertension and diabetes were adjusted for. Those patients using a statin, ACE inhibitor, aspirin or beta blocker did not have a significantly different IL-10 level when compared to patients not using medication.

Chapter 7

Case Control Study: Interleukin-10 Gene Polymorphisms and AAA

Contents

Introduction

In order to investigate the association between the interleukin-10 gene polymorphisms at the -1082, -819 and -592 positions and the presence of an AAA a case control study was conducted, the design of which is given in Chapter 5.

Three hundred and ninety patients with an aortic diameter $\geq$ 2.5 cm were recruited as cases. This group had a median age of 71 years (range 52-94) and a median aortic diameter of 5.3 cm, range 2.5 to 12 cm (mean 5.3 cm). Ninety nine (24%) of the 390 patients had previously undergone AAA repair. In these patients aortic diameter was recorded as the size when the AAA was repaired. Four hundred and four control patients with an aortic diameter < 2.5 cm, with a mean age of 65 years (range 52-94) and median aortic diameter of 1.8 cm, and range 1.4 to 2.4 cm (mean 1.8 cm) were recruited.

Table 7.1 displays patient demographic details and associated pathology in the two groups. As expected hypertension, smoking, ischaemic heart disease and chronic obstructive pulmonary disease were more common in those patients with an AAA. There was a significant difference in age distribution in the 2 groups as a consequence of the 'taking all comers' method of patient selection discussed in Chapter 5.

	Control (n=404)		AAA (n=390)		
	Number	**Percentage**	**Number**	**Percentage**	**P value**
Female	9	2%	36	9%	<0.01
Male	395	98%	353	91%	
Median age	65		71		<0.01*
Range	51-96		52-94		
Mean	67		72		
Age group (years):					
50-59	2	0 %	10	3 %	<0.01
60-69	338	84 %	160	41 %	
70-79	44	11 %	168	43 %	
80+	20	5 %	52	13 %	
Current smoker	57	14 %	93	24 %	<0.01
Ever smoked	266	66 %	361	93 %	<0.01
Family history	11	3 %	17	4 %	0.29
Hypertension	179	44 %	286	73 %	<0.01
IHD	63	16 %	118	30 %	<0.01
MI	45	11 %	95	24 %	<0.01
CABG	18	4 %	46	12 %	<0.01
CVA	33	8 %	62	16 %	<0.01
PVD	23	6 %	92	24 %	<0.01
Diabetes	37	9 %	30	8 %	0.54
Cancer	29	6 %	29	7 %	0.95
COPD	32	8 %	47	12 %	0.07

Table 7.1. Numbers and percentages of cases and controls with associated pathology along with P values (all calculated using Chi-squared test except * which used Mann Whitney test). Abbreviations: IHD, ischaemic heart disease. MI, myocardial infarction. CABG, coronary artery bypass graft. CVA, stroke. PVD, peripheral vascular disease. COPD, chronic obstructive pulmonary disease.

As would be expected from the high prevalence of cardiovascular pathology in the cases, they had also been prescribed more cardiovascular medication. Table 7.2 displays associated medication use in the two groups.

	Control (n=404)		AAA (n=390)	
	Number	**Percentage**	**Number**	**Percentage**
Aspirin	111	27 %	203	52 %
Warfarin	19	5 %	36	9 %
Beta-blocker	82	20 %	128	33 %
Nitrate	24	6 %	45	12 %
Diuretic	67	17 %	119	31 %
Statin	76	19 %	143	37 %
Cacium channel blocker	48	12 %	94	24 %

Table 7.2. Cardiovascular medication use in patients with and without AAA.

IL-10 -1082 polymorphism in patients with AAA and control patients

The 3 IL-10 genotypes (GG, GA and AA) and allele frequencies obtained by induced heteroduplex genotyping at the -1082 position were calculated in all 794 patients (Table 7.3). The frequencies of both were in keeping with those previously reported and discussed in Chapter 3.

	All patients (n=794)	
Genotype	**Number**	**Percentage**
GG	204	26 %
GA	405	51 %
AA	185	23 %
G allele	813	51 %
A allele	775	49 %

Table 7.3. -1082 genotype and allele numbers and percentages in all 794 patients.

Table 7.4 displays genotype and allele frequencies in cases and controls when the 2 groups were analysed separately. There were significant differences in genotype and allele frequencies between cases and controls; the GG genotype being more prevalent in controls and the AA genotype more prevalent in subjects with AAA (P=0.02, Chi-squared=7.70, 2df). The G allele was more prevalent in controls and the A allele more prevalent in subjects with AAA (P=0.01, Fisher's Exact test). The odds ratio for possession of the A allele being a risk factor for an AAA was 1.3 (95% confidence interval, 1.08-1.60).

	Control (n=404)		AAA (n=390)		
Genotype/allele	**Number**	**Percentage**	**Number**	**Percentage**	**P-value**
GG	118	29 %	86	22 %	
GA	205	51 %	200	51 %	
AA	81	20 %	104	27 %	0.02
G allele	441	55 %	372	48 %	
A allele	367	45 %	408	52 %	0.01

Table 7.4. -1082 genotype and allele frequencies and percentages in cases and controls along with P-values.

Knowing the genotype frequencies in the control group allows the Hardy-Weinberg equilibrium to be calculated i.e. the deviation of actual genotype frequency from expected frequency. Table 7.5 displays observed and expected genotypes calculated from the Hardy-Weinberg equation (P=0.95, Chi-squared=0.10, 2df) showing that the control patients were in Hardy-Weinberg equilibrium.

	Controls (n=404)		
Genotype	**Observed**	**Expected**	**P-value**
GG	118	120	
GA	205	200	
AA	81	83	0.95

Table 7.5. Observed and expected -1082 genotype frequencies in the control group.

Although a significant difference in genotype and allele frequencies between the case and control groups was demonstrated there were a number of demographic differences in the 2 groups which may confound the result; particularly sex and age distribution. As the numbers of female patients was so small, representing 2% and 9% of the controls and cases respectively I excluded these subjects from the analysis and restricted the analysis to men only. Table 7.6 displays genotype and allele frequencies in the male cases and controls. Again a significant difference in genotype frequency (P=0.02, Chi-squared=8.28, 2df) and allele frequency (P=0.01) (Fisher's Exact test) was demonstrated. The odds ratio for the A allele being a risk factor for AAA was 1.3 (95% confidence interval,1.10–1.65).

	Control (n=395)		AAA (n=353)		
Genotype/allele	**Number**	**Percentage**	**Number**	**Percentage**	**P-value**
GG	116	29 %	79	22 %	
GA	201	51 %	177	50 %	
AA	78	20 %	97	27 %	0.02
G allele	433	55 %	335	47 %	
A allele	357	45 %	371	53 %	0.01

Table 7.6. -1082 genotype and allele frequencies and percentages in male cases and controls.

Using this data to compare the high IL-10 producer GG genotype to the low IL-10 producer AA genotype in cases and controls (Table 7.7) shows that the AA genotype was significantly more common than the GG genotype in the subjects with an AAA (P=0.01) (Fisher's Exact test) with an odds ratio of 1.83 for this genotype being a risk factor for AAA (95% confidence interval,1.21-2.76).

	Control (n=395)		AAA (n=353)		
Genotype	**Number**	**Percentage**	**Number**	**Percentage**	**P-value**
GG	116	29 %	79	22 %	
AA	78	20 %	97	27 %	P=0.01 OR=1.83

Table 7.7 -1082 GG versus AA genotype in cases and controls. (OR, odds ratio).

Similarly comparing the intermediate producer GA genotype to the low producer AA genotype in cases and controls (Table 7.8) revealed a trend towards the AA genotype being more prevalent in subjects with an AAA, however this was not statistically significant (P=0.07) (Fisher's Exact test) with an odds ratio of 1.41 (95% confidence interval, 0.99-2.03).

	Control (n=395)		AAA (n=353)		
Genotype	**Number**	**Percentage**	**Number**	**Percentage**	**P-value**
GA	201	51 %	177	50 %	
AA	78	20 %	97	27 %	P=0.07 OR=1.41

Table 7.8. -1082 GA versus AA genotype in cases and controls. (OR, odds ratio).

IL-10 -1082 polymorphism and age

So far, a significant difference in genotype and allele frequency has been demonstrated in the overall group and the male subjects. However, significant differences in age distribution remained. I therefore subdivided the subjects into age groups (50-59, 60-69, 70-79 and >80 years). Table 7.9 shows the genotype and allele frequencies in these age groups in all male subjects (cases and controls combined). There was no statistically significant difference in genotypes in the age groups (P=1.56, Chi squared=9.28, 6df). A significant difference was found in allele frequencies (P<0.0001, Chi squared=54.74, 2df).

	50-59 (n=10)	60-69 (n=482)	70-79 (n=195)	80+ (n=61)
Genotype/allele	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**
GG	5 (50)	134 (28)	40 (21)	16 (26)
GA	5 (50)	240 (50)	105 (54)	28 (46)
AA	0	108 (22)	50 (26)	17 (28)
G allele	15 (75)	508 (53)	185 (47)	60 (49)
A allele	5 (25)	456 (47)	53 (53)	62 (51)

Table 7.9. -1082 genotype and allele distribution in all male subjects.
For genotype: P=1.56, Chi-squared=9.28, 6df
For allele: P<0.0001, Chi-squared=54.74, 2df (Test for independence)

Next, the genotype frequencies were examined in male AAA patients (Table 7.10). No difference was demonstrated in genotype frequency in the different age groups (P=0.13, Chi-squared=9.82, 6df). A significant difference was found in allele frequencies with the A allele becoming increasingly prevalent with increasing age (P=0.04, Chi-squared=8.34, 2df) (Test for independence). Test for trend was significant (P=0.04, Chi squared=4.18, 1df) (Test for trend) with the A allele becoming increasingly more prevalent with increasing age.

	50-59 (n=9)	60-69 (n=149)	70-79 (n=154)	80+ (n=41)
Genotype/allele	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**
GG	5 (56)	38 (26)	28 (18)	8 (20)
GA	4 (44)	71 (48)	82 (53)	20 (49)
AA	0 (0)	40 (27)	44 (29)	13 (32)
G allele	14 (78)	147 (49)	138 (45)	36 (44)
A allele	4 (22)	151 (51)	170 (55)	46(56)

Table 7.10. Genotype and allele distribution in male AAA subjects.
For genotype: P=0.13, Chi-squared=9.82, 6df
For allele: P=0.04, Chi-squared=8.34, 2df (Test for independence)
P=0.04, Chi-squared=4.18, 1df (Test for trend).

Similarly the results of analysing genotypes and alleles in male control patients by age group are displayed in Table 7.11. No significant association was found between age group and genotype or allele; P=0.68 (Chi-squared=3.99, 6df) and P=0.46 (Chi-squared=2.56, 3df test for independence) respectively.

	50-59 (n=1)	**60-69 (n=333)**	**70-79 (n=44)**	**80+ (n=17)**
Genotype/allele	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**
GG	0 (0)	96 (29)	12 (27)	8 (47)
GA	1 (100)	169 (51)	24 (55)	7 (41)
AA	0 (0)	68 (20)	8 (18)	2 (12)
G allele	1 (50)	361 (54)	44 (55)	23 (68)
A allele	1 (50)	305 (46)	40 (45)	11 (32)

Table 7.11. Genotype and allele distribution in male control subjects.
For genotype: P= 0.68, Chi-squared=3.99, 6df
For allele: P=0.46, Chi-squared=2.56, 3df (Test for independence)
P=0.30, Chi-squared=1.06, 1df (Test for trend)

Next, I restricted the analysis to men aged 60 to 69 years (the age group with most subjects) and comparing genotype and allele frequencies in cases and controls (Table 7.12). No difference was found between genotype or allele frequencies in cases and controls, P=0.29 (Chi squared=2.51, 2df) and P=0.16 (Chi squared=1.96, 1df) respectively.

	Control (n=333)		AAA (n=149)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
GA	96	29 %	38	26 %	
GA	169	51 %	71	48 %	
AA	68	20 %	40	27 %	0.29
G allele	361	54 %	147	49 %	
A allele	305	46 %	151	51 %	0.16

Table 7.12. Genotype and allele distribution in male case and control subjects aged 60-69 along with P-values.

In the same way, restricting the analysis to men aged 70 to 79 years revealed no difference in genotype or allele frequencies between cases and controls; P=0.24 (Chi squared=2.82, 2df) and P=0.14 (Chi squared=2.23, 1df) respectively (Table 7.13).

	Control (n=44)		AAA (n=154)		
Genotype/allele	**number**	**%**	**Number**	**%**	**P-value**
GA	12	27 %	28	18 %	
GA	24	55 %	82	53 %	
AA	8	18 %	44	29 %	0.24
G allele	48	55 %	138	45 %	
A allele	40	45 %	170	55 %	0.14

Table 7.13. Genotype and allele distribution in male case and control subjects aged 70-79 along with P-values.

IL-10 -592 polymorphism in patients with AAA and control patients

The 3 IL-10 genotypes (CC, CA and AA) and allele frequencies at the -592 position were calculated in all 794 patients (Table 7.14) and analysed in the same way as the -1082 polymorphism. Again, genotype and allele frequencies were in keeping with previously reported frequencies.

	All patients (n=794)	
Genotype/allele	**Number**	**%**
CC	483	61 %
CA	280	35 %
AA	31	4 %
C allele	1246	78 %
A allele	342	22 %

Table 7.14. -592 genotype and allele numbers and percentages in all 794 patients.

The observed genotype and allele frequencies in the case and control groups are displayed in Table 7.15. No significant difference was demonstrated between the case and control groups. P=0.38 (Chi squared=1.91, 2df) for genotype and P=0.18 (Fisher's Exact test) for alleles.

	Control (n=404)		AAA (n=390)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
CC	255	63 %	228	59 %	
CA	135	33 %	145	37 %	
AA	14	3 %	17	4 %	0.38
C allele	645	80 %	601	77 %	
A allele	163	20 %	179	23 %	0.18

Table 7.15. Genotype and allele frequencies and percentages in cases and controls along with P-values.

Table 7.16 displays observed and expected genotypes for the control group (calculated from Hardy-Weinberg equation). P=0.95 (Chi-squared test) showing that the control patients were in Hardy-Weinberg equilibrium.

	Control (n=404)		
Genotype	**Observed**	**Expected**	**P-value**
CC	255	257	
CA	135	130	
AA	14	16	0.95

Table 7.16. Observed and expected genotypes in the control group.

As discussed above, due to the small numbers of patients involved, the female subjects were removed from the analysis. Table 7.17 shows the -592 genotype and allele frequencies for the male subjects. No significant difference was found in genotype (P=0.47, Chi squared=1.50, 2df) or allele frequencies (P=0.26) (Fisher's exact test) between the groups.

	Control (n=395)		AAA (n=353)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
CC	249	63 %	208	59 %	
CA	133	34 %	130	37 %	
AA	13	3 %	15	4 %	0.47
C allele	631	80 %	546	77 %	
A allele	159	20 %	160	23 %	0.26

Table 7.17. -592 genotype and allele frequencies and percentages in male cases and controls along with P values.

-592 polymorphism and age

The results of analysing genotypes and alleles in all male patients by age group are displayed in Table 7.18. No significant association was found between age group and genotype, P=0.98 (Chi-squared=1.23, 6df) or allele frequency, P=0.81 (Chi-squared=0.93, 3df).

Genotype/allele	50-59 (n=10) Number (%)	60-69 (n=482) Number (%)	70-79 (n=195) Number (%)	80+ (n=61) Number (%)
CC	7 (70)	295 (61)	120 (62)	35 (57)
CA	3 (30)	170 (35)	67 (34)	23 (38)
AA	0 (0)	17 (4)	8 (4)	3 (5)
C allele	17 (85)	760 (79)	307 (79)	93 (76)
A allele	3 (15)	204 (21)	83 (21)	29 (24)

Table 7.18. Genotype and allele distribution in all male subjects by age group.
For genotype: P=0.98, Chi-squared=1.23, 6df
For allele: P=0.81, Chi-squared=0.93, 3df (Test for independence)
P=0.50, Chi-squared=0.46, 1df (Test for trend)

In the same way, looking at the genotype and allele frequencies in male AAA patients by age group revealed no difference, P=0.66 (Chi-squared=4.09, 6df) and P=0.42 (Chi-squared=2.84, 3df) respectively (Table 7.19).

Genotype/allele	50-59 (n=9) Number (%)	60-69 (n=149) Number (%)	70-79 (n=154) Number (%)	80+ (n=41) Number (%)
CC	6 (67)	86 (58)	96 (62)	20 (49)
CA	3 (33)	58 (39)	51 (33)	18 (44)
AA	0 (0)	5 (3)	7 (5)	3 (7)
C allele	15 (83)	230 (77)	243 (79)	58 (71)
A allele	3 (17)	68 (23)	65 (21)	24 (29)

Table 7.19. -592 genotype and allele distribution in male subjects with AAA by age group.
For genotype: P=0.75, Chi-squared=3.42, 6df
For allele: P=0.42, Chi-squared=2.84, 3df (Test for independence)
P=0.37, Chi-squared=0.80, 1df (Test for trend)

Analysing genotype and allele frequencies in male control patients by age group again revealed no significant difference, P=0.83 (Chi-squared=2.92, 6df) and P=0.54 (Chi-squared=2.16, 3df) respectively (Table 7.20).

Genotype/allele	50-59 (n=1) Number (%)	60-69 (n=333) Number (%)	70-79 (n=41) Number (%)	80+ (n=20) Number (%)
CC	1 (100)	209 (63)	24 (59)	15 (75)
CA	0 (0)	112 (34)	16 (39)	5 (25)
AA	0 (0)	12 (4)	1 (2)	0 (0)
C allele	2 (100)	530 (80)	64 (78)	35 (88)
A allele	0 (0)	136 (20)	18 (22)	5 (13)

Table 7.20. -592 genotype and allele distribution in male control subjects by age group.
For genotype: P=0.83, Chi-squared=2.92, 6df
For allele: P=0.54, Chi-squared=2.16, 3df (Test for independence)
P=0.47, Chi-squared=0.52, 1df (Test for trend)

Tables 7.21 and 7.22 show no significant difference in genotype or allele frequency between cases and controls in age groups 60-69 and 70-79. In age group 60-69; P=0.52 (Chi-squared=1.26, 2 df) for genotype and P=0.40 (Fisher's Exact test) for allele frequency. In age group 70-79: P=0.69 (Chi-squared=0.76, 2 df) for genotype and P=0.88 (Fisher's Exact test) for allele frequency.

	Control (n=333)		AAA (n=149)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
CC	209	63 %	86	58 %	
CA	112	34 %	58	39 %	
AA	12	4 %	5	3 %	0.52
C allele	530	80 %	230	77 %	
A allele	136	20 %	68	23 %	0.40

Table 7.21. -592 genotype and allele distribution in male cases and controls aged 60-69 along with P-values.

	Control (n=41)		AAA (n=160)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
CC	24	55 %	96	62 %	
CA	16	36 %	51	33 %	
AA	1	2 %	7	5 %	0.69
C allele	64	78 %	243	79 %	
A allele	18	22 %	65	21 %	0.88

Table 7.22. -592 genotype and allele distribution in male cases and controls aged 70-79 along with P-values.

-1082/-819/-592 haplotype in patients with and without AAA

Combined genotypes for the -1082 and -592 positions were calculated along with haplotypes for all cases and controls (Table 7.23). Although there was no significant difference in combined genotype frequency, P=0.12 (Chi-squared=8.77, 5df), there was a significant difference in haplotype with the ATA haplotype being more common in patients with AAA and the GCC haplotype less common, P=0.02 (Chi-squared=8.23, 2df).

	Control (n=395)		AAA (n=353)		
Combined genotype / haplotype	**Number**	**%**	**Number**	**%**	**P-value**
GCC/GCC	116	29 %	79	22 %	
GCC/ACC	107	27 %	92	26 %	
GCC/ATA	94	24 %	85	24 %	
ACC/ACC	26	7 %	37	10 %	
ACC/ATA	39	10 %	45	13 %	
ATA/ATA	13	3 %	15	4 %	0.12
GCC	433	55 %	335	47 %	
ACC	198	25 %	211	30 %	
ATA	159	20 %	160	23 %	0.02

Table 7.23. Combined -1082 and -592 genotype and haplotype frequencies in case and control groups along with P-values.

No significant difference was seen in combined -1082/-819/-592 genotype or haplotype when male AAA and control patients of different age groups were analysed separately (Tables 7.24 and 7.25). In subjects with AAA, P=0.29 (Chi-squared=17.45, 15df) for genotype and P=0.05 (Chi-squared=12.64, 6df) for haplotype. In control subjects P=0.87 (Chi-squared=9.12, 15df) for genotype and P=0.78 (Chi-squared=3.21, 6df) for haplotype.

	50-59 (n=9)	**60-69 (n=149)**	**70-79 (n=154)**	**80+ (n=41)**
Combined genotype / haplotype	**Number (%)**	**Number (%)**	**Number (%)**	**Number (%)**
GCC/GCC	5 (56)	38 (26)	28 (18)	8 (20)
GCC/ACC	1 (11)	32 (21)	50 (32)	9 (22)
GCC/ATA	3 (33)	39 (26)	32 (21)	11 (27)
ACC/ACC	0 (0)	16 (11)	18 (12)	3 (7)
ACC/ATA	0 (0)	19 (13)	19 (12)	7 (17)
ATA/ATA	0 (0)	5 (3)	7 (5)	3 (7)
GCC	14 (78)	147 (49)	138 (45)	36 (44)
ACC	1 (6)	83 (28)	105 (34)	22 (27)
ATA	3 (17)	68 (23)	65 (21)	24 (29)

Table 7.24. Combined -1082 and -592 genotype and haplotype frequencies in AAA patients by age group.
For genotype: P=0.29, Chi-squared=17.45, 15df
For haplotype:P=0.05, Chi-squared=12.64, 6df (Test for independence)

Combined genotype / haplotype	50-59 (n=1) Number (%)	60-69 (n=333) Number (%)	70-79 (n=41) Number (%)	80+ (n=20) Number (%)
GCC/GCC	0 (0)	96 (29)	12 (29)	8 (40)
GCC/ACC	1 (100)	91 (27)	11 (27)	4 (20)
GCC/ATA	0 (0)	78 (23)	12 (29)	4 (20)
ACC/ACC	0 (0)	22 (7)	1 (2)	3 (15)
ACC/ATA	0 (0)	34 (10)	4 (10)	1 (5)
ATA/ATA	0 (0)	12 (4)	1 (2)	0 (0)
GCC	1 (100)	361 (54)	47 (57)	24 (60)
ACC	0 (0)	169 (25)	17 (21)	11 (28)
ATA	0 (0)	136 (20)	18 (22)	5 (13)

Table 7.25. Combined -1082 and -592 genotype and haplotype frequencies in control patients by age group.
For genotype: P=0.87, Chi-squared=9.12, 15df
For haplotype:P=0.78, Chi-squared=3.21, 6df (Test for independence)

Age matched case control study

In order to match the groups exactly for age, the analysis was restricted. Subjects were matched for age +/- 1 year on a 1:1 basis having excluding females prior to matching. In order to do this objectively the earliest recruited controls were matched with the earliest recruited AAA patients of the same age. Any subjects without an appropriate age match were excluded. Table 7.26 displays age details of the 2 groups. There was no significant difference in age, P=0.50 (Mann-Whitney test).

	AAA	Control
Number	148	148
Mean	70.2	70.1
S.D.	6.6	6.7
Median	77	66
Range	52-91	51-91

Table 7.26. Age details of matched case and control groups. (SD, standard deviation).

IL-10 -1082 polymorphism in age matched study

Table 7.27 demonstrates genotype and allele frequencies at the -1082 position in the age matched cases and controls. In common with the age unmatched analysis there was significant difference in genotype with the AA genotype more common and the GG genotype less common in subjects with an AAA, P=0.0009 (Chi-squared=13.97, 2df). A similar trend was apparent for allele frequencies, the A allele being more common in subjects with an AAA, P=0.0002 (Fisher's Exact test). The odds ratio for the A allele being a risk factor for AAA was 1.87 (96% confidence interval, 1.35-2.59).

	Control (n=148)		AAA (n=148)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
GG	53	36 %	27	18 %	
GA	67	45 %	73	49 %	
AA	28	19 %	48	32 %	0.0009
G allele	173	58 %	127	43 %	
A allele	123	42 %	169	57 %	0.0002

Table 7.27. -1082 Genotype and allele frequencies in age matched cases and controls along with P-values.

Using this data to compare the high producer GG genotype to the low producer AA genotype in cases and controls (Table 7.28) shows that the AA genotype was significantly more common than the GG genotype in the AAA subjects, P=0.0004 (Fisher's Exact test) with an odds ratio of 3.37 (95% confidence interval: 1.74 to 6.49).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
GG	53	36 %	27	18 %	
AA	28	19 %	48	32 %	0.0004

Table 7.28. GG versus AA genotype in cases and controls.

Similarly comparing the intermediate producer GA genotype to the high producer GG genotype in cases and controls (Table 7.29) showed that the GA genotype is significantly more prevalent in AAA subjects, P=0.001 (Fisher's Exact test) with an odds ratio of 2.14 (95% confidence interval 1.21 to 3.78).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
GA	67	45 %	73	49 %	
GG	53	36 %	27	18 %	0.001

Table 7.29. GG versus GA genotype in cases and controls.

Next, comparing those subjects with the presence of an A allele i.e. those with AA or GA genotype to those without an A allele (GG genotype) in cases and controls showed a significantly higher prevalence of subjects with an A allele in the AAA

group, P=0.001 (Fisher's Exact test) with an odds ratio of 2.50 (95% confidence interval 1.46-4.27) (Table 7.30).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
GG	53	36 %	27	18 %	
GA+AA	95	64 %	121	82 %	0.001

Table 7.30. GG versus GA and AA genotype in cases and controls.

IL-10 -592 polymorphism in age matched study

Table 7.31 demonstrates genotype and allele frequencies at the -592 position in the age matched cases and controls. There was no significant difference in genotype, P=0.15 (Chi squared=3.76, 2df) or allele frequencies, P=0.09 (Fisher's Exact test). Odds ratio was 1.45 (96% confidence interval, 1.96-2.20).

	Control (n=148)		AAA (n=148)		
Genotype/allele	**Number**	**%**	**Number**	**%**	**P-value**
CC	103	70 %	87	59 %	
CA	42	28 %	57	39 %	
AA	3	2 %	4	3 %	0.15
C allele	248	84 %	231	78 %	
A allele	48	16 %	65	22 %	0.09

Table 7.31. -592 Genotype and allele frequencies in age matched cases and controls.

Comparing CC to AA genotype in cases and controls (Table 7.32) did not show a significant difference P=0.70 (Fisher's Exact test). Odds ratio 1.58 (95% confidence interval 0.34-7.25).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
CC	103	70 %	87	59 %	
AA	3	2 %	4	3 %	0.70

Table 7.32. -592 CC versus AA genotype in cases and controls.

Next, comparing the CC to CA genotype in cases and controls (Table 7.33) did not show a significant difference, P=0.06 (Fisher's Exact test), odds ratio 1.61 (95% confidence interval 0.98-2.62).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
CC	103	70 %	87	59 %	
CA	42	28 %	57	39 %	0.06

Table 7.33. -592 CC versus CA genotype in cases and controls.

Comparing those subjects with the presence of an A allele i.e. those with AA or CA genotype to those without an A allele (CC genotype) in cases and controls (Table 7.34) showed no significant difference, P=0.07 (Fisher's Exact test) with an odds ratio of 1.61 (95% confidence interval 0.99-2.59).

	Control		AAA		
Genotype	**Number**	**%**	**Number**	**%**	**P-value**
CC	103	70 %	87	59 %	
CA + AA	45	30 %	61	41 %	0.07

Table 7.34. -592 CC versus AA and CA genotype in cases and controls.

-1082/-819/-592 combined genotype and haplotype in age matched study

Combined genotypes for the -1082 and -592 positions were calculated along with haplotypes for all cases and controls (Table 7.35). A significant difference in combined genotype frequency was found, P=0.01 (Chi-squared=14.42, 5df), there was a significant difference in haplotype with the ATA haplotype being more common in patients with AAA and the GCC haplotype less common, P=0.0008 (Chi-squared=14.31, 2df).

	Control (n=148)		AAA (n=148)		
Combined genotype / haplotype	**Number**	**%**	**Number**	**%**	**P-value**
GCC/GCC	53	36 %	27	18 %	
GCC/ACC	41	28 %	42	28 %	
GCC/ATA	26	18 %	31	21 %	
ACC/ACC	9	6 %	18	12 %	
ACC/ATA	16	11 %	26	18 %	
ATA/ATA	3	2 %	4	3 %	0.01
GCC	173	58 %	127	43 %	
ACC	75	25 %	104	35 %	
ATA	48	16 %	65	22 %	0.0008

Table 7.35. -1082/-819/-592 combined genotype and haplotype frequencies in age matched cases and controls.

Adjusting for smoking and hypertension for -1082 polymorphism

In order to adjust for the main confounding variables; smoking and hypertension, data was stratified on the basis of the cases and controls either having or not having the risk factor. Table 7.36 shows -1082 genotype and allele frequencies in cases and control with and without a smoking history i.e. those patients who have ever smoked or not. As only 7 patients with AAA had never smoked the comparison between cases and controls without a history of smoking is not informative. In the analysis of cases and controls with a smoking history the significant trend towards patients with AAA having a high prevalence of AA genotype and low prevalence of GG genotype persisted, P=0.001 (Chi squared=13.60, 2 df). In this group the A allele was significantly more common in patients with AAA, P=0.0002 (Fisher's Exact test). Odds ratio for the A allele being a risk factor for AAA was 2.03, 95% confidence interval 1.39 to 2.97.

	No smoking history			**Smoking history**		
Genotype	**Control (n=55)**	**AAA (n=8)**	**P-value**	**Control (n=93)**	**AAA (n=140)**	**P-value**
GG	17	1		36	26	
GA	26	4		41	69	
AA	12	3	0.46	16	45	0.001
G	60	6		113	121	
A	50	10	0.28	73	159	0.0002

Table 7.36. -1082 genotype and allele frequencies in age matched cases and controls with and without a smoking history.

Table 7.37 shows similar data for patients currently being treated or not for hypertension. Comparing genotype in cases and controls with no history of hypertension did not reveal a significant difference in genotype, P=0.62 (Chi-squared=0.95, 2df). Similarly no difference was demonstrated in allele frequency in non-hypertensive subjects, P=0.38 (Fisher's Exact test). In contrast a highly significant difference in genotype, P=0.0004 (Chi squared=15.55, 2df) and allele frequency, P<0.0001 (Fisher's Exact test), odds ratio of 2.43 (95% confidence interval of 1.56 to 3.79) was demonstrated in hypertensive subjects.

	No hypertension			**Hypertension**		
Genotype	**Control (n=74)**	**AAA (n=54)**	**P-value**	**Control (n=74)**	**AAA (n=94)**	**P-value**
GG	25	14		28	13	
GA	34	27		33	46	
AA	15	13	0.62	13	35	0.0004
G	84	55		89	72	
A	64	53	0.38	59	116	<0.0001

Table 7.37. -1082 genotype and allele frequencies in age matched cases and controls with and without hypertension.

Adjusting for smoking and hypertension for -592 polymorphism

Table 7.38 shows -592 genotype and allele frequencies in cases and control with and without a smoking history. As only 7 patients with AAA had never smoked the comparison between cases and controls without a history of smoking is not informative. In the analysis of cases and controls with a smoking history there was no significant difference in genotype between cases and controls, P=0.27 (Chi

squared=2.61, 2 df). Nor was there a difference in allele frequency, P=0.19 (Fisher's Exact test).

	No smoking history			Smoking history		
Genotype	**Control (n=55)**	**AAA (n=8)**	**P-value**	**Control (n=93)**	**AAA (n=140)**	**P-value**
CC	39	5		64	82	
CA	15	2		27	55	
AA	1	1	0.27	2	3	0.27
C	93	12		155	219	
A	17	4	0.31	31	61	0.19

Table 7.38. -592 genotype and allele frequencies in age matched cases and controls with and without a smoking history.

Table 7.39 shows genotype and allele frequencies at the -592 position for patients currently being treated or not for hypertension. Comparing genotype in cases and controls with no history of hypertension did not reveal a significant difference in genotype, P=0.67 (Chi squared=0.81, 2df). Similarly no difference was demonstrated in allele frequency in non hypertensive subjects, P=0.61 (Fisher's Exact test). No significant difference in genotype, P=0.13 (Chi squared=4.13, 2df) and allele frequency, P=0.14 (Fisher's Exact test) was demonstrated in hypertensive subjects.

	No hypertension			Hypertension		
Genotype	**Control (n=74)**	**AAA (n=54)**	**P-value**	**Control (n=74)**	**AAA (n=94)**	**P-value**
CC	52	36		51	51	
CA	21	16		21	41	
AA	1	2	0.67	2	2	0.13
C	125	88		123	143	
A	23	20	0.61	25	45	0.14

Table 7.39. -592 genotype and allele frequencies in age matched cases and controls with and without hypertension.

IL-10 plasma concentration and genotype

In 250 subjects; 119 controls and 131 with AAA the IL-10 concentration was measured by ELISA as described in Chapter 5. There was no significant difference in IL-10 levels between these two groups, those with AAA had a mean IL-10 level of 2.24pg/ml (95% confidence interval 0.95pg/ml to 3.54pg/ml) and those in the control group 1.74pg/ml (95% confidence interval 1.07pg/ml to 2.42pg/ml) (P=0.49, Mann-Whitney Test) (Figure 7.1).

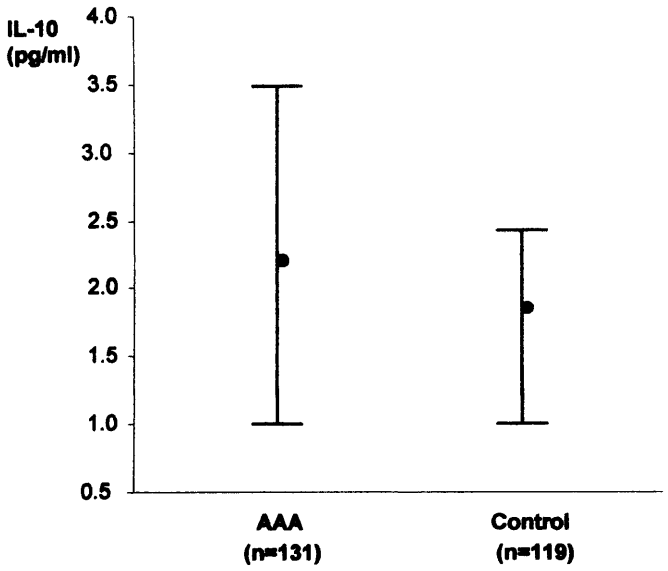


Figure 7.1. Plasma IL-10 levels (pg/ml) in the control and AAA groups. Data points represent mean IL-10 and error bars represent 95% confidence intervals.

There was no difference in IL-10 levels across the three -1082 genotypes (P=0.41, Kruskal-Wallis Test) with IL-10 levels as follows: 'GG' genotype 1.55pg/ml (95% confidence interval 1.10pg/ml to 2.00pg/ml, n=67), 'GA' genotype 2.01pg/ml (95% confidence interval 1.23pg/ml to 2.89pg/ml, n=113), 'AA' genotype 2.36pg/ml (95% confidence interval 0.01pg/ml to 4.70pg/ml, n=70) (Figure 7.2).

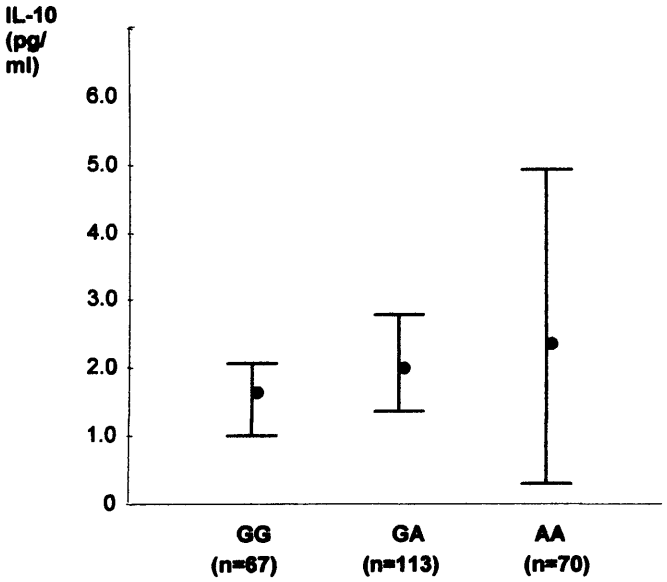


Figure 7.2. Plasma IL-10 levels (pg/ml) in both cases and controls according to -1082 genotype. Data points represent mean IL-10 and error bars represent 95% confidence intervals.

The presence of one or more A alleles had no effect on plasma IL-10 levels (P=0.35, Mann-Whitney Test) (GG genotype 1.55pg/ml (95% confidence interval 1.10pg/ml to 2.00pg/ml, n=67), GA or AA genotype 2.18pg/ml (95% confidence interval 1.17pg/ml to 3.20pg/ml, n=183) (Figure 7.3).

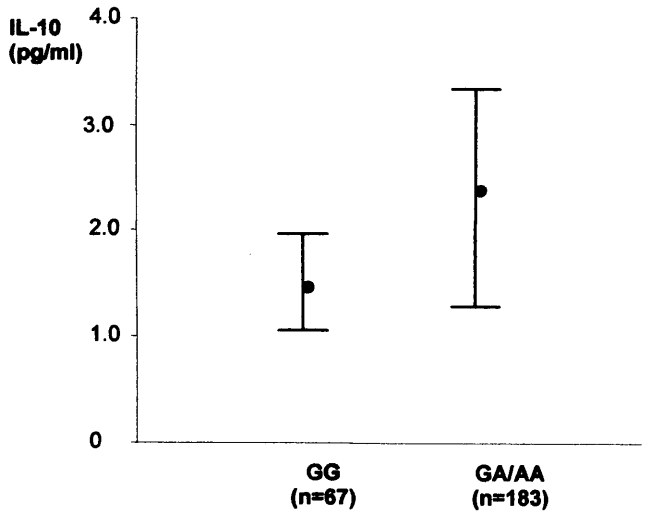


Figure 7.3. Plasma IL-10 levels (pg/ml) in both cases and controls according to presence/absence of an A allele. Data points represent mean IL-10 and error bars represent 95% confidence intervals.

There was no difference in IL-10 levels across the three -592 genotypes (P=0.11, Mann-Whitney Test) with IL-10 levels as follows: 'CC' genotype 1.82pg/ml (95% confidence interval 1.32pg/ml to 2.32pg/ml, n=160), 'CA' genotype 2.49pg/ml (95% confidence interval 0.36pg/ml to 4.62pg/ml, n=81), 'AA' genotype 1.02pg/ml (95% confidence interval 0.03pg/ml to 2.01pg/ml, n=9) (Figure 7.4).

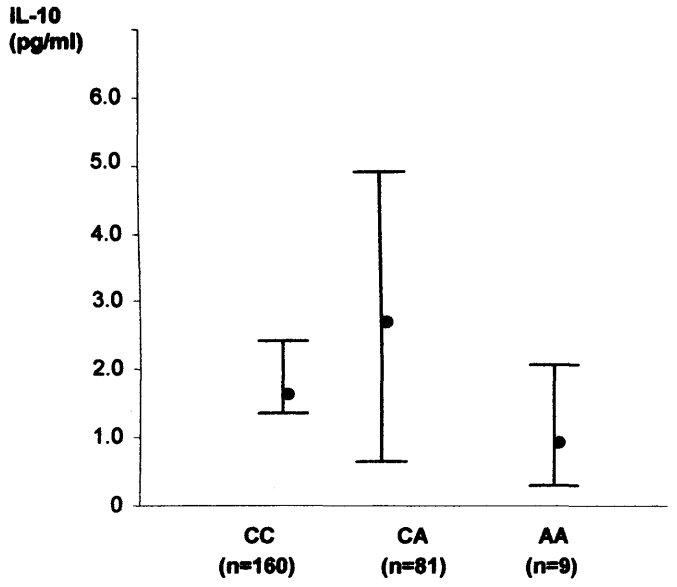


Figure 7.4. Plasma IL-10 levels (pg/ml) in both cases and controls according to -592 genotype. Data points represent mean IL-10 and error bars represent 95% confidence intervals.

Interleukin-10 in the aneurysm wall

In 22 patients undergoing open AAA repair a small anterior portion of the aortic sac was excised, homogenised and manipulated to a protein concentration of 1mg/ml before an ELISA was performed to determine IL-10 concentration. In all samples IL-10 was detectable. Table 7.40 shows the IL-10 concentration.

Mean IL-10 g/dl	2.38
SD	0.83
Range	1.75-4.39

Table 7.40. Mean, standard deviation (SD) and range of IL-10 concentration in AAA wall.

Summary

Initially an unmatched analysis was performed to compare genotype and allele frequencies in cases and controls. The -1082 G allele and GG genotype were found to be significantly more prevalent in controls and the A allele and AA genotype more prevalent in AAA subjects (P=0.01 and P=0.02 respectively). No significant difference in allele or genotype frequency at the -592 position was demonstrated. The ATA haplotype was significantly more common in patients with AAA and the GCC haplotype less common (P=0.02).

When one-to-one age matching was performed the -1082 AA genotype was significantly more common than the GG genotype in the AAA subjects (P=0.0004, Fisher's Exact test) with an odds ratio of 3.37 (95% confidence interval: 1.74 to 6.49). Similarly the intermediate producer GA genotype was significantly more prevalent than the GG genotype in subjects with AAA, P=0.001 (Fisher's Exact test) with an odds ratio of 2.14 (95% confidence interval 1.21 to 3.78). Overall the A allele was significantly more prevalent in subjects with AAA (P=0.0002) with an odds ratio of 1.87 (96% confidence interval, 1.35-2.59) for the A allele being a risk factor for AAA. There was no significant difference in -592 genotype between cases and controls in the age matched analysis.

The significant difference in -1082 genotype and allele frequency persisted in the age matched analysis when subjects with a smoking history and hypertensive subjects were analysed separately. However, the difference disappeared when non hypertensive subjects were analysed separately.

There was a significant difference in haplotype with the ATA haplotype being more common in patients with AAA and the GCC haplotype less common, P=0.0008.

The A allele was found to be increasingly prevalent with increasing age in subjects with AAA (P=0.04), a trend that was not seen with other alleles or genotypes.

No correlation was demonstrated between plasma IL-10 concentration and the presence or absence of AAA or between plasma IL-10 concentration and IL-10 -1082 or -592 genotype. IL-10 is detectable in the aneurysm wall.

Chapter 8

The Effect of IL-10 Polymorphisms on Rate of Abdominal Aortic Aneurysm Expansion

Contents

Abdominal aortic aneurysm expansion

Abdominal aortic aneurysms are characterised by chronic inflammation, elastin and collagen degradation and smooth muscle cell loss, as was discussed previously. Weakening of the aortic wall due to loss of the structural proteins leads to progressive dilatation. Loss of elastin appears to be responsible for initial aneurysmal dilatation and collagen loss for later rapid expansion and eventual rupture. Current understanding of the mechanisms responsible for these changes puts emphasis on proteases and an inflammatory milieu, regulated in part by cytokines.

The increasing numbers of patients undergoing abdominal ultrasound examination in AAA screening programmes combined with contemporary evidence that elective repair of AAA < 5.5 cm confers no survival benefit has resulted in the identification of large numbers of patients with AAA too small to justify surgery (UK Small Aneurysm Trial Participants 1998) (Lederle FA 2002). When a patient with an AAA is identified, cardiovascular risk factors can be addressed; however there is no medical therapy that curtails AAA expansion. Therefore a 'watchful waiting' approach is employed with patients undergoing regular ultrasound scans to monitor expansion until elective repair is indicated.

A number of investigators have attempted to quantify the rate of AAA expansion. In general, older studies were limited by small sample size and few serial aneurysm size measurements. More recently, data from the UK Small Aneurysm Trial and the ADAM study have clarified average expansion rates of AAA by providing large numbers of patients with long follow up. Table 8.1 displays AAA expansion rates reported in different series.

Study	Number of Subjects	AAA Size (cm)	Expansion (mm/year)
Eriksson P (2005)	455	4.0 – 5.5	3.08
Yeung JM (2002)	58	4.3	3.5
UK Small Aneurysm Trial (2002)	1090	4.62	2.6
Lederle FA (2003)	1134	4.7	3.2
Sharp MA (2003)	277	4.6	2.8
Brady AR (2004)	1743	4.3	2.6
Norman P (2004)	433	3.0-3.9	1.0
Norman P (2004)	112	4.0-5.4	2.2

Table 8.1. AAA expansion rate and mean or range of AAA diameter reported in previous studies.

Consistently initial aneurysm size has been found to correlate with rate of expansion and it is accepted that large aneurysms expand more rapidly than smaller ones (Brady AR 2004). Conflicting evidence exists as to the importance of other factors. Current smoking (MacSweeney ST 1994) (Brady AR 2004), hypertension (Schewe CK 1994), advanced age (Chang JB 1997) and female sex have been found to influence growth rate but these are not universal findings.

Prediction of aneurysm expansion rate is useful for 2 reasons; it allows the timing of serial scans to be planned in patients with small AAA and may also help predict risk of rupture. Rapid growth has generally been regarded as a risk factor for rupture and therefore an indication for elective repair (Brown PM 1996) (Limet RJ 1991). The UK Small Aneurysm Trial used an expansion rate > 1cm per year as an indication for

elective repair (UK Small Aneurysm Trial Participants 2002). However, this is controversial; Sharp found no correlation between expansion rate and rupture risk and Cronenwett convincingly argued that large size is associated with both rapid expansion and high rupture risk (Sharp MA 2003) (Cronenwett JL 1996).

A difficulty in studying AAA expansion is predicting rate of growth. Expansion is irregular; frequently periods of growth are interspersed with periods of inactivity (Brady AR 2004) and rate of expansion varies markedly between individuals. No consistent growth pattern has been found to be universally applicable with linear and exponential curves both being applied. Limet argued that as larger aneurysms expand more rapidly than smaller ones an exponential model is most appropriate (Limet R 1991). In a study of 277 patients Sharp found no evidence of an exponential growth rate. A straight line with the equation $y=0.059x-0.11$ best fitted the expansion, where y was growth in cm per 6 months and x was the AP diameter in cm. The R^2 value was 0.23 (Sharp MA 2003). Linear regression and size of aneurysm were significant. Chang in his study of 514 aneurysms calculated rate of expansion by drawing a straight line of best fit between the points (Chang AR 1997). Vardulaki used a 'random effects' exponential model to allow for measurement error, individual variation in growth rate and the step-wise progression (Vardulaki KA 1998). However it was found that when a linear pattern of growth was assumed very similar results were obtained. Brady in analysing expansion rate of the UK Small Aneurysm Trial and MASS data found that many patients have a linear or accelerated rate of expansion (Brady AR 2004).

A spin off from the UK Small Aneurysm Trial has been a number of studies investigating the effect of genotype on AAA expansion. A large sample size is needed to provide adequately powered studies. Indeed, Eriksson in a study of 455 patients looking at MMP gene polymorphisms in AAA expansion would only have identified factors that influence growth rate by 25% (Eriksson P 2005).

It has been reported that the atherogenic apolipoprotein E genotype influences the rate of AAA expansion in a small study (Gerdes LU 2000). The Cystatin C gene polymorphisms +148 G to A and -82 G to C were studied by Eriksson (Eriksson P 2004). Cystatin C is an inhibitor of the elastinolytic cysteine proteases cathepsins S and K which was discussed in Chapter 2. It was found that patients with the +148 AA genotype had significantly slower aneurysm expansion. Table 8.2 displays the effect of different gene polymorphisms on AAA expansion.

Study	**Number of subjects**	**Gene**	**Polymorphism**	**Effect**
Eriksson P (2005)	455	MMP	MMP-2,	NS
			MMP-3,	NS
			MMP-9,	NS
			MMP-12,	NS
		PAI	PAI-1	NS
Eriksson P (2004)	424	Cystatin C	+148 G to A	P=0.027
			-82 G to C	P=0.055
Yeung JC (2002)	58	ACE	DD	P=0.6
			DI	
			II	

Table 8.2. Effect of different gene polymorphisms on rate of AAA expansion (NS, not significant).

Overall, at present, little is known about biochemical or genetic mechanisms that may influence rate of expansion. Better understanding could potentially identify medical or genetic therapy to curtail expansion.

The effect of IL-10 genotype on AAA expansion rate

It is accepted that the majority of abdominal aortic aneurysms increase in size over time and it is possible that similar pathogenetic pathways are responsible for the development and growth of AAA. I have demonstrated that a single nucleotide polymorphism (IL-10 -1082 G/A) in the promoter sequence of the Interleukin-10 (IL-10) gene is associated with the presence of an AAA. The presence of the A allele, which has previously been associated with reduced IL-10 production, was found to be more common in patients with AAA (Eskdale J 1998). This is biologically plausible as reduced IL-10 production is associated with a proinflammatory milieu and MMP activation.

I therefore postulated that the A allele may also be associated with increased AAA growth rate. A study was therefore conducted to determine whether this polymorphism influences the rate of AAA expansion.

Methods

As discussed in the previous chapters 390 patients with AAA were recruited into the study and the IL-10 genotype at the -1082 and -592 positions was determined by induced heteroduplex genotyping. At the time of recruitment the aortic diameter measured by ultrasound scanning was recorded. In addition, the date of any previous scans and aortic diameter at these times was recorded. One year after patient

recruitment ceased hospital records and data from the Leicestershire Aortic Aneurysm Screening Programme were examined to determine subsequent measurements of aortic diameter.

Of the 390 patients, 178 had 2 or more serial measurements of aortic diameter, thereby allowing expansion rates to be calculated. Ultrasound detection of AAA is sensitive (>98%) and specific (almost 100%) (Lindholt JS 1999). However measurement accuracy is variable and repeatability may range by approximately 3mm (Chang AR 1997) (Ernst CB 1993). I therefore used only measurements taken by 2 experienced ultrasonographers involved in the Leicester AAA screening programme.

Expansion rate

Curve fitting software (SPSS Version 11.0) was employed to determine the curve of best fit. I discovered however that no single curve model would fit the data. Best fit was obtained equally with compound, growth, exponential and logistic models (R^2 = 0.73 for all). Figure 8.1 is a XY scatter graph showing expansion over time for all subjects and Figures 8.2 – 8.7 show expansion rate and linear and exponential curves of best fit for 6 representative subjects.

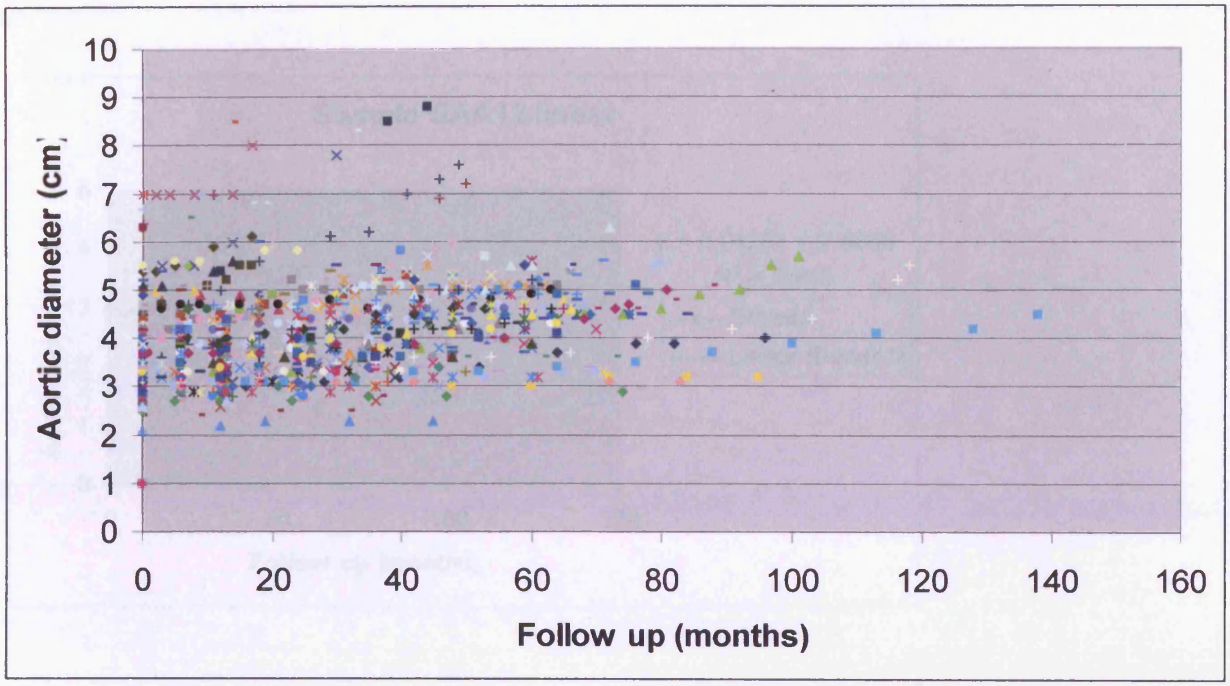
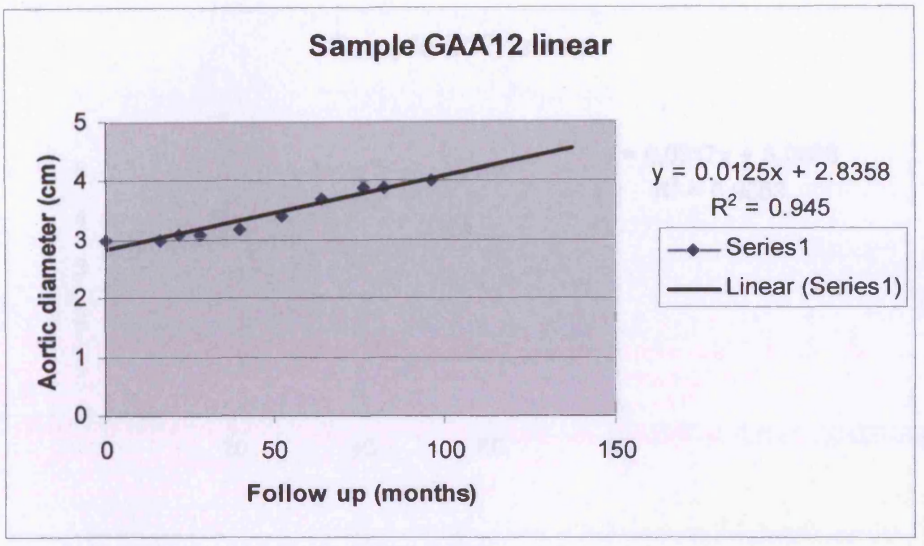


Figure 8.1. XY scatter graph demonstrating aortic diameter over time for all 178 patients studied.

i.



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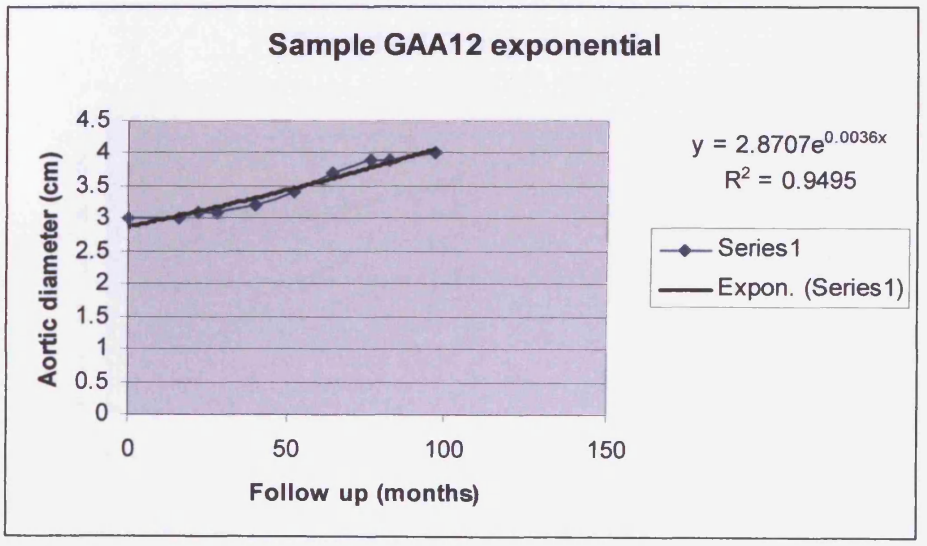
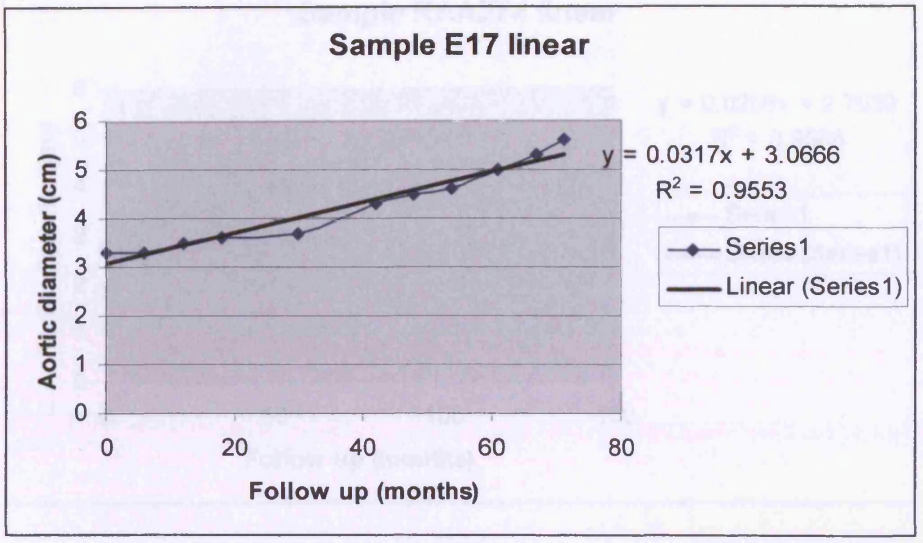


Figure 8.2. Expansion over time for subject GAA12.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



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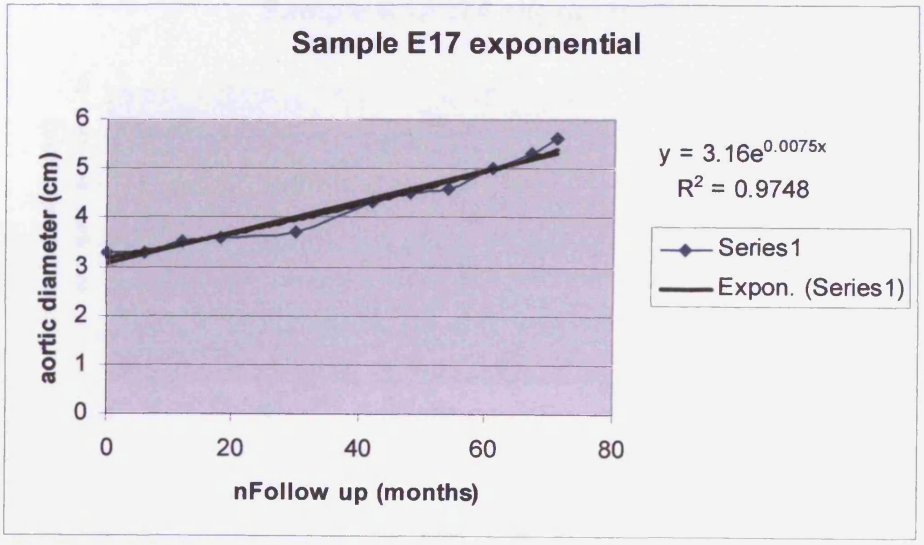
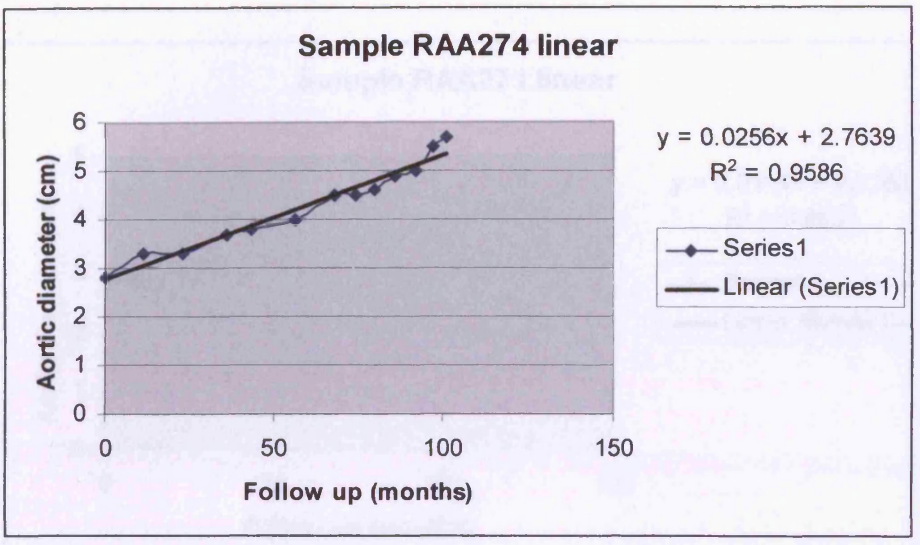


Figure 8.3. Expansion over time for subject E17.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



ii.

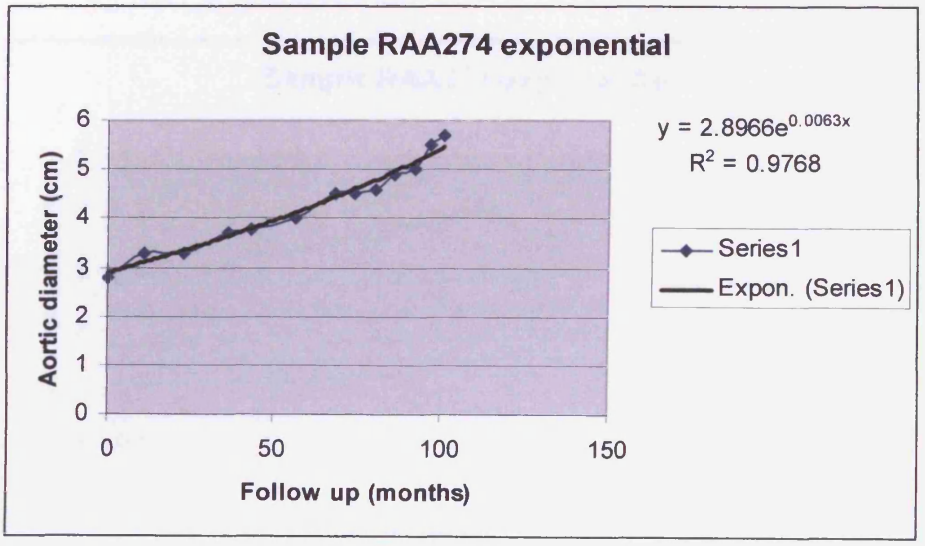
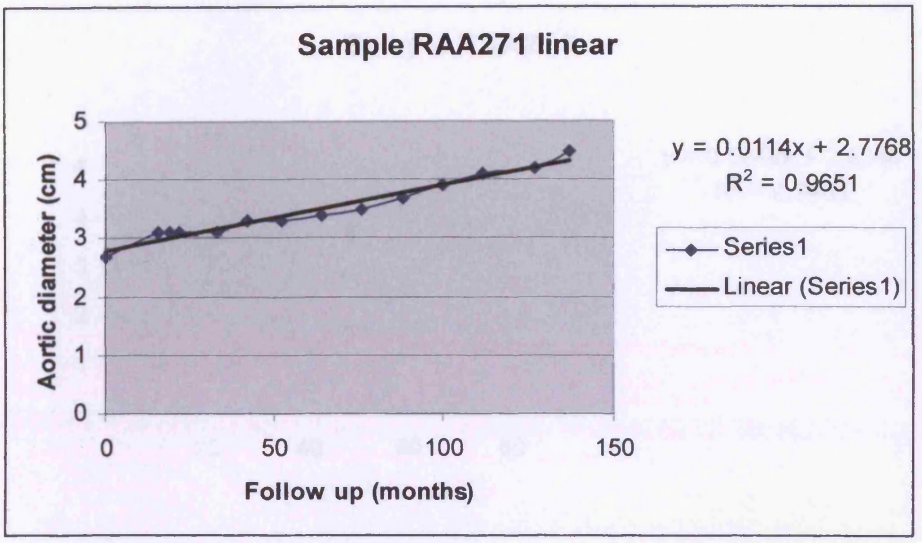


Figure 8.4. Expansion over time for subject RAA274.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



ii.

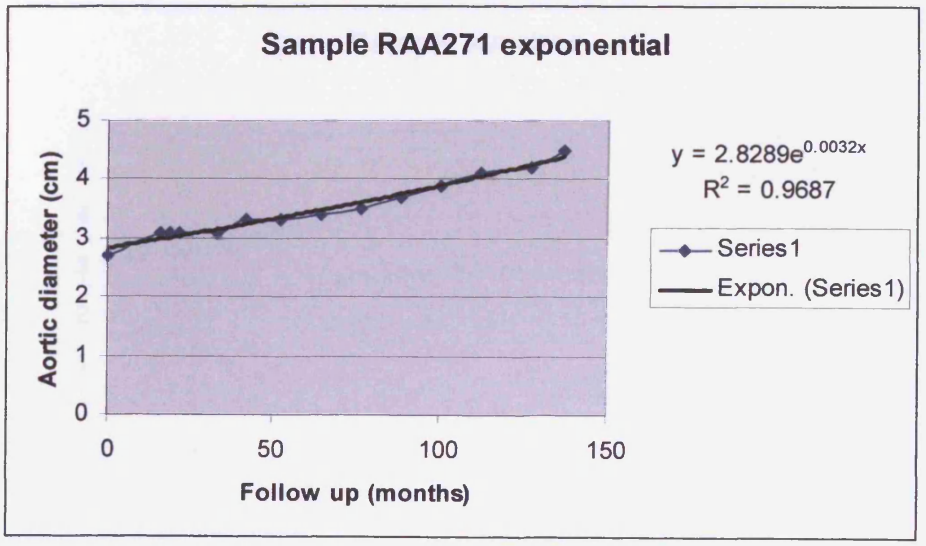
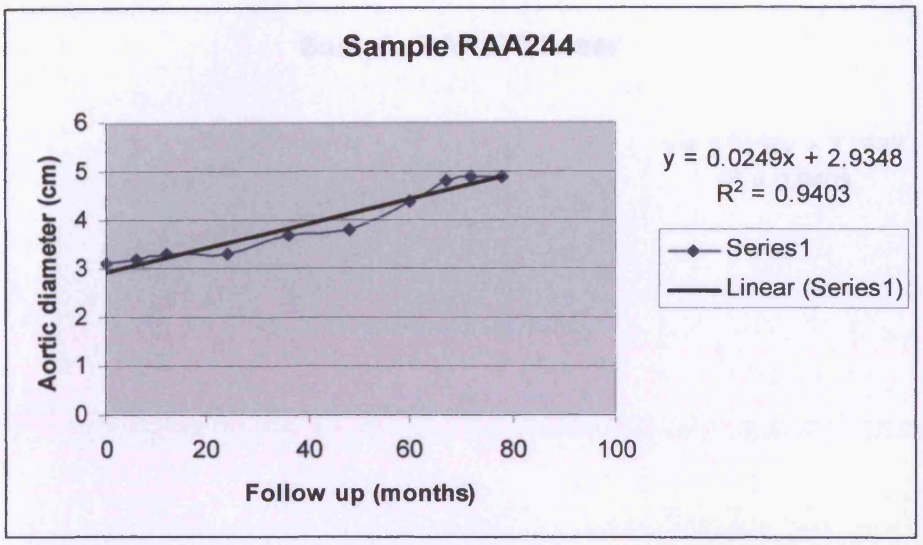


Figure 8.5. Expansion over time for subject RAA271.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



ii.

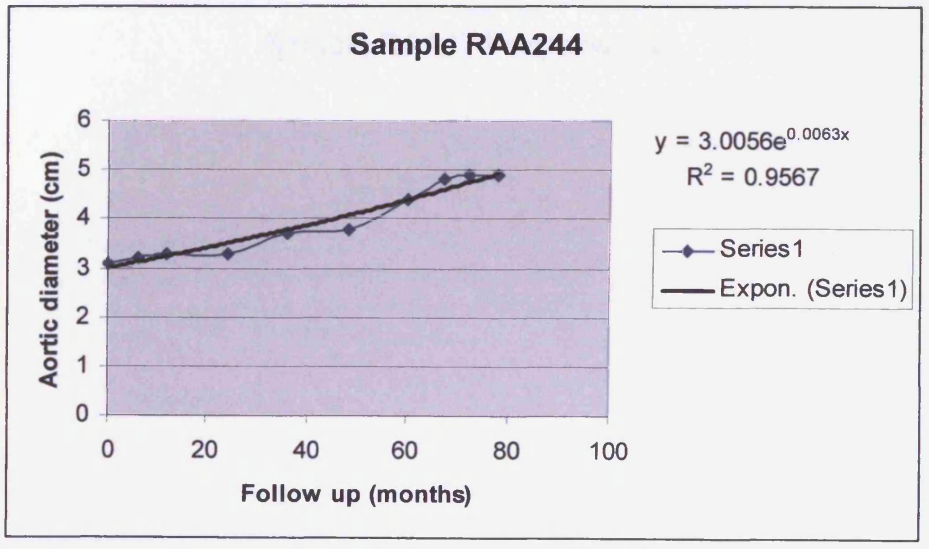
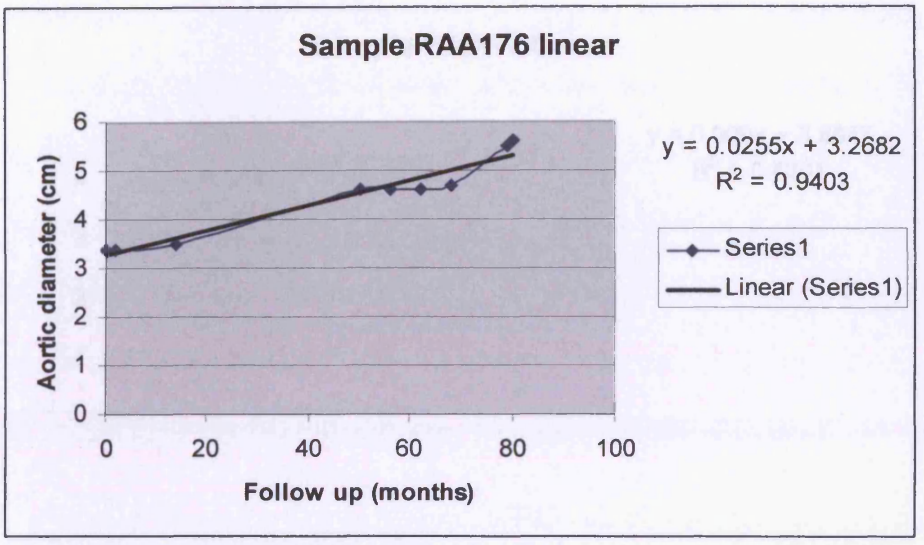


Figure 8.6. Expansion over time for subject RAA244.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



ii.

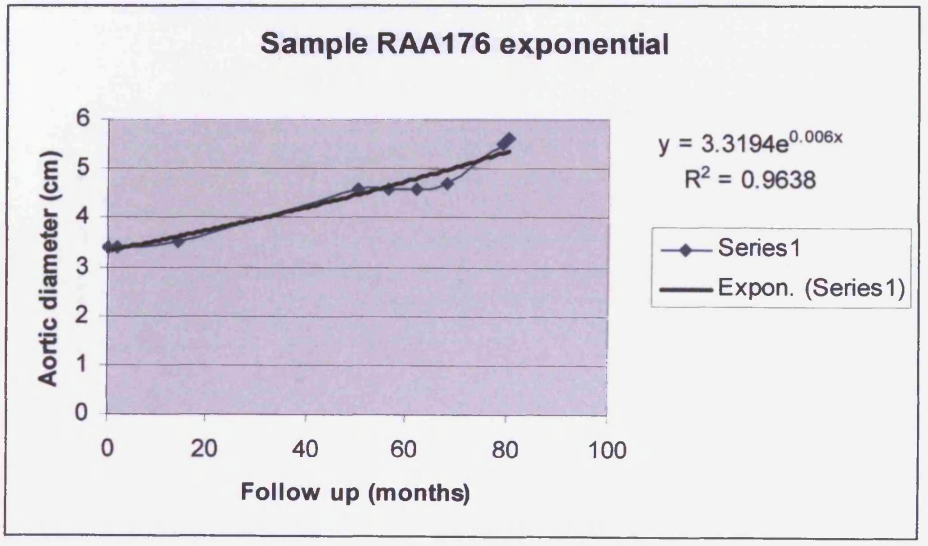
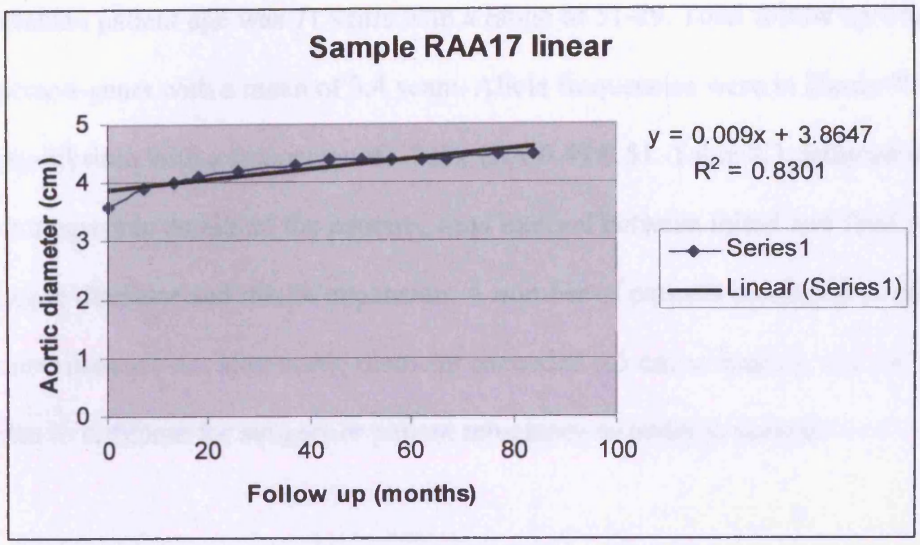


Figure 8.7. Expansion over time for subject RAA176.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

i.



ii.

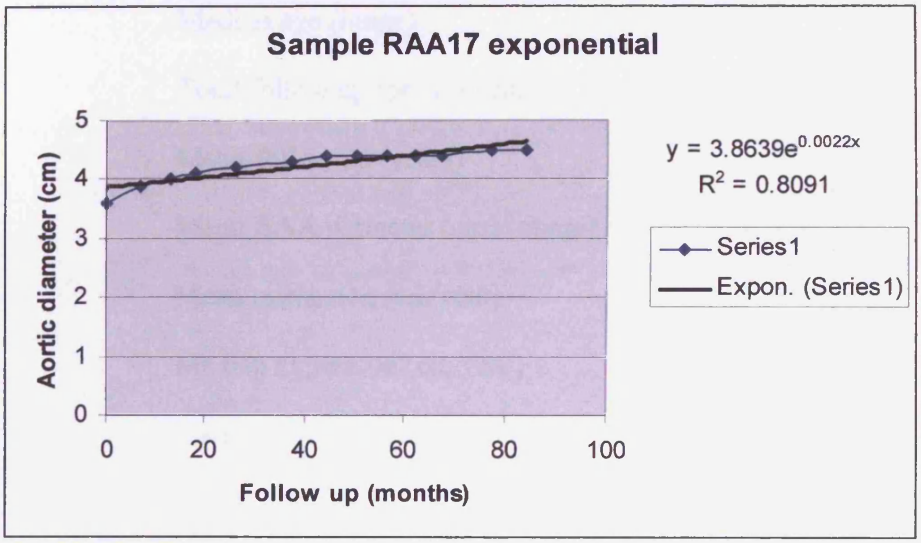


Figure 8.8. Expansion over time for subject RAA17.
i. R^2 value and equation if linear expansion is assumed.
ii. R^2 value and equation if exponential expansion is assumed.

Patients

Median patient age was 71 years with a range of 51-89. Total follow up was 604 person-years with a mean of 3.4 years. Allele frequencies were in Hardy-Weinberg equilibrium with a frequency of: -1082 G:A 0.49:0.51. Table 8.3 demonstrates the demographic details of the patients, time interval between initial and final scan, initial aortic diameter and rate of expansion. A number of patients continued to undergo surveillance even after aortic diameter exceeded 5.5 cm as surgery was not performed due to unfitness for surgery or patient reluctance to undergo surgery.

Number of subjects	178
Number of men (%)	167 (94)
Median age (range)	71 (51-89)
Total follow up (person years)	604
Mean follow up (years)	3.4
Mean AAA diameter (cm) (range)	3.7 (3.0-5.5)
Mean expansion (cm/year)	0.27
Median expansion (cm/year)	0.20
Standard deviation (cm/year)	0.28
Range (cm/year)	0.0 – 2.4

Table 8.3. Details of patients, follow up, initial aortic diameter and expansion rate.

Results

Table 8.4 shows the mean expansion rate in patients with and without the A allele ie those who are GG homozygotes versus those with the AA or GA genotype. Mean growth rate was not significantly different between patients with an IL-10 -1082 A allele (associated with AAA) (mean 0.28 cm/yr, SD 0.29) and those without (0.22 cm/yr SD 0.24, P=0.28 Student's t-test).

Allele	Subjects	Expansion (cm/year)	SD	P-value
No A allele	37	0.22	0.24	
A allele	141	0.28	0.290	0.28

Table 8.4. Mean expansion rate in patients with and without the -1082 A allele (SD, standard deviation).

Table 8.5 shows the mean expansion rate in patients with each genotype: GG, GA and AA. Mean growth rate increased stepwise with each additional A allele present although this finding was not statistically significant. Growth rates for each genotype were as follows: GG (mean 0.22 cm/yr, SD 0.24), GA (0.27 cm/yr SD 0.23), AA (0.30 cm/yr, SD 0.40), P=0.47, one-way ANOVA.

Genotype	Subjects	Expansion (cm/year)	SD	P-value
GG	37	0.22	0.24	
GA	99	0.27	0.23	
AA	42	0.30	0.40	0.468

Table 8.5. Mean expansion rate in patients with the -1082 GG, GA and AA genotypes (SD, standard deviation).

Mean growth rate was significantly higher in those patients who were current cigarette smokers compared to those who were not (0.40 cm/yr (SD 0.21, n=36) vs 0.23 cm/yr (SD 0.43, n=142), P=0.02, Student's t-test) (Table 8.6).

	Subjects	Expansion (cm/year)	SD	P-value
Current smoker	36	0.40	0.212	
Non smoker	142	0.23	0.43	0.02

Table 8.6. Mean expansion rate in current smokers and non smokers (SD, standard deviation).

Growth rate also correlated positively with initial size P=0.00 (Pearson) (r^2=0.2) and patient age (P=0.025 Pearson). Figure 8.9 shows the correlation between initial aneurysm size and growth rate. Figure 8.10 shows the correlation between patient age and growth rate.

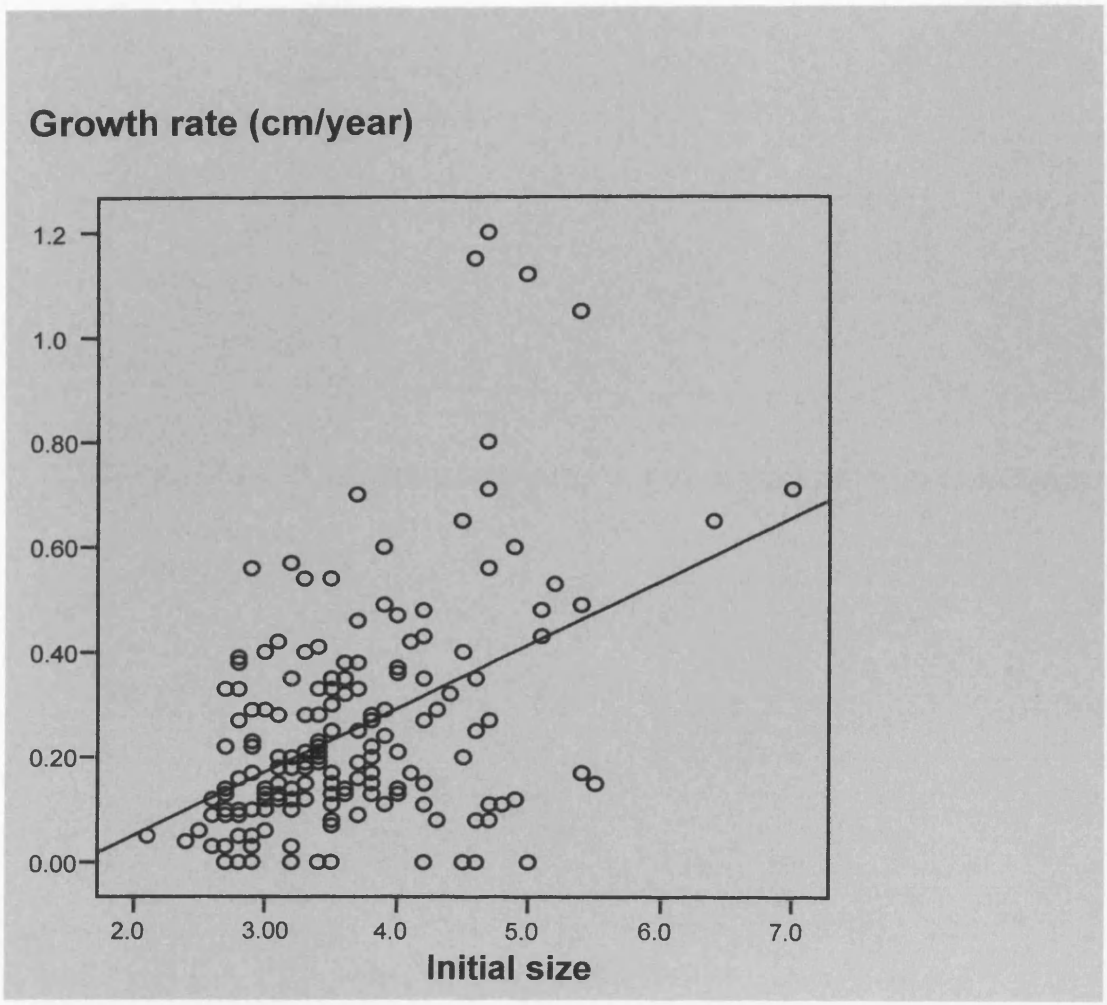


Figure 8.9 Correlation between initial AAA size (cm) and growth rate (cm/year).

Figure 8.10 Correlation between initial AAA size (cm) and growth rate (cm/year).

Summary

Mean growth rate was not significantly different between those individuals with an IL-10 -1082 A allele and those without. Whilst mean growth rate increased stepwise with each additional A allele present this finding was not statistically significant. AAA growth rate varies in individuals over time and it is possible that the 3.4 year mean follow up period was not long enough to reveal an association between genotype and expansion rate.

Mean growth rate was significantly higher in those patients who were current cigarette smokers compared to those who were not. Both patient age and size of AAA at the time of entering the study significantly correlated with growth rate.

Chapter 9

Discussion

Contents

Introduction

Abdominal aortic aneurysms (AAA) account for approximately 9000 deaths annually in the United Kingdom. The incidence of AAA is increasing, partly as a consequence of the ageing population (Naylor AR 1988) (Samy AK 1994) (Melton L 1984). This, combined with the recent inception of AAA screening programmes has resulted in increasing numbers of patients with incidental AAA being identified. Many of these patients have small AAA (less than 5.5 cm), that are too small to justify surgical repair.

In marked contrast to ischaemic heart disease and cerebrovascular disease, manifestations of stenotic arterial disease, there is no medical treatment for AAA. A number of pharmaceutical agents have been investigated but none has been shown to convincingly curtail AAA expansion or prevent rupture. Consequently, the only means by which mortality in AAA can currently be improved is by population screening to identify patients with subclinical AAA, the provision of better cardiovascular risk reduction and by developments in operative technique. Elective endovascular aneurysm repair (EVAR) has lower operative mortality and morbidity than open repair and may prove, in the future, to markedly improve the operative mortality in ruptured AAA (RAAA). In Chapter 1 I presented the results of the Leicester experience of EVAR in symptomatic or RAAA. Although only 21 patients were treated (11 symptomatic AAA and 10 RAAA) the results are promising with only 1 peri-operative and 3 late deaths. Clearly better understanding of the pathogenesis of AAA formation and expansion may provide an opportunity to develop a treatment for small AAA, an innovation that would be of enormous benefit.

Feasibility of preoperative computed tomography in RAAA

A widespread concern in performing EVAR in patients with RAAA is the need to obtain an abdominal CT scan in order to determine anatomical suitability for EVAR. This inevitably causes significant delay in commencing surgery and potentially increases mortality. In Chapter 1 I sought to ascertain the natural history of RAAA after admission to hospital in 56 patients who did not undergo surgery due to associated pathology. Interestingly, it was apparent that the majority of the patients remained haemodynamically stable and survived for a number of hours following admission. Importantly almost 90% were alive and relatively haemodynamically stable 2 hours after admission, a time interval during which a patient with a RAAA could easily undergo a CT scan and be transferred to theatre.

Although this was a small retrospective study that provides no information on the true effect preoperative delay has on the outcome of surgery, the evidence provided may indicate that there is more time than previously thought to facilitate EVAR in RAAA.

Adequacy of cardiovascular risk reduction in AAA

Landmark studies such as The Heart Protection Study, The Heart Outcomes Prevention Evaluation Study and The Antithrombotic Trialists' Collaboration have provided unequivocal evidence of the huge benefit of statins, ACE inhibitors and antiplatelet agents in patients with occlusive arterial disease. However, patients with aneurysmal disease were not analysed as a subgroup, although undoubtedly some of the patients studied would have had an AAA in addition to occlusive disease. Consequently direct evidence for the benefit of these agents in patients with AAA is

lacking. This has resulted in little mention of patients with AAA in recommendations aimed at appropriately targeting cardiovascular risk reduction.

In Chapter 6, in a study of 313 patients with AAA, I demonstrated that the majority have associated manifestations of occlusive arterial disease and therefore have a clear indication for a statin, ACE inhibitor, beta-blocker and antiplatelet agent. However, it was apparent that suboptimal cardiovascular risk management was prevalent with only 60% of patients using an antiplatelet agent, 41% a statin, 38% a beta-blocker and 39% an ACE inhibitor when indicated.

This is a significant finding as many patients with an AAA are not under the care of a general physician or cardiologist. The vascular surgery clinic would be an appropriate setting for the commencement of treatment, a strategy that would markedly improve cardiovascular survival in patients with AAA.

Employing logistic regression analysis revealed no association between IL-10 genotype at the -1082 or -592 positions and history of hypertension, MI, CVA or diabetes when considered as independent variables or when cardiovascular risk factors were adjusted for. Similarly, the -1082 and -592 genotypes were not associated with IL-10 level when considered as independent variables or when age, sex, smoking history, hypertension and diabetes were adjusted for. Those patients using a statin, ACE inhibitor, aspirin or beta blocker did not have a significantly different IL-10 level when compared to patients not using medication.

The role of Interleukin-10 gene polymorphisms in AAA

In Chapter 2 the pathogenesis of AAA was discussed. Degradation of collagen and elastin occurs in an intricate inflammatory process regulated in part by MMPs, TIMPs and cytokines. The pro- / anti- inflammatory cytokine balance appears to be instrumental in this process. In addition, there is undoubtedly a genetic component to AAA formation, the nature of which is currently unknown.

Interleukin 10 (IL-10) is a potent antiinflammatory cytokine that has been demonstrated to inhibit a number of pro-aneurysmal processes including the inhibition of proinflammatory cytokines, MMP inhibition, TIMP stimulation, iNOS, NO and uPA inhibition and apoptosis inhibition. These diverse actions may imply that IL-10 participates in the inhibition of numerous AAA - causing pathways. Although numerous proinflammatory cytokines have been implicated in AAA formation, relatively few antiinflammatory cytokines have been identified. IL-10 is a critically important antiinflammatory cytokine and it may therefore be the case that IL-10 has a key role in inhibiting numerous inflammatory cytokines. This hypothesis is supported by the important role IL-10 has been shown to have in other disease states.

The IL-10 gene is polymorphic and the presence of an A allele at the -1082 position is associated with a 'low producer' genotype (Eskdale J 1998). The effect of the linked –819/-592 polymorphism is less clear although the A allele at the –592 position has been associated with lower IL-10 production (Rosenwasser LJ 1997) (Lowe PR 2003). The 3 polymorphisms are closely linked with only 3 out of the 8 possible haplotypes existing in the Caucasian population; G/C/C, A/C/C and A/T/A at positions -1082/-819/-592 respectively (Turner DM 1997). The GCC, ACC and ATA haplotypes have

been associated with high, intermediate and low IL-10 production (Edwards-Smith CJ 1999).

In Chapter 7, I performed a case control study to investigate the IL-10 polymorphism frequencies at -1082 and -592 positions in patients with and without AAA. In the initial unmatched analysis of all 794 recruited patients I demonstrated that the low IL-10 producing A allele and AA genotype were more prevalent in patients with AAA than those with normal diameter aortas. No difference in –592 genotype or allele frequencies was demonstrated although the low IL-10 producing ATA haplotype was more common in patients with AAA and the high IL-10 producing GCC haplotype less common. Interestingly, I found a significant trend towards increasing -1082 A allele frequency with age in patients with AAA.

When a strictly age matched analysis was performed I again demonstrated that the –1082 A allele was significantly more common in patients with AAA. A dose dependent mechanism appeared to exist with the number of A alleles present increasing the risk of AAA in a stepwise fashion. In common with the unmatched analysis I demonstrated no difference in –592 genotype or allele frequency between cases and controls. Again, as in the unmatched analysis the ATA haplotype was more common in patients with AAA and the high IL-10 producing GCC haplotype less common.

I attempted to adjust for smoking history but the very few patients with AAA who had never smoked made this analysis meaningless. When genotype was compared in cases and controls undergoing treatment for hypertension the -1082 AA genotype and A

allele remained significantly more common in patients with AAA. Interestingly, this difference disappeared in subjects without hypertension and no difference in genotype or allele frequencies was found.

In critically evaluating the results of this study a number of factors must be considered. The design of a case control study is of vital importance. I sought to minimise selection bias as much as possible by initially selecting all the control patients from the Leicestershire AAA screening programme. This ensured a control group selected only by age (65-66 years), having a general practitioner who agreed to participate in the programme, the patient's participation in the programme and the patient's consent to taking part in the study. A further 63 patients aged over 70 without an AAA (after abdominal imaging) were recruited from the vascular surgery ward to provide a source of older control patients. Although this is a less randomly selected group of patients, many of the cases were discovered to have an AAA while on the vascular ward and they therefore represent a group that would have been recruited as cases if they had an AAA. As the patient selection process allowed recruitment of few female patients the associations I have described can only be applied to men.

The cases were selected from a variety of sources; the AAA screening programme, vascular surgery outpatient clinics and admission to hospital for AAA repair. During the period of subject recruitment I recruited all patients with AAA I encountered who consented to taking part in the study. There were no selection criteria other than having an aortic diameter > 2.5 cm. The cases were not consecutive as recruiting all patients from these sources during the period of study was clearly impossible. However, it can be reasonably assumed that the recruited patients were a

representative sample of patients known to have an AAA in the region. Many of the cases had been diagnosed as having an AAA some years previously and were therefore prevalent cases. Ideally only incident cases would have been used i.e. those patients in the screening programme who were discovered to have an AAA at the time of recruitment. However, as only 4% of the screened population were found to have an AAA this was impractical. In practice this is unlikely to be important given the long latent period of AAA and relatively long survival.

The 'taking all comers' approach to recruiting cases and controls minimised selection bias but resulted in a degree of mismatch between the 2 groups. As expected there was a marked difference in medical history with hypertension, manifestations of occlusive arterial disease, chronic obstructive pulmonary disease and smoking history being higher in cases. This potentially introduces confounding factors but nonetheless this is not a difficulty if the objective of the study is to investigate the association between the IL-10 gene and the presence of an AAA. The wide range of ages in the AAA group allowed genotype frequencies to be examined in different age groups.

Great care was taken to prevent DNA contamination of the samples and I proved the accuracy of the genotyping method, IHG. The technique had previously been optimised and in order to recheck accuracy I genotyped 100 samples of known genotype. In addition I rechecked 373 reactions during the genotyping process and demonstrated 100% reproducibility. In each run of samples genotyped I ran water blanks to identify DNA contamination of the reagents. No contamination was ever detected. The samples genotyped in each run were randomly selected so that case and

control DNA was used. This meant that if any undetected error in the genotyping process occurred the bias would not affect one group exclusively.

The obvious conclusion to draw would be that the low IL-10 producing -1082 A allele may play a role in increasing an individual's susceptibility to AAA formation. This is biologically plausible given the reported direct effect of the polymorphisms on the IL-10 gene function and the potential role IL-10 could play in inhibiting mechanisms implicated in AAA formation. The findings of the study are supported by the similar results obtained in 200 different subjects in the pilot study, thereby suggesting reproducibility (Bown MJ 2003). Alternative explanations are that the -1082 A allele causes another risk factor for AAA formation such as hypertension or that it is linked to another gene that is the true risk factor gene for AAA. Unfortunately the case control study design cannot elucidate the relationship further.

It is of interest that the significant differences in genotype and allele frequencies disappeared when non-hypertensive subjects were analysed separately. Possible explanations are that the A allele only increase the risk of AAA formation in hypertensive subjects or even that the alleles are instrumental in the development of hypertension, a risk factor for AAA formation. Caution must be exercised in interpreting this finding as the presence of hypertension in the study was based on a previous diagnosis having been made. There were almost certainly many hypertensive patients which I classified as non-hypertensive on the basis that their hypertension had not previously been recognised.

The mechanism of AAA formation is highly complex and multifactoral with environmental factors involved. As discussed in Chapter 3, there are more than 2 million single nucleotide polymorphisms (SNP) in the human genome, each with a modest effect on gene transcription (Marian AJ 2001). It would appear somewhat fortuitous that any one SNP would be clearly associated with such a complex disease state given the huge numbers of other SNPs present, perhaps contributing with additive or subtractive effect. Nonetheless numerous SNP-disease associations have been reported and SNPs do appear to contribute to susceptibility to disease.

No association was found between IL-10 genotype and plasma IL-10 concentration or the presence of an AAA and IL-10 concentration. Although this may appear to contradict the effect of the IL-10 polymorphisms I have proposed there are a number of alternative explanations. It is possible that despite all reasonable precautions being taken to reduce inaccuracy in measuring IL-10 concentration the experimental method was not sufficiently accurate to detect small differences. Alternatively it may be the case that genotype predicts lifetime exposure to IL-10 or IL-10 response to a stimulus and the power of the study was too small to detect this.

The effect of IL-10 polymorphisms on rate of AAA expansion

In the preceding chapter I investigated the role of the Il-10 -1082 polymorphism on the rate of AAA expansion. There was a trend towards the A allele being associated with more rapid expansion although statistical significance was not reached. It may be the case that the effect of the polymorphism was too small to be detected by the power of the study. However, as in previous studies initial AAA size and current smoking correlated with rate of expansion indicating that if any association between IL-10

genotype and AAA growth exists it is far less significant than initial AAA size and smoking.

Results of thesis

1. The majority of patients admitted to hospital with RAAA remain haemodynamically stable and survive long enough to undergo CT imaging and consideration for EVAR.

2. Most patients with AAA have manifestations of occlusive arterial disease and have clear indication for cardiovascular risk reducing medication. Current treatment of cardiovascular risk in patients with AAA is suboptimal.

3. IL-10 -1082 A allele frequency increases with age in patients with AAA.

4. In an age matched analysis:

a. -1082 AA genotype was significantly more common than the GG genotype in the AAA subjects (P=0.0004, Fisher's Exact test) with an odds ratio of 3.37 (95% confidence interval, 1.74 to 6.49).

b. The intermediate producer GA genotype was significantly more prevalent than the GG genotype in AAA subjects, P=0.001 (Fisher's Exact test) with an odds ratio of 2.14 (95% confidence interval, 1.21 to 3.78).

c. The -1082 A allele was significantly more prevalent in subjects with AAA (P=0.0002) with an odds ratio of 1.87 (96% confidence interval, 1.35-2.59) for the A allele being a risk factor for AAA.

5. In an age matched analysis:

a. There was no difference in -592 genotype between cases and controls, P=0.15 (Chi squared=3.76, 2df)

b. There was no difference in -592 allele frequency between cases and controls, P=0.09 (Fisher's Exact test).

6. There was a significant difference in haplotype with the ATA haplotype being more common in patients with AAA and the GCC haplotype less common, P=0.0008 (Chi-squared=14.31, 2df).

7. The association between the -1082 genotypes and allele frequencies and AAA disappeared when non-hypertensive subjects were analysed separately.

8. Plasma IL-10 concentration is not different in patients with or without an AAA.

9. No association was found between the IL-10 -1082 or -592 polymorphisms and plasma IL-10 concentration.

Future Work

This thesis goes some way towards proving an association between IL-10 gene polymorphisms and AAA. However, a number of uncertainties exist. The increasing A allele prevalence with increasing age in patients with AAA warrants further investigation. By recruiting older control patients it will be determined whether this association persists for subjects with normal aortic diameter and allow a comparison of genotype and allele frequency in age matched elderly subjects.

I demonstrated an association between the gene polymorphisms studied and hypertension. The numbers of subjects studied were relatively small and the classification of hypertension was based on a prior diagnosis having been made. A larger study with a more robust definition of hypertension is required to clarify the association.

Cytokines do not operate in isolation. Numerous cytokines participate in a complex pathway and many have functionally important polymorphisms. A similar study to determine the effect of combinations of cytokine polymorphisms may provide far more insight into the mechanism of AAA formation than simply studying the effect of one.

There is an established genetic component to AAA formation, the nature of which is still unknown. There is growing interest in the possibility of gene polymorphisms perhaps mediating genetic susceptibility to disease. It is often difficult to accurately determine family history of AAA due to AAA frequently being asymptomatic, the late age of onset and infrequent post mortem examinations. It was impossible to

accurately determine family history in this study. It is hoped that with increasing numbers of patients undergoing AAA screening it may be possible to compare cytokine gene polymorphisms in patients with and without family history of AAA.

Appendix 1

Names and addresses of suppliers of laboratory consumables

BD Biosciences
21 Between Towns Road
Cowley
Oxford
OX4 3LY
Tel: 01865 748 844
www.bdbiosciences.com

Bio - Rad Laboratories Ltd
Bio - Rad House,
Maylands Avenue.
Hemel Hempst.
Herts HP2 7TD
Tel: 08 328 2000
www.bio-rad.com

Biosource UK Ltd
Rue de l'Industrie 8,
B - 1400 Nivelles
BELGUIM
Tel: +44 800 1691547
www.biosource.co.uk

Gentra Systems, Inc.
13355 10th Avenue N
Suite 120
Minneapolis, MN 55441
USA

Eurogentec Ltd.
P.C. House, 2 South Street, Hythe
Southampton
Hampshire SO45 6EB
UK
Tel.: + 44 (0) 1794 511 411
info.uk@eurogentec.com

Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley
UK
PA4 9RF
Tel: 0141 814 6100
euroinfo@invitrogen.com

Sarstedt Ltd.
68 Boston Road
Beaumont Leys
Leicester LE4 1AW
United Kingdom
Tel.: +44 1162 359023
info@sarstedt.com

Sigma-Aldrich Company Ltd.
The Old Brickyard
New Road
Gillingham
Dorset
SP8 4XT
0800 717181
ukorders@europe.sial.com

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