AN INVESTIGATION INTO THE CYTOKINE EXPRESSION PROFILE OF THE ABDOMINAL AORTIC ANEURYSM WALL

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by

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Statement of Originality

I hereby declare that the work contained in this thesis is entirely my own, except where acknowledged, and has not been submitted for a degree to this or any other University.

R. K. Middleton

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This thesis is dedicated to my family
for all the support and encouragement they have given me.

Abstract

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Abdominal aortic aneurysm is a disease commonly found in elderly males involving dilatation of the abdominal aorta. The aneurysm wall is characterised by a decrease in the elastin/collagen ratio, apoptosis of smooth muscle cells and a prominent inflammatory infiltrate. Degradation of the extracellular matrix has been attributed to matrix metalloproteinases (MMP), the expression and activation of which is tightly regulated by inflammatory mediators such as cytokines. The presence of an inflammatory infiltrate is a potential source of cytokines within the aneurysm wall.

The aim of this thesis is to investigate the nature of the cytokines that are expressed within the aneurysm. Further work aims to characterise the expression of key cytokines and to investigate their effect on MMP expression.

The cytokine profile of the aneurysm wall was examined using a 42-cytokine protein array. Overall, a number of pro-inflammatory cytokines, chemokines and growth factors were raised within the aneurysm wall. Interleukin-6 (IL-), IL-8, monocyte chemoattractant protein-1 (MCP-) and MCP-2 were highly elevated in the aneurysm above the physiological levels found in abdominal and thoracic aorta.

The expression of chemokines within the aneurysm wall was characterised further using immunohistochemistry. IL-8 was found to co-localise with the infiltrate, which was predominantly formed from $CD20^+$ B-lymphocytes and $CD3^+$ T-lymphocytes, whilst MCP-1 co-localised to $CD68^+$ macrophages.

The effect of IL-8 on the expression of MMP-2 and MMP-9 was investigated through an *in vitro* aneurysm model. The results showed that MMP-2 was expressed constitutively, whilst MMP-9 expression from the same culture decreased with time. IL-8 did not effect MMP-2 and MMP-9 expression.

In conclusion, the abdominal aortic aneurysm wall is a highly pro-inflammatory and chemotactic environment. Co-localisation of IL-8 and MCP-1 with the infiltrating cells suggests a role for these cytokines in aneurysm pathogenesis. However, a direct involvement between IL-8 and MMP expression is unlikely.

Publications and Presentations Arising from this Thesis

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Middleton RK, Lloyd GM, Bown MJ, Jones LJ, London NJ, Sayers RD. Characterisation of Interleukin-8 and Monocyte Chemoattractant Protein-1 Expression within the Abdominal Aortic Aneurysm and their Association with Mural Inflammation. *Submitted to the European Journal of Vascular and Endovascular Surgery.*

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List of Abbreviations

AAA	Abdominal aortic aneurysm
ACE	Angiotensin converting enzyme
ADAM	Aneurysm detection and management veteran affairs cooperative study
Ang I	Angiotensin I
Ang II	Angiotensin II
AOD	Arterial occlusive disease
APC	Antigen presenting cell
APMA	p-Aminophenylmercuric acetate
Apo E	Apolipoprotein E
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca	Calcium
Capture AB	Capture antibody
CCR	CC receptor
CD	Cluster of differentiation
CI	Confidence interval
Cox	Cyclooxygenase
CSF	Colony stimulating factor
Cu	Copper
CXCR	CXC receptor
DAB	3,3'-Diaminobenzidine
Detect AB	Detection antibody
DMEM	Dulbeccos modification of Eagles medium
ECM	Extracellular matrix
EDP	Elastin degradation peptide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ENA-78	Epithelial neutrophil activating peptide-78
EVAR	Endovascular AAA repair

EVG	Elastin van Gieson
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte/macrophage colony stimulating factor
GRO	Growth-regulated oncogene
H&E	Haematoxylin & eosin
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HRP-SA	Horseradish peroxidase-streptavidin
HUVECs	Human umbilical vein endothelial cells
IAAA	Inflammatory abdominal aortic aneurysm
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL-	Interleukin-
IL-1Rα	Interleukin-1 receptor antagonist
IMS	Industrial methylated spirit
γ-IP	γ-Interferon inducible protein
IQR	Interquartile range
ISCVS	International society for cardiovascular surgery
-/-	Knockout
MASS	Multicentre aneurysm screening study
MCP-	Monocyte chemoattractant protein-
M-CSF	Macrophage colony stimulating factor
MDC	Macrophage derived chemokine
MHC	Major histocompatibility complex
MIG	Monokine-induced by interferon-γ
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MT1-MMP	Membrane type-1 matrix metalloproteinase
NGF	Nerve growth factor

NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density
OSM	Oncostatin M
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear leukocytes
RANTES	Regulated upon activation, normal T-cell expressed and secreted
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Streptavidin
SCF	Stem cell factor
SD	Standard deviation
SDF-	Stromal cell derived factor-
SMC	Smooth muscle cell
SVS	Society for vascular surgery
TARC	Thymus & activation regulated chemokine
TBS	Tris buffered saline
T_C-lymphocyte	Cytotoxic T-lymphocytes
TCR	T-cell receptor
TGF-	Transforming growth factor-
T_H-lymphocyte	T_{Helper}-lymphocyte
TIMP	Tissue inhibitor of metalloproteinases
TNF-	Tumour necrosis factor-
t-PA	Tissue-plasminogen activator
Tris-HCl	Tris (hydroxymethylaminomethane) hydrochloric acid
UKSAT	UK small aneurysm trial
u-PA	Urokinase-plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
vs.	Versus

Contents

Chapter One

Abdominal Aortic Aneurysms

Chapter 1. Abdominal Aortic Aneurysms

1. Abdominal Aortic Aneurysms

1.1 The History of Abdominal Aortic Aneurysms

The first written description of an arterial aneurysm was reported by the Egyptians in the Ebers Papyrus, 2725 B.C.[1]. The term aneurysm is thought to come from the Greek word "aneurysma", meaning to widen or dilate. In the second century A.D. the first accurate description of an aneurysm was described by Galen, who wrote, "When the arteries are enlarged, the disease is called an aneurysm... If the aneurysm is injured the blood gushes forth and it is difficult to staunch it"[2]. Later in the second century the Greek surgeon, Antyllus, developed a treatment which involved proximal and distal ligation of the artery, followed by an incision into the aneurysm sac and evacuation of the thrombus, due to his belief that the thrombus caused the dilatation[3]. This technique, though it was seldom used, represented the first detailed procedure in the history of vascular surgery to attempt to treat aneurysms.

The bipolar ligation remained the only available treatment until 1710 when simple proximal ligation was advocated by the French surgeon, Anel. In 1785, John Hunter used the single ligature technique to ligate the proximal femoral artery to successfully treat a popliteal aneurysm. The first attempt to ligate an aortic aneurysm was performed in 1817 by Hunter's protégé, Sir Astley Cooper. Unfortunately the patient died three days later[4]. The treatment was based on the theory that ligation would slow or arrest circulation within the aneurysm sac, causing thrombosis, eventually alleviating any symptoms and preventing rupture of the aneurysm. However, ligating the aneurysm rendered the extremities vulnerable to ischaemic damage.

In 1888, Rudolph Matas performed his first endoaneurysmorrhaphy for a large traumatic aneurysm of the brachial artery (Figure 1-1). Encompassing the work of Antyllus he reported a method for restoring the continuity of luminal blood flow down the affected artery. The technique involved proximal and distal ligation, followed by an incision into the sac, removal of the thrombus and intravascular suturing of the arterial openings within the aneurysm sac[5]. However, with the need to sacrifice the branching arteries this technique was limited to treatment of aneurysms where the branching arteries were not

essential to prevent ischaemia. Its use was therefore limited in the treatment of aortic aneurysms and other major arteries.

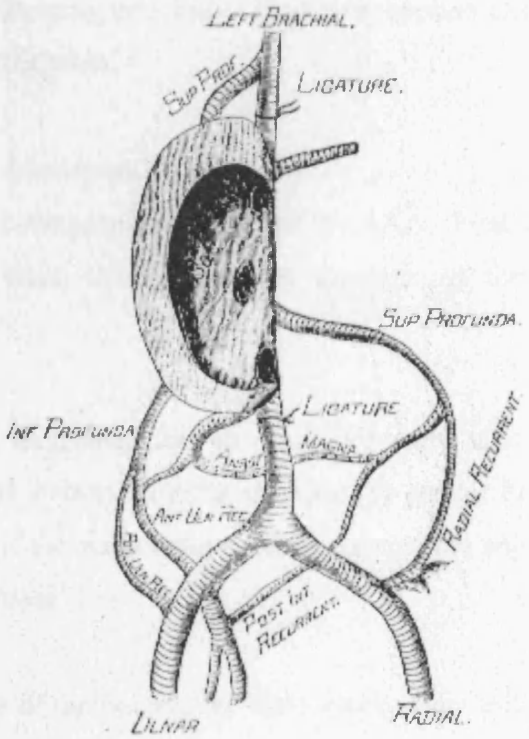

Figure 1-1. Matas' original drawing of his initial endoaneurysmorrhaphy in 1888[6].

At the beginning of the twentieth century, Carrel and Guthrie began experimenting on the use of homograft aortic replacements. The first successful resection of an abdominal aortic aneurysm (AAA) using an arterial graft replacement was performed by Dubost in Paris, 1951 and the principle of AAA repair has remained largely unchanged[4]. Subsequently with the development of synthetic, prosthetic grafts the operative mortality of open AAA repair has fallen to 5-6%[7]. An impressive result when compared to Hunter's period where aneurysm repair, using ligation, had only a 5% chance of success[4].

The endovascular AAA repair (EVAR) technique was introduced in 1991 by Parodi, presenting surgeons with the ability to repair AAAs with less physical trauma to the patient[8]. Recently, the results of a 4-year trial into the long-term benefits of EVAR versus open AAA repair showed that EVAR in fit patients led to a 3% decrease in aneurysm-related deaths[9].

1.2 Definition

An aneurysm is an abnormal focal dilatation of an artery and is classified according to its site, morphology and underlying aetiology. However, applied clinically this description is vague and requires quantification.

1.2.1 Definition of an Aneurysm

Several definitions have been proposed to define the AAA. Firstly, Sterpetti *et. al.* defined an AAA according to when the ratio of the diameter of the infrarenal aorta to the suprarenal aorta is ≥1.5[10].

An alternative definition suggested that an AAA is present when the maximum external diameter of the infrarenal abdominal aorta is either (1) greater than 40mm or (2) exceeds the maximum diameter of the aorta between the origin of the superior mesenteric and left renal arteries by at least 5mm[11].

In 1991 the joint councils of the Society for Vascular Surgery and the International Society for Cardiovascular Surgery (SVS/ISCVS) sought to standardise the definition of an arterial aneurysm. They recommended an aneurysm be defined as 'a focal dilation of an artery involving an increase in diameter of 50% or more compared with the normal expected diameter'[12].

Application of this definition to the AAA means that for the normal male infrarenal aorta, with a mean diameter of 14.1-20.5mm (range, 0.4-3.7mm)[12], an AAA exists when the luminal diameter of the infrarenal aorta is greater than 30mm.

To date, whilst the generally accepted definition is that of the SVS/ISCVS there is a still a degree of variation in the definitions used for reporting AAAs.

1.2.2 Aneurysm Site

An aneurysm can occur anywhere along the arterial tree and can be classed according to site as either thoracoabdominal, abdominal or peripheral aneurysms. The thoracoabdominal aneurysm is classified as types I-IV depending on the length of the

descending thoracic/abdominal aorta involved[13]. The most common aneurysm variant is the AAA, of which 89% affect the infrarenal abdominal aorta[14] (Figure 1-2) and only 5% involve the suprarenal aorta[15].

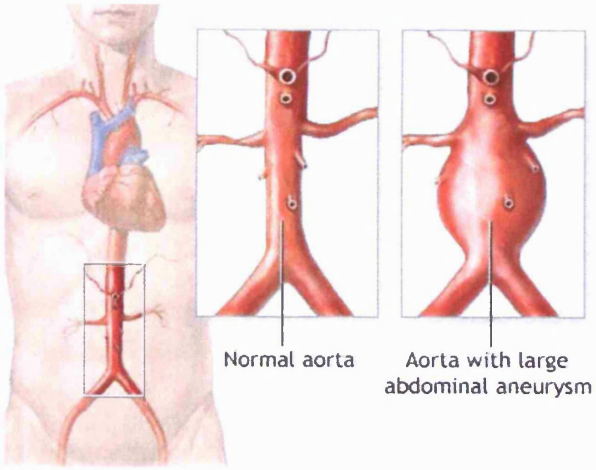

Figure 1-2. Diagrammatic representation of an infrarenal AAA[16].

Approximately 20% of AAAs extend into one or both common iliac arteries and about a third of these affect the internal iliac artery. AAA may also be associated with other peripheral aneurysms. The peripheral arteries that are most commonly affected are the iliac, popliteal, femoral and carotid arteries with popliteal aneurysms comprising 80% of all peripheral aneurysms[17].

1.2.3 Morphology

Aneurysms are usually 'true aneurysms' where the dilatation involves all three layers of the artery wall. These aneurysms may be saccular (where the aneurysm involves dilatation of a section of the arterial wall to form a 'sac-like' protrusion) or fusiform (symmetrical dilatation of the artery giving the aneurysm a 'balloon-like' appearance); the latter form being the more common variant.

Alternatively, an aneurysm may be classed as a false aneurysm where the aneurysm does not involve all three layers of the artery wall. These aneurysms are caused by penetrating trauma or iatrogenic injury, for example from the use of an arterial monitoring line or infection. The resulting haematoma sometimes remains in continuity with the lumen

giving rise to a false aneurysm. This false aneurysm is essentially formed from compacted thrombus and surrounding connective tissue.

An additional form of aneurysm is the dissecting aneurysm where the intima, the innermost layer of the aortic wall, of the aorta is stripped away from the media, the central layer, resulting in blood flowing through the vessel wall in addition to the luminal flow[13, 18]. Dissecting aneurysms usually occur in the descending thoracic aorta or aortic arch, secondary to hypertension or connective tissue disease.

1.3 Natural History of the Abdominal Aortic Aneurysm

The infrarenal aortic diameter has been reported to increase with age in both men and women[19-22]. However, Wilmink *et. al.* reported that only a small proportion of aortas expand with age[23]. Of those aortas that become aneurysmal the natural history of the disease is to progressively expand, the rate varying amongst individuals, until eventually the aneurysm wall ruptures. AAAs may rupture posteriorly into the retroperitoneum, producing a contained leak and allowing the possibility of surgical repair, or rupture anteriorly, which usually results in exsanguination and death[17]. The clinical goal is therefore to prevent rupture through early intervention. The two main features used to predict AAA rupture are aortic diameter and expansion rates.

1.3.1 Aneurysm Diameter

The principle feature that determines AAA rupture is aortic diameter. The natural history of aneurysm rupture is difficult to ascertain because large AAAs (>55mm) are usually repaired and previous autopsy studies were unable to reflect diameter *in vivo* or were not reproducible. This led to a reported range of rupture rates from 1%[24] to 6%[25] per annum for an AAA of <50mm and from 1.0%[26] to 9.4%[27] for aneurysms 55-59mm[28]. The UK small aneurysm trial (UKSAT) followed the survival of patients with small aneurysms (40-55mm) for a mean surveillance period of 4.6 years and reported a mean risk of rupture of small aneurysms of 1% per year[7]. The increased risk of rupture with increased aortic size is summarised in Table 1-1 (from pooled data[28]). Surgical intervention is indicated at a diameter of >55mm and the sudden rise in the risk of rupture is most notable at this size.

Table 1-1. Annual rupture rates of AAAs according to size[28]

Initial AAA diameter (mm)	Annual risk of rupture (%)
30	0.2-0.4[29]
40	0.8-1.1[29]
40-55	0.6[30]-1.0[7]
55-59	9.4[27]
60-69	10.2[27]
>70	30.5[31]-32.5[27]

1.3.2 Expansion

AAA expansion is a continual process and rapid expansion is associated with an increased risk in rupture[27, 32]. Brown *et. al.* found that patients with aneurysms (50-59mm) who ruptured had a mean expansion rate of 4.4mm/year compared to the non-ruptured group whose mean expansion rate was 2.1mm/year. When they compared the expansion rate of patients with aneurysms >60mm the expansion rate of the ruptured group was 8.4mm/year compared to 3.9mm/year[26]. However, it has been debated if the apparent associated risk merely stems from the increased diameter size, which is a known risk[33]. Nevitt *et. al.* found that it was increased aneurysm size not expansion rate that was associated with the increased risk of rupture[34].

It has been reported that there is a linear relationship between initial size and the rate of expansion[35]. Limet *et. al.* found that aneurysms with a diameter of <40mm had an expansion rate of 5.3mm/year, increasing to 6.9mm/year for a diameter of 40-49mm and 7.4mm/year for diameters >50mm[32]. Small aneurysms expand more slowly. A study of 790 males with an aneurysm between 30-39mm (mean size, 33mm) showed a median expansion rate of 1.1mm/year[36]. The results of the surveillance group in the UKSAT showed that with a mean AAA diameter of 46.1mm the median small aneurysm growth rate was 3.3mm/year (interquartile range 2.0-5.3mm)[7]. The expansion rate of AAAs has been described using an exponential model[32], which explains the pattern that large aneurysms expand at a much greater rate than small aneurysms. Rapid expansion is often a step preceding rupture and therefore surgical repair is also indicated for aneurysms that have an expansion rate of >10mm/year.

1.4 Epidemiology

Aortic aneurysms are very rare in people under the age of 55 years and those occurring under this age limit are virtually confined to patients with connective tissue disorders, such as Ehler-Danlos and Marfans syndrome. The majority of aneurysms are idiopathic AAAs in elderly males.

1.4.1 Incidence

The annual incidence (number of new AAA cases in a specific population over a given time period) of AAAs in Western society is estimated as being between 11.3-117.2 per 100,000 person-years in men and between 2.2-33.9 per 100,000 person-years in women[37-42] (Table 1-2).

Table 1-2. Reported incidence of AAAs

Location	Incidence reported for year:	Incidence per 100,000 person-years		
		Men	Women	Total
England and Wales[37]	1983	11.3	3.0	--
Oxford[38]	1999	14.9	2.2	--
Scotland[39]	1984	--	--	63.6
The Netherlands[40]	1992	37.6	5.5	--
Western Australia[41]	1980-1981	117.2	33.9	--
Rochester, USA[42]	1971-1980	--	--	36.5

The incidence of AAA has increased over the past decades[37-43], despite a downward trend in deaths from other cardiovascular diseases. A recent report on AAA incidence in England and Wales for the period 1979-1999 found that mortality rates had increased from 80 to 115 per million in men and from 13 to 25 per million in women. The admission rates for all AAAs in the Oxford region were found to have increased from 52 to 149 per million per year in men and from 3 to 22 admissions per million per year in women. This represented an annual rise of 8.2% (95% confidence interval (CI) 7.1-9.2) in men and a 12.1% rise (95% CI 9.1-15.1) in women[38].

The reported incidence of ruptured AAAs varies from 1-21 per 100,000 person-years[44] and its incidence is increasing[45]. UK statistics show that ruptured AAAs cause 2.1% of all deaths in males and 0.75% of all deaths in women over the age of 65 years in England and Wales[46]. This accounts for approximately 8,000 deaths in England and Wales per annum in males over 55 years and it is now the 13th commonest cause of death in the Western world[28].

1.4.2 Prevalence

With the increase in incidence and an ageing population the prevalence of AAAs (the total number of AAA cases existing at a given moment in time) has increased[47]. Estimating the prevalence and incidence of AAA within a population is difficult to measure accurately due to the asymptomatic nature of the disease. However, ultrasonic screenings of the male population throughout England has estimated a prevalence of between 1.3-12.7%[44]. Autopsy reports have estimated male AAA prevalence to be between 2.3-4.3%[47-49]. Table 1-3 shows the prevalence of large AAAs (>40mm) in males in England over the age of

Table 1-3. Prevalence of AAAs in screening surveys of the male UK population

Location	AAA diameter (mm)	Age (years)	Number of patients screened	Prevalence (%)
Large AAA: >40mm				
Northumberland[50]	>49	65-79	628	1.6
Huntingdon[51]	≥46	≥50	3,030	1.3
Oxford[52]	>40	65-74	426	2.3
Gloucestershire[53]	>40	65	4,232	1.3
Birmingham[54]	>40	65-75	2,669	3.0
Small AAA: >29mm				
Northumberland[50]	>29	65-79	628	6.7
Huntingdon[51]	≥30	≥50	3,030	5.2
Huntingdon[44]	>29	≥50	7,493	5.2
Oxford[52]	*	65-74	426	5.4
Birmingham[54]	>29	65-75	2,669	8.4
Chichester[55]	>29	65-80	2,342	7.6

*5mm greater than the diameter of the suprarenal aorta.

50 years to be between 1.3-3.0% and small AAAs (29–40mm) to be prevalent in 5.2-8.4% of males.

With the male predominance of AAAs fewer studies have been conducted on the prevalence of AAAs in females. The estimated prevalence of AAAs (>30mm) in women is between 0.6-1.3%[55-57].

1.5 Aetiology

AAAs have several distinct aetiologies which can be grouped either as congenital or acquired. Most aneurysms are classified as acquired and result either from connective tissue disorders, trauma, infection, inflammatory disorders, genetics or a non-specific, degenerative disease. The majority of AAAs are non-specific and this thesis concerns itself with this latter condition.

1.5.1 Congenital

AAAs in infants are uncommon although they are usually acquired through infection, iatrogenic trauma, vasculitis, a connective tissue disorder or tuberous sclerosis. True congenital "primary" neonatal AAA is exceedingly rare with unknown aetiology[58]. In 2001, Mehall *et. al.* reported only the fifth case of a congenital AAA in a male infant of six weeks old with a 60mm diameter infrarenal AAA[59].

1.5.2 Connective Tissue Disorders

AAAs are associated with two inheritable disorders of connective tissue: Marfans syndrome and Ehlers-Danlos syndrome type IV.

Marfans syndrome is a systemic disorder of connective tissue caused by mutations in the fibrillin-1 gene[60]. The clinical manifestations of this disorder are dislocation of the ocular lens, long-bone overgrowth and aortic aneurysms, typically of the aortic root. It is inherited in an autosomal dominant manner and begins in the first or second decade of life. It leads to premature death caused by aortic rupture or dissection[61, 62].

Normally fibrillin-1 protein monomers aggregate to form microfibrils which surround a core of elastin to produce elastic fibres[63]. This provides elastic arteries and the aorta with extensibility. In Marfans syndrome the mutation in fibrillin results in a decreased elastin content and fragmentation of the elastic fibres within the aortic wall. Apart from the structural role of fibrillin, it has been suggested that fibrillin may have a dynamic role in the regulation of the proteolytic activity within the extracellular matrix. It has been suggested that fibrillin is involved in the regulation of transforming growth factor-β (TGF-β), which in turn has a role in matrix metabolism[61, 62].

Ehlers-Danlos syndrome represents a rare group of connective tissue disorders but only type IV is associated with arterial rupture. This vascular form of Ehlers-Danlos syndrome is caused by mutations in the type III procollagen gene leading to reduced quantities or abnormalities of the protein. The reduction in collagen results in arterial dissection or rupture[62].

1.5.3 Trauma

As mentioned in *1.2.3 Morphology*, trauma to the vessel wall can lead to the development of a false aneurysm. The site most commonly affected is the femoral artery after invasive procedures. A false aneurysm occurs in 0.1% to 0.2% of diagnostic angiograms and 3.5% to 5.5% of interventional procedures[64]. Longer procedures, large-bore catheters, anticoagulation and a poor puncture technique are associated with a higher incidence of false femoral aneurysms. False aneurysms of the aorta are rare and usually associated with previous aortic bypass surgery, but may occasionally occur from a non-medical penetrating injury.

1.5.4 Infection

Infected (mycotic) aortic aneurysms are uncommon. Before 1960 infected aortic aneurysms were reported to represent 2.6%-3.4% of all aortic aneurysms[65, 66]. Since then the frequency of infected aneurysms has fallen to between 0.5%-1.3%[67-69]. The aetiology has also changed. In the preantibiotic era, the most common cause of infected aortic aneurysm was bacterial endocarditis with *Streptococcus pyogenes*. In modern times, intravascular procedures and depressed immunocompetence have become more common risk factors[70]. Concomitantly, with the decrease in *Streptococcus* infections

Staphylococcus aureus and *Salmonella sp.* have become more frequent within infected aneurysms[71].

As will be discussed in *Chapter 2.5.2 Inflammation, Chlamydia pneumoniae* has also been found within the aortic wall of some AAA specimens[72, 73] and has been proposed as a mechanism involved in the initiation of AAA formation.

Syphilis was once a major cause of infected aneurysms. With the decrease in the incidence of syphilis the prevalence of syphilitic aneurysms has accordingly decreased. Syphilis is caused by *Treponema pallidum* and in tertiary syphilis this results in obliterative endarteritis of the vasa vasorum leading to aneurysm formation.

Aortic aneurysms are a rare complication of tuberculosis. With the worldwide resurgence of tuberculosis, due to antibiotic resistance, the clinical presentation of this rare condition may increase in the future[74].

1.5.5 Inflammatory Disorders

Inflammatory abdominal aortic aneurysms (IAAA) form 2.5-10.0% of all aneurysms[75]. An IAAA was first described in 1972 by Walker *et. al.* as a distinct form of aneurysm with a "thick, firm, smooth wall... which is shiny white in appearance"[76]. An IAAA is defined by the triad of a thickened aneurysm wall, extensive perianeurysmal and retroperitoneal fibrosis and dense adhesion to adjacent abdominal organs[77]. The aetiology of the IAAA is not fully understood and there is some controversy over whether the IAAA truly is a distinct clinical and pathological entity or simply an inflammatory variant of the non-specific aneurysm[78].

Other inflammatory disorders of the aorta which result in aneurysm formation include polyarteritis nodosa, Kawasaki and Takayasu's arteritis. The aetiology of these conditions is incompletely understood.

1.5.6 Familial Genetics

In 1977, Clifton reported the first case of familial clustering of AAAs[79] and it is now known that some AAAs have a familial component to their aetiology.

Formal segregation studies have shown that AAAs are likely to be inherited in an autosomal dominant or recessive manner. Kuivaniemi *et. al.* investigated familial inheritance in 233 families and reported that 72% of the families appeared to show autosomal recessive inheritance, whereas 25% showed autosomal dominant inheritance and in eight families the familial aggregation could be explained by autosomal dominant inheritance with incomplete penetrance[80]. This variation in the mode of inheritance would suggest that the various familial clusters result from different genes.

Candidate genes that have been investigated include those encoding for type III collagen, matrix metalloproteinases (MMPs), protease inhibitors, angiotensin converting enzyme (ACE) and various cytokines. It seems unlike that a single gene is responsible for the initiation of AAA formation. Whilst the genetic component may render the individual susceptible to the disease, AAA aetiology is likely to be a complex, multifactorial process involving environmental and genetic factors. A detailed discussion of the role of genetics in the development of AAAs is given in *Chapter 2.5.3 Genetics*.

1.5.7 Non-specific, Degenerative Disease

Previously aneurysms were termed as "atherosclerotic" based on the considered aetiology of the disease. It was thought that atherosclerosis caused the arterial wall to weaken, resulting in the dilatation of the vessel in response to arterial pressure. The basis for this assumption was that the aortic wall of the aneurysm is frequently affected by atherosclerosis and that aneurysmal disease shares some of the risk factors associated with arterial occlusive disease (AOD) (smoking, male sex, advanced age, hyperlipidaemia and hypertension). However, the majority of patients who develop AOD do not develop an aneurysm. Other discrepancies are that operable aneurysm patients are generally ten years older than those with occlusive disease[81] and that diabetes, which is a major risk factor for AOD, is rare in AAA patients[82]. Subsequently it has been shown that biochemically these diseases differ with regards to inflammatory and proteolytic mediators[83-87]. These discrepancies have resulted in the realisation that these are distinct pathological processes and these aneurysms are now termed "non-specific".

1.6 Risk Factors

Risk factors associated with AAAs are advanced age, male sex, family history, hypertension, smoking, previous cardiovascular disease and hypercholesterolaemia (Figure 1-3). Diabetes and chronic limb ischaemia are negatively associated[44, 88-90].

1.6.1 Advanced Age

Non-specific, degenerative AAAs are rare below the age of 50 and then the incidence and prevalence of AAAs increases steadily with age[51, 55]. Vardulaki *et. al.* reported that the incidence of AAA in the male population at the age of 50 years was 0.08%, which increased to 0.67% by the age of 65 years. The annual incidence of AAA was found to fall after 65 years, however the prevalence continued to rise with age[91]. Morris *et. al.* found that between the ages of 50-64 years 2.3% of the male population had an AAA ($\geq$30mm), which increased to 8.8% of the male population aged 65-79 years and in the population aged over 80 years it affected 11.9% of males[51].

1.6.2 Male Sex

The prevalence of AAAs in males is 3 to 6 times higher than in females[55, 92, 93]. Vardulaki *et. al.* reported that the prevalence of AAAs was 6.8% in males compared to females at 1.1%[93]. A recent meta-analysis of population based screening studies showed that male sex was strongly associated with AAAs with a combined odds ratio of 5.69[88]. The aneurysm detection and management (ADAM) veteran affairs cooperative study group determined that female sex was negatively associated with AAAs, with an odds ratio of 0.22 (CI, 0.07-0.68)[89]. Interestingly, in necropsy studies the age standardised mortality rate of AAA is only twice as high in men than women[94]. Whilst AAAs are more prevalent in males it has been suggested that the risk of rupture is greater in women[26] and women are more likely to die from rupture[95]. With increasing age the male to female ratio of death from aneurysm rupture decreases[95].

1.6.3 Family History

As discussed in *1.5.6 Familial Genetics*, a family history of AAA is positively associated with AAAs. Screening studies have shown that there is a high prevalence of AAAs amongst first-degree relatives of AAA patients, with up to 18% of brothers and 5% of

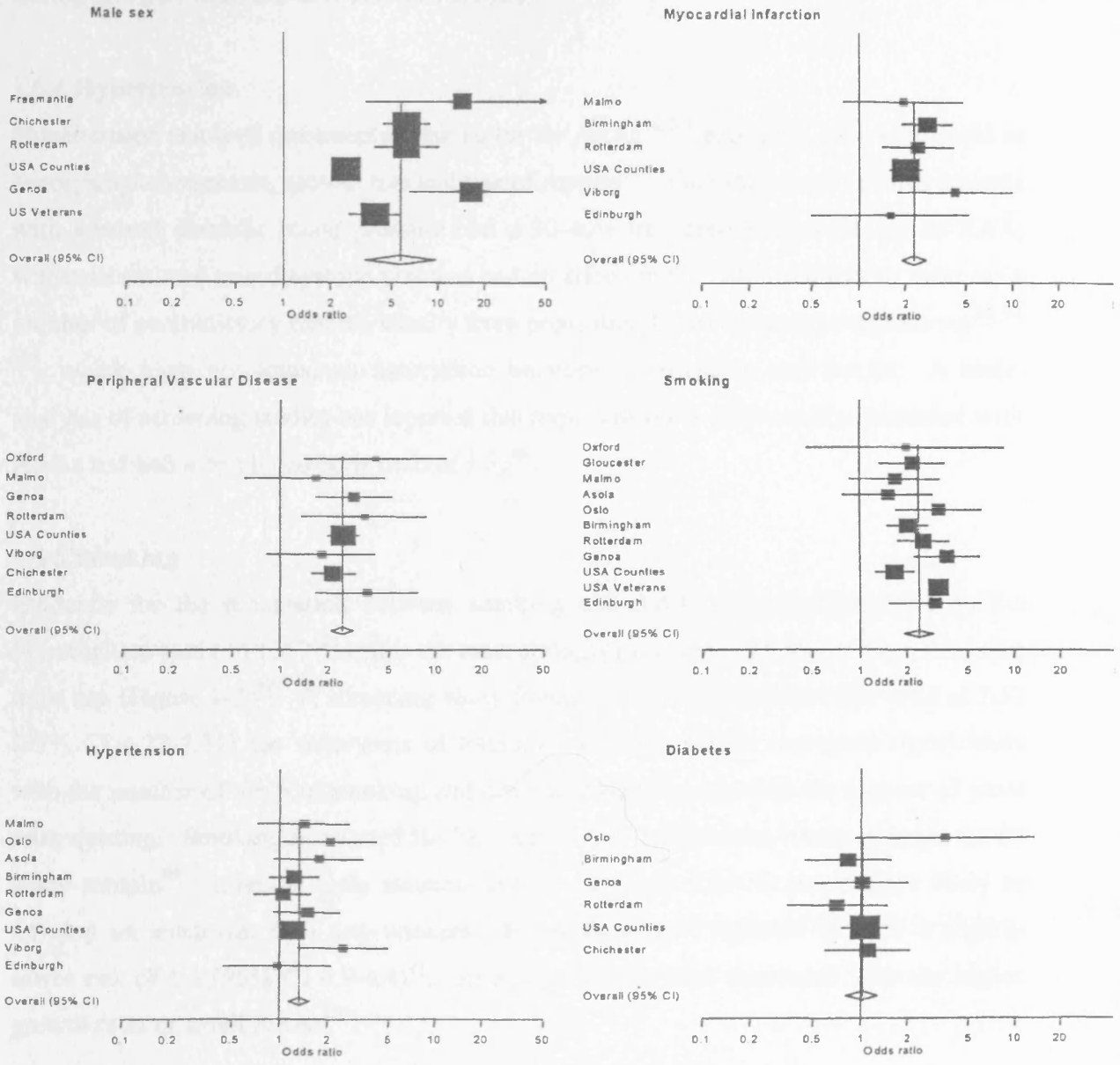

Figure 1-3. Forest plot of studies of risk factors for AAAs. Black squares indicate odds ratio and 95% confidence intervals from individual studies and diamonds represent combined odds ratio and 95% confidence intervals from random-effects models. The size of the black squares are proportional to the weight of the study in the analysis[88].

sisters having an aneurysm[80]. The odds ratio of a first-degree relative of an AAA patient having an AAA themselves is between 2.6-4.4[96-98].

1.6.4 Hypertension

Hypertension is a well documented risk factor for AAAs[99-101] and it has been implicated in aneurysm pathogenesis, growth rate and risk of rupture[102]. One study reported that patients with a raised diastolic blood pressure had a 30-40% increased risk of having an AAA, whilst an isolated raised systolic pressure had no affect on the risk[93]. However, there are a number of contradictory reports, usually from population based screening programmes[54, 98, 103], which have not found an association between hypertension and AAAs. A meta-analysis of screening studies has reported that hypertension is only weakly associated with AAAs and had a combined odds ratio of 1.33[88].

1.6.5 Smoking

Evidence for the association between smoking and AAAs was first provided by the Framingham study in 1967[104] and is the most strongly associated risk factor for AAAs after male sex (Figure 1-3)[88]. A screening study found that smoking had an odds ratio of 5.57 (95% CI 4.27-7.31) for aneurysms of >40mm. This association increased significantly with the number of years of smoking and decreased significantly with the number of years after quitting. Smoking accounted for 78% of all AAAs that were 40mm or larger in the study sample[89]. Lifetime male smokers are 2.5 (95% CI 1.5-4.4) times more likely to develop an aneurysm than non-smokers. Ex-smokers were reported to have a slightly lower risk of 1.5 (95% CI 0.9-4.4)[93]. Smoking has also been associated with the higher growth rates of small AAAs[105].

1.6.6 Cardiovascular Disease

Patients with cardiovascular disease and peripheral vascular disease are twice as likely to have an AAA than those without[44]. Cornuz *et. al.* reported a moderate association of myocardial infarction (odds ratio 2.28) and peripheral vascular disease (odds ratio 2.50) with AAAs[88]. A screening programme of 2,669 males aged 65-75 years found that patients with AAAs had a significant increase in ischaemic heart disease (21.9% vs. 11.6%), history of myocardial infarction (18.3% vs. 7.4%) and peripheral vascular disease (13.2% vs. 8.0%) compared to males without AAAs[54]. A case-control study of AAA prevalence in

patients with coronary artery disease showed that the prevalence of AAAs was greater in the coronary artery disease group than in the age-matched control group (14% vs. 3%)[106].

1.6.7 Hypercholesterolaemia

Some evidence suggests that AAA are associated with raised lipids[89, 98, 107, 108]. Singh *et. al.* reported, in a population-based study of 6,386 males and females, that a low serum level of high density lipoprotein was associated with an increased risk of AAAs[107]. However, other studies have not found an association with hypercholesterolaemia[44, 100, 109]. Blanchard *et. al.* reported that there was no association between AAAs and clinical hypercholesterolaemia, serum levels of total cholesterol, low density lipoprotein or high density lipoprotein[109].

1.7 Clinical Presentation

An AAA may present as either asymptomatic, symptomatic or ruptured. Approximately three quarters of AAAs are asymptomatic at initial diagnosis[17] and, in the absence of a local screening programme, are found incidentally during routine examination or through the patient's observation of a pulsatile mass in the abdomen. Aneurysms become symptomatic when they expand rapidly or leak and pain indicates an increased likelihood of rupture. Symptomatic AAAs may cause symptoms through the pressure they exert on surrounding structures and present as vague abdominal or back pain. Alternatively, symptoms may result from embolisation of mural thrombus from the AAA causing ischaemia or infarcts of the lower limbs. As described in *1.3. Natural History of the Abdominal Aortic Aneurysm*, the final stage in AAA expansion is rupture. The diagnostic triad of a ruptured AAA is hypovolaemic shock, pulsatile abdominal mass and abdominal or back pain. If the rupture occurs into the peritoneal cavity or results in a large haemorrhage the patient may present with circulatory collapse or sudden death. Approximately 50% of patients with ruptured aneurysms die before reaching hospital. The mortality rate for emergency repair is between 30-70% and therefore the overall mortality rate for a ruptured aneurysm is between 65-85%[110].

1.7.1 Diagnostic Methods

Routine screening for AAAs is performed using B mode ultrasonography, which gives an accurate assessment of both diameter and site of aneurysm. The image acquired can be affected by excessive bowel gas and is less clear in obese patients. Ultrasound measurements are reproducible within a range of 6mm, however it may not yield sufficient detail of the visceral and renal vessels for it to be used for aortic reconstructive surgery. A more detailed image of the AAA and its morphology can be obtained through computed tomography or magnetic resonance angiography[17, 111].

1.7.2 Screening

With the risk of mortality associated with rupture being between 65-85%[110] compared to a 30-day operative mortality risk of 5-6% for elective repair[7] it has been debated if there should be a nationwide screening programme of the at risk group, i.e. males over the age of 65 years.

The purpose of a screening programme is to reduce aneurysm-related deaths by identifying AAAs before they rupture and to treat electively those aneurysms deemed at risk of rupturing. The effect of screening on mortality was recently assessed by the multicentre aneurysm screening study (MASS) as a randomised controlled trial of males between the ages of 65-74 years. Comparison of the screened group to the non-screened control group showed a reduction of 53% in aneurysm-related deaths in the screened group over a mean period of 4.1 years[110].

Within the Leicestershire region screening is offered as a one-off screening to males at the age of 65 years. Those males with aortas around 25mm are not screened again. It has been shown that males over the age of 65 years with an aortic diameter <26mm do not significantly develop a clinically relevant AAA over a 12 year period[112].

1.7.3 Treatment

At present the only treatment available is elective surgery to prevent further AAA growth and rupture. The decision to treat an asymptomatic aneurysm occurs when the risk of rupture outweighs that of operative mortality. Surgery is required once the AAA is

≥55mm, or has an expansion rate of ≥10mm per year, or displays symptoms; providing that the patient is fit enough to undergo surgery.

With screening programmes identifying a large number of small, asymptomatic aneurysms it has been debated if small aneurysms should be immediately repaired or monitored until they reach an operable size. Two prospective trials, the UKSAT and the ADAM study, have determined that survival is not improved by elective repair of small aneurysms and that ultrasonographic surveillance for small aneurysms is safe[7, 30].

Open Repair

Conventional "open" repair involves major abdominal surgery with the exposure of the AAA, clamping proximally and distally to the aneurysm and replacement of the aneurysmal section with a prosthetic graft. Most centres report a 30-day mortality of 6%[110]. The mortality associated with open repair is affected by the cardiovascular fitness of the patient and other pre-existing co-morbidities. Complications arising from open repair include myocardial infarction, multi-organ failure, graft infection and lower limb ischaemia due to embolisation. The physical trauma of the laparotomy and ischaemia induced by aortic cross-clamping alone means that in a fit patient it will be several months before they are restored to full health.

Endovascular AAA Repair

The alternative surgical treatment with minimal invasiveness is EVAR. This procedure involves the placement of a stent-graft inside the aneurysm sac via the femoral arteries to exclude the aneurysm from the circulation. EVAR has the advantage that it avoids a major laparotomy, is less physically traumatising so that the majority of patients do not require time in intensive care, patients mobilise earlier and on average are discharged in less than 4 days. This type of repair may be better for patients with co-exisiting disease, who would be considered to be at high risk from open repair.

The question of the long-term prospects of patients who have EVAR has recently been addressed through the randomised controlled EVAR I and II trials[9, 113]. The EVAR I trial randomised patients physically suitable for EVAR to receive either an open repair or an

EVAR. The results showed that EVAR resulted in a 3% reduction in aneurysm-related mortality compared to open repair and this reduction was sustained at 4 years[9].

The benefits of EVAR for those patients deemed unfit for open repair compared to non-intervention was assessed through EVAR trial II. At the end of the four year follow-up period EVAR had not improved survival over non-intervention and was associated with a need for continual surveillance and reinterventions[113].

1.7.4 Prognosis

Whilst successful aneurysm repair extends the patients life expectancy it does not return the patient to the survival rate of the normal age- and sex-matched population[114-116]. A Norwegian study found that the 10-year survival rate for all patients after operation was 38%, compared with an expected rate of 52%. The standardised mortality was approximately 30% higher in the operated group than in the demographically matched population. Older patients and those with known cardiac disease had the lowest long-term survival[114]. Late deaths from cardiovascular or cerebrovascular causes are more frequent in AAA patients with repaired AAAs than in the age- and sex- matched population[116]. This may be because AAAs are positively associated with smoking and hypertension and AAA patients frequently have co-existing morbidities such as coronary heart disease. Therefore patients who develop AAAs and undergo repair tend to be less healthy than the normal population, have a reduced life expectancy and often die from a cardiovascular event unrelated to the AAA following successful AAA repair[44].

1.7.5 Pharmacological Therapy

Screening programmes are identifying a large number of small AAAs which are treated on a "wait and see" basis. Currently there is no alternative therapy to surgery, however it would be desirable if a drug therapy could be used to reduce or halt AAA growth and prevent rupture. Previous attempts at drug therapy have used β-blockers, non-steroidal anti-inflammatory drugs (NSAID) and MMP inhibitors.

Animal and human studies have suggested that the β-blocker propranolol may decrease the growth rate of small aneurysms[117, 118]. Propranolol delays the formation of aortic aneurysms in the blotchy mouse aneurysm model. In this model propranolol decreased the

solubility of the dermal connective tissue, suggesting propranolol has a direct effect on connective tissue metabolism[119]. In the hypertensive rat model, propranolol reduced the size of experimental AAAs[118]. However, this treatment proved to be untenable in human trials. One trial was stopped after two years because of statistically high mortality in the propranolol group and prior to this 60% of the propranolol group had had to stop treatment. The study was unable to conclude whether AAA expansion had been reduced in the propranolol group due to the small number of participants remaining in the trial[120]. A larger study found that the annual AAA growth rate was similar between the propranolol and the placebo groups. Additionally, the side effects of propranolol were not well tolerated, with patients in the propranolol group having a significantly poorer quality of life[121].

Indomethacin, a NSAID, has been shown to attenuate aneurysm growth in animal models[122, 123]. Indomethacin is a non-specific cyclooxygenase inhibitor. Cyclooxygenases (Cox 1 or Cox 2 isoforms) control the synthesis of prostaglandin E2, which regulates the expression of MMP-9. Miralles *et. al.*, using an elastase-induced rat AAA model, showed that indomethacin decreased prostaglandin E2 synthesis, and therefore MMP-9 expression, through inhibition of the Cox 2 isoform resulting in preservation of medial elastin[122]. A recent study of NSAID drugs in a rat aneurysm model found that indomethacin alone statistically decreased MMP-9 expression, however it did not limit aneurysm expansion, suggesting that MMP-9 inhibition was not sufficient to reduce aneurysm expansion[124].

With the known up-regulation of MMPs within the aneurysm wall a number of studies have investigated a variety of MMP inhibitors. Doxycycline, a tetracycline derivative, has been shown to reduce the expression of MMP-2 and MMP-9 by AAA tissue explants[125]. Rat aneurysm model systems have shown that doxycycline treatment diminishes the extent of aortic dilatation, preserves medial elastin and reduces MMP-9 activity. It does not affect MMP-2 expression[126]. A phase II trial of doxycycline found that this drug was well tolerated by patients and was associated with a gradual decrease in plasma MMP-9 levels. However, in a 6-month period no significant changes were noted in AAA diameter[127].

To date there is insufficient experimental evidence to support a suitable pharmacological use for any of these drugs. With a better understanding of the pathogenesis of AAAs a

future therapeutic target may be identified and provide a pharmacological treatment for this condition.

1.8 Summary

The AAA is a common degenerative disease of the elderly, which is most prevalent in Caucasian males between the ages of 65-80 years. An aneurysm is a dilatation of the aorta which progressively expands, often asymptomatically, until it ruptures leading to extensive intra-abdominal haemorrhage, circulatory collapse and death. Aneurysm rupture accounts for 8,000 deaths in England and Wales per annum and is a leading cause of death in the Western world[28].

The only treatment available is elective, prophylactic surgery to prevent further expansion of the aneurysm and remove the risk of rupture. However, this surgery is not suitable for all patients and there is a risk of post-operative complications, including mortality. There is a need for an alternative medical treatment but previous attempted drug therapies have not been successful. In order to design a suitable treatment a greater understanding of the pathology of the disease is necessary.

Chapter Two

Pathogenesis of Abdominal Aortic Aneurysms

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2.1 Anatomy of the Aorta

The aorta is the main artery of the body and acts as a conduit carrying oxygenated blood out of the heart and distributing it around the body via branching-off vessels. It exits the heart from the left ventricle and traverses from the thoracic area down towards the pelvis, where it terminates at the bifurcation when the aorta divides to become the left and right common iliac arteries. The aorta is sub-divided into the ascending, aortic arch, descending, thoracic and abdominal aorta.

2.1.1 Abdominal Aorta

The abdominal aorta begins at the aortic hiatus of the diaphragm, anterior to the twelfth thoracic vertebra, and descends to the fourth lumbar vertebra where it ends at the aortic bifurcation.

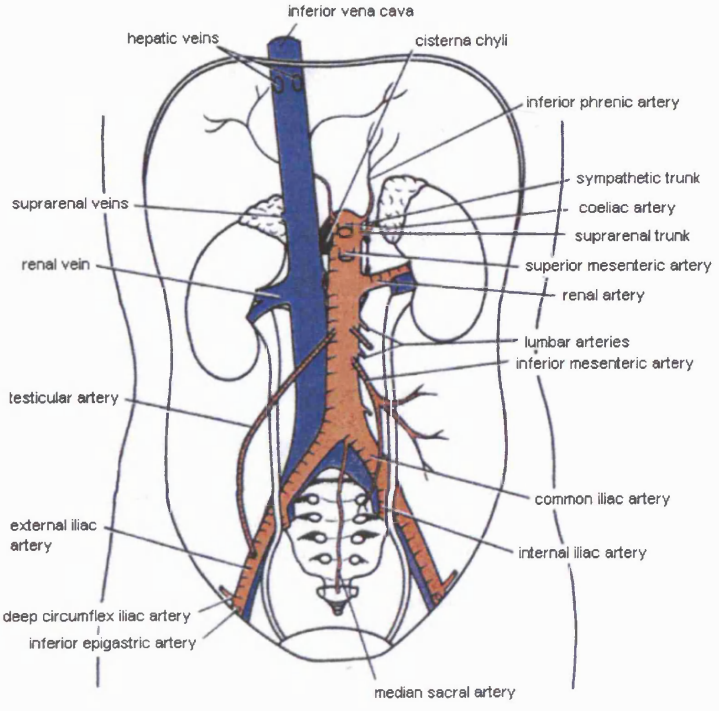

Figure 2-1. Diagrammatic representation of the abdominal aorta and its branches[128].

The abdominal aorta tapers as it descends reflecting the number of large branches emanating from it. Anteriorly branching from the abdominal aorta are three visceral arteries: the coeliac artery, superior mesenteric artery and inferior mesenteric artery. Laterally branching from the abdominal aorta are a further three visceral arteries: the suprarenal artery, renal artery and testicular/ovarian artery. Also laterally branching are abdominal wall branches - the inferior phrenic artery and four lumbar arteries. The abdominal aorta terminates when it branches to form the two common iliac arteries and the median sacral artery[128]. The branching of the abdominal aorta is depicted in Figure 2-1.

2.2 Histology of the Aorta

The aorta is classed as an elastic artery due to the large quantity of elastin within its wall. Rather than just functioning as a conducting tube the aorta also facilitates the continuous movement of blood down the arterial tree through passive distension during systole and elastic recoil during diastole. To perform this function the three-dimensional structure of the aortic wall confers the vessel with tensile strength and elasticity. In common with all elastic arteries the aortic wall is arranged into three concentric layers: the tunica intima, the tunica media and the tunica adventitia (Figure 2-2).

2.2.1 Tunica Intima

The tunica intima is the innermost layer of the aorta and surrounds the lumen with a single cellular layer of endothelial cells, which are anchored on a thin basal lamina. The endothelium controls the passage of substances to and from the lumen by restricted transendothelium diffusion through tight junctions and by pinocytosis. The subendothelial layer of the intima is comprised of connective tissue components, such as collagen and elastin. The main cell type of the subendothelium is the smooth muscle cell (SMC), which secretes extracellular matrix (ECM) components. Fibroblasts are also present. The tunica intima is separated from the tunica media, the central layer, by the internal elastic lamina[129].

2.2.2 Tunica Media

The tunica media is the thickest layer in a healthy aorta, containing a large amount of elastin and is chiefly responsible for the elasticity of the aorta. It is a highly organised layer of connective tissue with concentric elastic lamellae embedded between layers of SMCs. Collagen fibres are arranged in a regular alignment with the elastic lamellae to provide the aorta with the properties of high tensile strength. There are no fibroblasts present in this layer, therefore the SMCs alone are responsible for the maintenance of the medial ECM. Fenestrations in the lamellae facilitate the diffusion of substances through the arterial wall. In the adult the aorta has between 40-70 lamellae; the number of elastic lamellae decreases along the descending length of the aorta[129]. The tunica media is separated from the tunica adventitia by the external elastic lamina.

2.2.3 Tunica Adventitia

The tunica adventitia is the external layer of the aorta and is less than half the thickness of the tunica media. It consists of connective tissue but is less well organised than the tunica media. The adventitia consists predominantly of collagen fibres, although some elastic fibres are present. The elastin found in the adventitia is present as elastic fibres, not in structured lamellae. The collagen fibres in the adventitia serve to prevent the expansion of the aortic wall beyond its physical limits during systole. The principal cells of this layer are fibroblasts and macrophages. Being to far from the lumen to be supplied by pinocytosis the adventitia is supplied by its own blood vessels, *vasa vasorum*, and nerves, *nervi vascularis*[129].

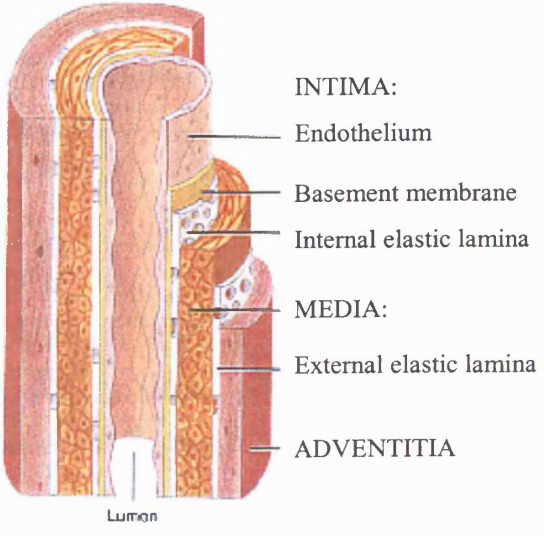

Figure 2-2. The layered structure of the aorta[129]

2.3 Extracellular Matrix of the Aorta

The layers of the aorta consist of a variety of cells surrounded by an extensive ECM. ECM is an intricate network of proteins and polysaccharides found in the extracellular space which are assembled into an organised meshwork. The ECM in the vascular wall acts as a supporting and structural framework. It provides a scaffold for the anchorage of vascular cells, determines the physical properties of the aorta and has roles in vessel maintenance. The macromolecules that constitute the ECM are mainly produced locally by endothelial cells, vascular SMCs and fibroblasts within the matrix. The two main component of the ECM of the aorta are the structural fibrous proteins, elastin and collagen, which account for the main physical properties of the aorta. Additionally, proteoglycans form a hydrated gel-like "ground substance" in which the fibrous proteins are embedded.

2.3.1 Elastin

Elastin is the most abundant protein of the aortic wall being the major component of the elastic fibres, which forms the elastic lamellae in the media. It is responsible for aortic distensibility during systole without damage to the vessel and the elastic recoil of the aorta during diastole[130].

Elastin is a highly insoluble protein and is composed largely of two alternating sequences along the length of the polypeptide: a hydrophobic sequence, which is responsible for the elastic properties of the molecule, and an alanine- and lysine-rich sequence, which forms the cross-links between adjacent elastin molecules[131]. Elastin is initially secreted as tropoelastin, a soluble precursor of elastin, into the extracellular space where desmosine and isodesmosine cross-links form to generate a network of insoluble elastic fibres. The cross-links are formed by the initial action of lysyl oxidase on lysine residues of the tropoelastin[132].

Elastin comprises 90% of the protein content of the elastic fibres. The elastic fibres are formed from a central core of elastin surrounded by a sheath of microfibrils. The microfibrils are composed of a number of distinct glycoproteins, including fibrillin[63]. The importance of fibrillin in elastic fibre integrity is demonstrated by patients with Marfans Syndrome. As discussed in *Chapter 1.5.2 Connective Tissue Disorders* these patients are defective in the fibrillin-1 gene and are prone to aortic dissection or aneurysm rupture. The

microfibrils are thought to be important in the assembly of elastic fibres by providing a scaffold for the deposition of secreted elastin[131].

2.3.2 Collagen

The tensile strength of the aorta, which prevents over distension during systole, is provided by the collagen network. Initially, the load on the aortic wall is borne by elastin, with the collagen fibres coiled up, resulting in a highly distensible vessel. However, with increasing load the elastic limit of the vessel wall is approached. To avoid potential wall damage the collagen fibres uncoil as the wall is stretched and progressively the load is borne by these fibres, preventing further distension of the aorta[130].

Collagen exists in various isoforms. Between 80-90% of the collagen found in the aortic wall consists of types I and III, with the remainder consisting of types IV, V, VI and VIII. Collagen type I predominates in the aorta and represents approximately 60% of the aortic collagen, whilst type III accounts for approximately 30%[132]. Both these collagen isoforms are present throughout the layers of the aorta[133].

The basic unit of fibrillar collagen is a 300nm triple helix. The triple helix is secreted as procollagen into the extracellular space and converted to collagen molecules through the cleavage of N- and C- terminal propeptides. The collagen molecules aggregate to form quarter-staggered fibrils. These newly synthesised collagen fibrils are soluble and have no tensile strength. These fibrils aggregate into larger collagen fibres and the mature collagen is stabilised by the formation of pyridinoline and deoxypyridinoline cross-links, initiated by the lysyl oxidase enzyme. The cross-linking of mature collagen results in an insoluble collagen network with high tensile strength[131, 132].

2.3.3 Proteoglycans & Structural Glycoproteins

Glycoproteins present within the vascular wall include: fibrillin, fibronectin, vitronectin, laminin, tenascin and thrombospondin[132]. Many of these glycoproteins have an adhesive role by simultaneously interacting with cells and the ECM. For example, fibronectin promotes the attachment of fibroblasts and various other cells to the ECM and laminin supports the attachment of epithelial cells to the basal lamina in the intimal layer[131]. As mentioned, fibrillin is involved in the formation of elastic fibres.

Proteoglycans consist of protein covalently linked to a class of polysaccharide chain known as the glycosaminoglycans. The proteoglycans provide the ground substance in which the elastic lamellae and collagen fibres are embedded and it is this polysaccharide gel that enables the matrix to resist compressive forces. Some of the proteoglycans present within the vascular wall are: aggrecan, versican, biglycan, decorin, fibromodulin, fibroglycan and glypican. The aqueous phase of the polysaccharide gel permits the diffusion of nutrients and metabolites through the ECM. These molecules also regulate cellular activities such as proliferation, migration, adhesion, differentiation and control the availability of cytokines stored in the ECM[131, 132].

2.3.4 Cells of the Extracellular Matrix

Not only are the structural properties of the ECM components important for the aorta to withstand systolic pressure and elastic recoil, but the orientation of these proteins is also important in achieving optimal functionality. Vascular SMCs and fibroblasts are essential for the maintenance of the ECM through the synthesis and secretion of the ECM components: collagen, elastin, laminin, gelatin and proteoglycans[134]. However, these cells are also important in controlling the orientation of the matrix components and therefore the overall three-dimensional structure of the ECM. The orientation of secreted ECM proteins is influenced by the orientation of the cell's cytoskeleton[131]. Whilst these cells have the ability to synthesis new ECM components in response to vascular injury, the deposition of the components is such that over time the optimal three-dimensional organisation of the ECM is lost and correspondingly the wall is weakened[132].

2.4 Pathological Changes in the Abdominal Aortic Aneurysm

The AAA is characterised by a structural disorganisation and loss of elastic lamellae, a decrease in medial SMC density and an intense inflammation of the adventitia. The inflammatory process is characterised by infiltration of the adventitia by mononuclear phagocytes and lymphocytes.

2.4.1 Elastin Degradation

Alterations in the elastin and collagen composition of the ECM was first described in 1970 by Sumner *et. al.* who reported a reduction in the elastin and collagen content of AAAs

compared to normal and atherosclerotic aortas[135]. It is now well established that an extensive loss of elastin occurs in AAAs[136-139] (Figure 2-3, *EVG*). Quantitative analysis of elastin in the healthy aorta showed that elastin accounted for 35% of the dry weight of the media whilst in AAAs this was reduced to 8%[136]. Carmo *et. al.* demonstrated that the desmosine and isodesmosine cross-links between elastic fibres were decreased by 90% in AAA samples compared to non-aneurysmal aorta and this corresponded with a 91% decrease in elastin[140]. Gandhi *et. al.* reported that the ratio of cross-links to the amount of elastin present was not significantly different between the AAA samples and the controls[138]. These reports suggest that AAAs do not suffer from an elastin cross-linking deficiency but simply a degradation of elastin. As mature elastin is not synthesised in the adult aorta, and elastin has a half-life of approximately 70 years[130], damage to the elastin content cannot be rectified.

Loss of elastin is considered to be an early event in AAA formation[141, 142]. Dobrin *et. al.* found that elastase treatment of arterial explants caused segments to dilate without rupturing and decreased vessel distensibility. In contrast, collagenase treatment of

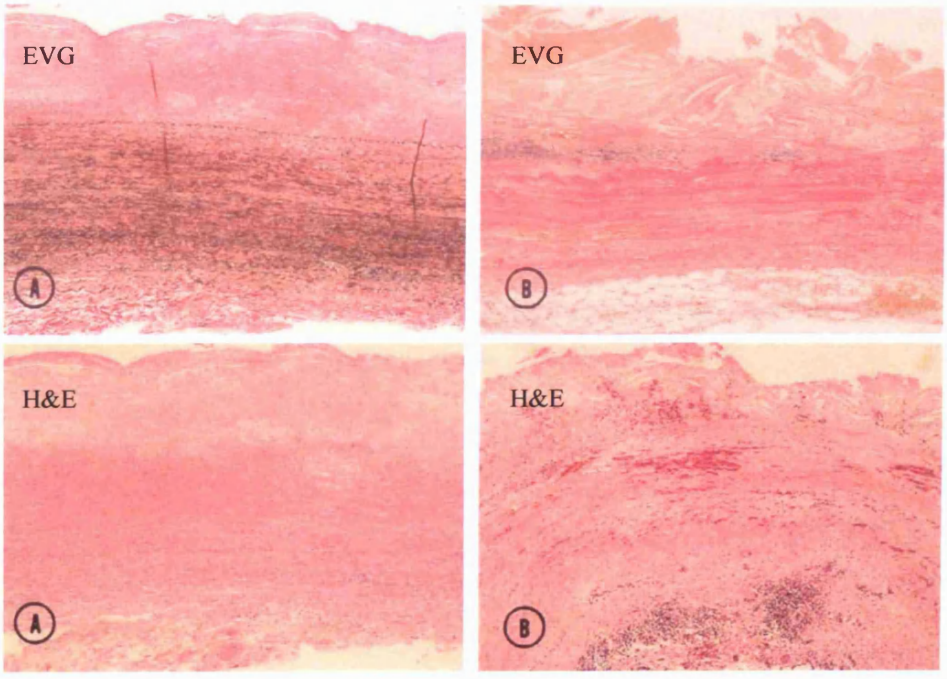

Figure. 2-3. Structural changes of the AAA wall. Elastin van Gieson (EVG) staining of the infrarenal aorta from (A) non-aneurysmal aorta and (B) AAA shows that the elastic fibres (stained black), which are layered in the control, are notably absent in the AAA. The haematoxylin & eosin (H&E) staining of the (A) control aorta and (B) AAA shows the inflammatory infiltrate found within the aneurysmal adventitia[137].

arterial segments resulted in increased vessel distensibility and wall rupture[143]. These results suggested that with the loss of elastin the arterial load is transferred onto the collagen network resulting in decreased distenstibility with the collagen preventing over expansion of the vessel. Dobrin *et. al.* concluded wall integrity depended on intact collagen, rather than elastin, and in order for the vessel to rupture it required the mechanical failure of the collagen network[143, 144].

2.4.2 Increased Collagen Metabolism

Unlike elastin, collagen is synthesised throughout life and the collagen content represents the net effect of synthesis and degradation. The total collagen content of the AAA wall has been reported to be reduced[135, 140], unaltered[138] or elevated[137, 145, 146]. Carmo *et. al.* observed a decrease in total collagen content, whilst there was an increase in collagen cross-linking. They suggested that whilst mature collagen accumulated, as shown by the increase in cross-links, the synthesis of new collagen was somehow defective leading to an overall loss of total collagen[140]. However, it has been reported that there is an increase in the newly synthesised, type III propeptide in AAAs[147]. Rather than decreasing Bode *et. al.* suggested that the collagen content was maintained, or even increased in AAAs due to an increased rate of collagen synthesis[147]. One study found that the proportion of medial collagen in the AAA wall increased from 62% to 84% of the total volume[146]. An increase in collagen turnover has been supported by messenger RNA (mRNA) studies, which have shown type I and III procollagen to be raised within the AAA[148, 149].

Subsequent to elastin degradation and aortic dilatation it has been suggested that the increase in wall stretch results in an increase in collagen synthesis as a compensatory mechanism[141] and has been observed *in vitro*[150]. A compensatory increase in collagen may stabilise the AAA, but is overcome in the case of AAA rupture. Ruptured AAAs express a higher level of collagenases than non-ruptured AAAs[146]. The mechanical failure of the collagen network in preventing AAA rupture may result from an increased proteolytic degradation of the collagen fibres.

2.4.3 Smooth Muscle Cell Apoptosis

The AAA is characterised by a thinning of the tunica media. In healthy aorta the elastic media is dominated by vascular SMCs, however in AAAs the SMC content of the media is substantially reduced. One study found that the medial SMC density was decreased by

74%[151]. This study also reported that there was no evidence of overt cellular necrosis but that many of the SMCs were apoptotic. Henderson *et. al.* also found a reduction in SMCs and evidence of increased apoptosis in AAAs[134]. They showed that infiltrating T-lymphocytes expressed apoptotic mediators, including FasL. Aneurysmal SMCs were found to express Fas and therefore, potentially, SMC apoptosis could be induced upon FasL-Fas binding[134]. SMCs from AAAs also exhibit altered biochemical properties distinct from other vascular SMCs. Liao *et. al.* cultured AAA and non-aneurysmal inferior mesenteric arterial explants from the same patient and found that the AAA-derived SMC had a diminished proliferative capacity and an accelerated replicative senescence[152]. This could result in a gradual loss of medial SMCs.

In the healthy aorta SMCs are responsible for the maintenance and repair of the ECM. SMCs, as a source of ECM components including collagen, may have a protective function and their loss by apoptosis could contribute to the development of AAAs[134]. However, these cells are also a source of MMP-2[153, 154] and MMP-9[153], suggesting that SMCs could participate in AAA formation. The use of a decellularised guinea pig xenograft transplanted into rats to induce AAA formation demonstrated that aortic seeding with synergistic vascular SMCs prevented AAA formation in the treated rats. The seeded aortas were protected against elastin degradation, displayed a reduction in inflammatory cell infiltration and up-regulated the expression of tissue inhibitors of metalloproteinases (TIMPs). They concluded that SMCs were important for aortic wall homeostasis and that failure of this protective mechanism resulted in AAA formation[155].

2.4.4 Inflammatory Infiltrate

AAAs are associated with a prominent inflammatory infiltrate (Figure 2-3, *H&E*), although the extent of inflammation varies amongst AAAs. Human AAAs demonstrate a large number of T-lymphocytes, B-lymphocytes, plasma cells, neutrophils, macrophages and dendritic cells within the outer media and adventitia[156-158]. Vascular dendritic cells have been shown to be in contact with both T- and B-lymphocytes and may act as an antigen presenting cell (APC) for both cellular and humoral responses within the vascular wall[157, 159]. Phenotypic analysis of the infiltrating T- and B-lymphocytes revealed that both cell types consisted of activated memory cells[160, 161]. Arguments for the source of this activation have suggested an autoimmune reaction[162] or an exogenous antigenic agent, such as *Chlamydia pneumomiae*[163].

The importance of the inflammatory infiltrate in AAA formation has been demonstrated in a murine AAA model where T_{Helper}-lymphocyte (T_H-lymphocyte) knockout mice were protected against aneurysm formation[164]. Similarly, the depletion of neutrophils in a murine AAA model inhibited AAA formation[165]. The possible role of the inflammatory infiltrate within the development of AAAs will be discussed in more detail in *2.5.2 Inflammation.*

2.5 Mechanisms of Abdominal Aortic Aneurysm Formation

The mechanism involved in the development of AAAs remains to be fully elucidated. Previously it was thought to be caused through atherosclerotic weakening of the aortic wall, however it is now considered to have a complex multifactorial aetiology. The four mechanisms that contribute to the induction and growth of AAAs are proteolytic degradation of the aortic wall, inflammation, genetics and biomechanical wall stress (Figure 2-4).

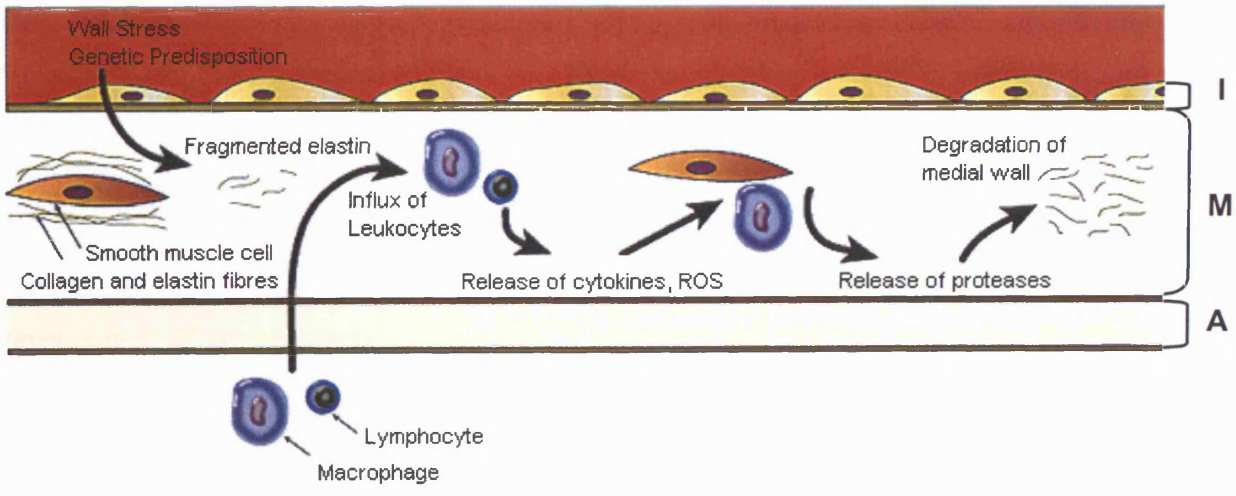

Figure 2-4. A potential mechanism for AAA development[166]. A combination of factors may be involved in the initial stages of aneurysm development, including a genetic predisposition, biomechanical wall stress and fragmentation of elastin, which through an unknown immunological pathway results in the recruitment of inflammatory cells into the aortic wall. The influxing leukocytes release inflammatory mediators, including cytokines, chemokines and reactive oxygen species (ROS). This results in further chemotaxis and induces the inflammatory cells to release and activate proteases, e.g. MMPs. The increase in active proteases leads to degradation of the media and aneurysm dilatation. *I*, intima; *M*, media; *A*, adventitia.

2.5.1 Proteolysis

Wall homeostasis requires the carefully regulated balance between tissue repair and degradation to maintain the optimal structure of the ECM. Loss of regulation of vascular remodelling can lead to AAA development where the degradation of the ECM overcomes the capacities of the repair mechanism. The extensive fibrolytic degradation of the ECM of the AAA wall is attributed to an increase in mural proteolysis.

Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases

MMPs are a family of zinc proteases that have substrate specificity for ECM components and are grouped as: collagenases, gelatinases, stromelysins and membrane type (MT1-MMP) (Table 2-1). MMPs are expressed in normal tissue as part of the natural process of wound healing and maintenance of the ECM. However, the expression of MMPs can be altered in certain pathological conditions, for example: rheumatoid arthritis[167], tumour metastasis and invasion[168, 169]. MMP expression is tightly regulated at three levels:

Firstly, regulation occurs at the transcriptional level and may be regulated by hormones, cytokines and growth factors. Studies have identified the expression of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12 in AAAs[154, 170-172]. However, the key MMPs associated with AAA development have been identified as MMP-2 and MMP-9[153], which are elastolytic and are considered to work in concert to produce AAAs[173]. Petersen *et. al.* demonstrated that MMP-2 levels correlated with aneurysm diameter and suggested it had a role in the early expansion of the unruptured AAA[174]. MMP-9 appears to be responsible for the continued expansion and rupture of AAA and is less prevalent in small aneurysms[174, 175]. Mice with deletion of the MMP-2 or MMP-9 gene are resistant to the development of elastase-induced AAAs[173, 176]. MMP-2 and MMP-9 expression has been localised to vascular SMCs and macrophages, respectively[85, 86].

Secondly, post-translational enzymatic activation of the latent proenzyme is required to produce a fully activated MMP. Sakalihasan *et. al.* found that activated MMP-2 and MMP-9 were significantly higher in the AAA wall than in the controls[177]. *In vivo* the principal activator of pro-MMP-2 is MT1-MMP; whilst the main physiological activator of pro-MMPs is the serine protease, plasmin. Subsequently, the activated MMPs can act directly on pro-MMPs resulting in their activation.

Thirdly, TIMPs inhibit MMPs by irreversibly binding to active MMPs. TIMPs are constitutively expressed within aortic tissue[85, 86], which may reflect their role as key modulators of proteolytic activity. TIMP-1, a major inhibitor of MMP-9, has been reported to be up-regulated two-fold at the protein level in AAAs[171], but was not significantly different at the mRNA level between AAA and AOD specimens[86, 170, 178]. TIMP-2, which complexes with MMP-2, has been reported to be significantly increased in AAAs[178], unaltered[85, 170] or decreased[171]. The overall increase in proteolysis within the AAA wall may reflect an alteration in the MMP/TIMP ratio. In the elastase-induced AAA rodent model deletion of the TIMP-1 gene resulted in a larger AAA compared to the wild-type, suggesting that TIMP-1 has a protective effect on damping down the proteolytic response[179].

Table 2-1. MMPs documented to be altered in AAA in humans[166]

MMPs	Alternative names	Primary substrate	Cells of origin
MMP-1	Collagenase–1, Interstitial collagenase	Collagen (type I, III)	Epithelial, inflammatory
MMP-2	72kDa Gelatinase A	Collagen (type IV), elastin	SMCs, fibroblasts
MMP-3	Stromelysin-1	Collagen	
MMP-9	92kDa Gelatinase B	Collagen, elastin	Macrophages, SMC
MMP-12	Macrophage elastase	Elastin	Macrophages
MMP-13	Collagenase-3	Collagen (type IV)	SMCs
MT-MMP-1	MMP-14	Activates pro-MMP-2	Membrane bound
TIMP-1		Inhibitor of MMPs	
TIMP-2		Inhibitor of MMP-2	

Serine Proteases

Plasmin is initially secreted as plasminogen, an inactive zymogen, and converted to plasmin by the actions of tissue-plasminogen activator (t-PA) or urokinase-plasminogen activator (u-PA). The active plasmin can activate pro-MMPs resulting in a proteolytic cascade and can also have a direct effect on ECM breakdown[180]. This pathway is controlled through the inhibitory actions of plasminogen activator inhibitor-1 (PAI-1),

which inhibits u-PA and t-PA. The targeted deletion of the u-PA gene prevented AAA formation in apolipoprotein E (apo E) deficient mice and in the absence of u-PA there appeared to be a limitation in the plasmin-dependent activation of MMPs[181]. Similarly, the over-expression of PAI-1 prevented AAA dilatation in aortic xenografts[182], a model system which usually results in aortic dilatation. The serine proteases, plasmin, u-PA and t-PA are increased within the AAA wall[87, 183, 184].

Cysteine Proteases

The cathepsins are a family of cysteine proteases. Cathepsin S and K regulate intracellular protein degradation and turnover, and are localised to SMCs and macrophages within atherosclerotic plaques[185]. They are also over-expressed at sites of arterial elastin damage[186]. Cathepsin D, H and L are involved in the degradation of structural proteins and were found to have a greater activity in the AAA wall compared to normal aorta[187, 188].

The major inhibitor of cathepsins is cystatin C. Cystatin C knockout, apo E deficient mice have a greater aortic diameter, increased elastin degradation and an increase in collagen content compared to apo E deficiency alone[189]. Increased human AAA size and expansion are associated with cystatin C deficiency[190].

2.5.2 Inflammation

Inflammation is a complex response to local injury or trauma. The initiation and persistence of inflammatory responses are tightly regulated to ensure an effective response against an infectious agent, minimal damage to the host and rapid clearance of the activated immune cells. In AAAs this control is lost and chronic inflammation results in extensive wall damage.

Initiation of Inflammatory Cell Infiltration

The AAA wall is characterised by a dense inflammatory infiltrate of macrophages and lymphocytes. A report on the phenotypes of the inflammatory infiltrate observed that the lymphocytes present in AAAs were of a different cluster of differentiation (CD) marker phenotype to those lymphocytes in the blood[160]. This suggested that the lymphocytes present in the AAAs were not just a result of extravasation from the blood but were specifically recruited into the adventitia by an unknown stimulus. The mechanism for this

recruitment remains to be elucidated. The following theories for the development of the inflammatory infiltrate have been proposed:

Elastin Degradation Peptides

Elastin degradation peptides (EDPs), resulting from the fragmentation of elastin within the AAA wall, may act as a chemotactic signal to infiltrating neutrophils and monocytes during the early stages of AAA formation[158]. The biological activities of EDPs are mediated through a 67kDa elastin binding receptor. EDPs were identified as having chemotactic properties through an experiment which showed that extracted soluble proteins from AAAs were chemotactic to differentiated mononuclear phagocytes, however if the EDP receptor was blocked chemotaxis was abolished[191].

Autoimmunity

The presence of B-lymphocytes and large amounts of immunoglobulins (IgG variety) which react with microfibrils of the aortic wall may be indicative of an autoimmune response to specific tissue antigens[162]. Haug *et. al.* described an association between autoimmune disease and IAAAs[192]. However, Walton *et. al* found that the repertoire of immunoglobulin variable heavy chain genes expressed by the infiltrating B-lymphocytes was unrestricted[193]. Ocana *et. al.* found that AAA-derived B-lymphocytes were, upon analysis of the kappa and lambda light chains expressed, polyclonal in nature[160]. Furthermore, analysis of T-cell receptor (TCR) gene expression found that the TCR Vβ gene expression in AAA tissue was also polyclonal[194]. The diversity in antigen specificity of the B- and T-lymphocyte population suggests that these lymphocytes are not the result of clonal expansion of a few autoantigen specific B- or T-lymphocytes, as would be expected in an autoimmune response.

Infectious Agent

There is some debate over whether *Chlamydia pneumoniae* is a causative agent of aortic aneurysm development. Initial reports suggested that *C. pneumoniae*[72, 73] and *C. pneumoniae* reactive T-lymphocytes[163] were found within the wall of AAAs. Conversely, other studies have been unable to detect this organism within the AAA wall[195, 196]. An investigation into the effects of *C. pneumoniae* on MMPs found that in asymptomatic AAAs there was no significant difference in MMP-2 and MMP-9 expression between the AAA group with *C. pneumonia* and those AAAs without[197].

Chronic Inflammation & Inflammatory Mediators

Once the immune response has been initiated within the AAA it develops into a chronic inflammatory response. The role of the inflammatory infiltrate in the development of AAAs was highlighted by an elastase-perfused animal study where the exposure of the aorta to elastase initially resulted in elastin degradation, accompanied with modest dilatation, but truly aneurysmal dilatation occurred several days post-perfusion and coincided with the medial infiltration of activated macrophages and T-lymphocytes[198]. Based on these findings, it has been postulated that an initial stimulus for remodelling results in an influx of inflammatory cells and the activation of the proteolytic system within the AAA wall (Figure 2-4). The effect of the infiltrate on aortic wall homeostasis and in the further development of the AAA has yet to be defined. The cells of the infiltrate, lymphocytes and macrophages, are known to produce a variety of inflammatory mediators.

Cytokines

Cytokines can regulate the transcription of MMP genes. The cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF) can induce the transcription of certain MMPs[199, 200], whilst the anti-inflammatory cytokine IL-10 is known to inhibit MMP transcription[201]. IL-1β can stimulate the up-regulation of PAI-1 in AAA-derived SMCs[183]. The possible role and evidence for cytokine involvement in AAA pathogenesis will be dealt with in more detail in *Chapter 3 Cytokines.*

Reactive Oxygen Species and Antioxidants

Inflammatory cells can produce reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide, which may promote aneurysmal wall degradation. The levels of superoxide in the human AAA wall was found to be 2.5 fold higher than in adjacent tissue and 10 fold higher when compared to control tissue[202]. Using an elastase-induced AAA model one study found that the inducible enzyme nitric oxidase synthase, which contributes to a pro-oxidant environment, was up-regulated over 50 fold by day 2 post-elastase infusion. By day 10 post-infusion the expression of superoxide dismutase, a potent antioxidant, was down-regulated more than 20 fold[203]. In addition to their toxicity, ROS have been shown to activate MMPs[204, 205] and induce SMC apoptosis[206].

Angiotensin II

Angiotensin II (Ang II), which is converted from angiotensin I (Ang I) by the proteolytic action of ACE, has been shown to accelerate the progression of atherosclerosis and in apo E-deficient mice administration of exogenous Ang II induces the formation and expansion of AAAs[207], possibly through the up-regulation of u-PA[208]. Further evidence for the involvement of Ang II was demonstrated by the inhibition of ACE in a rat elastase-induced AAA model, which blocked the formation of Ang II and reduced AAA expansion rate[209].

2.5.3 Genetics

Genetics factors may be involved in susceptibility to AAAs, as demonstrated by the familial aggregation of AAAs. The genes which render an individual susceptible to AAAs are unknown and vary amongst the different familial clusters[80]. The main candidate genes investigated have roles in ECM composition, proteolysis and inflammation.

Connective Tissue Components:

Collagen

In patients with a family history of AAAs lower quantities of type III collagen have been detected in 6-18% of the patients[146, 210, 211]. Several mutations have been described in the type III collagen gene (*COL3A1*)[212-214]. Mutations in this gene resulted in a 50% reduction in type III collagen in heterozygous mice; the homozygous mutant mice did not produce type III collagen and died from blood vessel rupture[215]. Analysis of gene expression of 50 patients with AAAs found that only 2% had a mutation in the type III collagen gene[213]. There is no evidence that mutations in the type I collagen gene are involved in AAA formation.

Fibrillin

Targeted deletion of the fibrillin-1 gene (*Fbn1*) in mice results in a Marfan-like phenotype, leads to an inflammatory response and formation of aortic aneurysms[63, 216]. Francke *et. al.* found a Gly1127Ser mutation in the fibrillin-1 gene in families with ascending aortic disease, aneurysms and dissection, but who did not have Marfans syndrome. This mutation was thought to predispose individuals to aortic dilatation later in life[217]. In the absence of Marfans syndrome, an association between a mutation in the fibrillin genes and familial AAAs has yet to be determined.

Proteases and their Inhibitors:

Matrix Metalloproteinases

There appears to be little evidence for their genetic involvement in the development or growth of AAAs. Eriksson *et. al.* genotyped 455 AAA patients for polymorphisms in MMP-2, MMP-3, MMP-9 and MMP-12 but found no significant association between growth rate and genotype[218]. Eighteen polymorphic sites were identified in MMP-2, however there were no significant differences in genotype or allele frequencies of any of the polymorphisms between the case and control groups[219]. Genotyping studies of the MMP-9 polymorphism, C-1562T, revealed that genotypes containing the T allele were significantly more frequent in AAA patients than in controls[220]. As this allele is more functionally active than the C-allele it may be responsible for the raised expression of MMP-9 found within the AAA wall.

Tissue Inhibitors of Metalloproteinases

Although decreased activity of TIMP has been suggested as a basis underlying AAA formation, little evidence suggests that TIMP-deficiency results from a genetic defect[221, 222]. However, two linked polymorphisms in TIMP-1 have been reported to be significantly associated with AAAs in male cases without a family history. No association was found between TIMP-2 and AAAs[223].

Plasminogen Activator Inhibitor-1

The PAI-1 gene has a single nucleotide polymorphism (-675 4G/5G) in its promoter. There is a higher frequency of the –675 5G insertion allele in patients with familial AAAs[224]. The 5G allele produces less PAI-1 compared to the 4G allele[225], which would suggest that familial AAAs may result from a reduction in PAI-1, which would lead to increased activation of plasmin and increased MMP activation. However, genotyping of 460 patients with small AAAs found no evidence that the frequency of the 4G/5G polymorphism is altered in patients with non-familial AAAs. A comparison of AAA growth rates did suggest that the 5G5G genotype may promote faster AAA growth, although this failed to reach significance[226].

Inflammation:

Cytokines

The IL-6 gene has a polymorphic site (-174 G>C) within its promoter region. Jones *et. al.* showed that the G/G genotype was associated with lower IL-6 plasma concentrations[227], although there was no association between IL-6 genotype and AAA growth. IL-10 contains a –1082 G>A polymorphism where the A-allele results in reduced IL-10 production and Bown *et. al.* showed that this A-allele was significantly more common in AAA patients[228]. IL-10 is an anti-inflammatory cytokine and individuals with reduced levels of IL-10 may be prone to AAA development due to an inability to mount a sufficient anti-inflammatory response. Other cytokine polymorphisms that have been investigated are: TNFα (–308 A>G), IL-1β (+3953 T>C), IL-6 (–174 G>C) and IL-10 (–592 C>A), however these were found not to be associated with AAAs[228].

Human Leukocyte Antigen Class II

The human leukocyte antigen (HLA) class II genes give rise to the major histocompatibility complex II (MHC II) expressed on T_H-lymphocytes, which enable the T_H-lymphocytes to present antigen to immune cells. These genes are highly polymorphic and several alleles have been suggested to predispose the carrier to AAA formation through an autoimmune response[229, 230]. Hirose *et. al.* have suggested that HLA-DQ3 antigen appears to have a protective effect in relation to AAAs[231], whereas HLA-DR2(15) promotes AAA disease[232].

Angiotensin Converting Enzyme

A polymorphism in the human ACE gene has been identified which exists as two alleles, the D-allele (deletion) and the I-allele (insertion). The D-allele lacks a 287bp sequence and is associated with higher ACE activity within tissues and therefore higher levels of Ang II, compared to the I-allele[233, 234]. However, the risk of carriers of the D-allele having an increased likelihood of developing an AAA is contentious with conflicting studies reporting either an association between the DD genotype and AAAs[235, 236], or a lack of association[237, 238].

2.5.4 Biomechanical Wall Stress

Approximately 89% of aortic aneurysms affect the infrarenal aorta[14]. Compared with proximal aorta, the infrarenal aorta is chronically exposed to the highest peripheral resistance, lowest flow and highest oscillatory wall sheer stress[239]. These conditions increase inflammatory and pro-apoptotic gene expression *in vitro* and *in vivo*[240, 241]. Low flow conditions have been shown in rodent AAA models to result in AAAs with fewer endothelial cells and SMCs than AAAs in rats with high flow[242]. In rats with high flow the increase in endothelial cells and SMCs had a stabilising effect on the AAA[242]. Additionally, conditions of increased aortic flow result in a reduction in mural macrophage infiltration[243]. However, whilst the infrarenal aorta may be predisposed to dilation the majority of elderly people do not develop AAAs suggesting that additional initiation factors may be involved.

One computer modelling study suggested that intraluminal thrombus may result in an attenuated oxygen flow from the lumen to the underlying AAA wall[244]. This problem is compounded by the scarcity of the vasa vasorum in the abdominal aorta, which are quite abundant in the thoracic aorta, resulting in the abdominal aorta's dependency on transluminal diffusion for oxygen and nutrients[245, 246]. Further investigation by Vorp *et. al.* revealed that the AAA wall adjacent to a thick layer of intraluminal thrombus was weaker, exhibited a greater degree of cellular hypoxia and inflammation when compared to wall specimens from the same individual but at a site with less thrombus[247]. Accordingly, hypoxia has been proposed as a possible mechanism for the initial injury to the pre-aneurysmal wall.

Once an aneurysm has developed increased wall stress aids AAA growth and increases the risk of rupture. The process of dilatation influences the load on the aortic wall. According to Laplace's law, for a cylindrical vessel, the circumferential wall stress is directly proportional to aortic diameter and blood pressure, and inversely proportional to the wall's thickness[28]. Therefore with aortic dilatation the load on the wall increases, producing further dilatation of the aneurysm. However, as the aorta dilates its shape changes from cylindrical to spherical this reduces the load on the wall and counteracts the effects of dilatation. The relationship between risk of rupture and wall stress was highlighted by one study where the ruptured or symptomatic AAAs had a higher peak wall stress than

diameter-matched asymptomatic AAAs[248]. However, computer modelling has been unable to reliably predict rupture.

2.6 Summary

The abdominal aorta is an elastic artery organised into three concentric layers: the tunica intima, tunica media and tunica adventitia. The organisation of these layers provides the aorta with elasticity and tensile strength, chiefly through the properties of its ECM components, elastin and collagen, respectively. In AAAs there is a prominent loss of elastin, increase in collagen turnover, increased SMC apoptosis and an inflammatory infiltrate. These changes result in a weakened aortic wall and dilatation. The mechanisms of proteolytic degradation, inflammation of the aortic wall, genetic predisposition and biomechanical wall stress are thought to contribute to the initiation and development of AAAs. Despite extensive research into the pathophysiology of the disease a detailed understanding of the mechanism involved in AAA formation remains to be determined.

Chapter Three

Cytokines

Chapter 3. Cytokines

3. Cytokines

3.1 Definition

Cytokines are soluble, low molecular weight extracellular signalling proteins, or glycoproteins, of the immune system and are involved in cell-to-cell communication. They are secreted by a range of leukocytes and other cells in response to a stimulus and transmit a signal to effector cells by binding to the cytokine specific receptor on the effector cell. The receptor-ligand binding results in intracellular signal transduction and altered gene transcription leading to a cellular response. Cytokines regulate the response of the immune system and have an important role in both health and disease states[249].

Cytokine nomenclature is mostly derived from the historical approach of naming cytokines based on biological activity and lacks a systematic basis for it. The term *cytokine* is all encompassing for these proteins and includes those cytokines previously termed *lymphokines* and *monokines*, which were cytokines secreted from lymphocytes and monocytes/macrophages, respectively. A systematic approach resulted in the introduction of the term *interleukin (IL)*, which indicated cytokines of leukocyte origin, followed by a number. However, it is not universally applied to new cytokines and has led to anomalies such as IL-8 which structurally belongs to the chemokine family, whilst other members of this family are named due to their main activities, e.g. monocyte chemoattractant protein-1 (MCP-1). Clarification of the chemokine family has led to the introduction of a naming system where chemokines are grouped according to subfamily. The two main chemokine subfamilies are the CC and the CXC chemokines. By this system IL-8 is named CXCL8 and MCP-1 is CCL2[249].

3.2 Cytokine Properties

The response of an effector cell to a particular cytokine is determined by the presence of specific membrane receptors. Generally, cytokines and their receptors have a high affinity for each other; therefore picomolar concentrations of cytokines can mediate a biological effect. Cytokines usually act locally in either an autocrine (acting on the cell that produced

it) or paracrine fashion (acting on a target cell in close proximity to the cytokine-secreting cell). Occasionally, cytokines act in an endocrine manner (targeting cells at a distant site)[250]. Most cytokines are secreted, but some can be expressed on the cell membrane, or stored in reservoirs in the ECM.

Cytokines exhibit the attributes of pleiotropy, redundancy, synergy, antagonism and cascade induction[251-253]. A pleiotropic cytokine produces different biological effects depending on the target cell it interacts with. Cytokine redundancy means that two or more cytokines can mediate a similar function on the same target cell. Cytokine synergism between two cytokines or more results in an augmented response to the cytokines, which is greater than the response to the individual cytokines alone; whereas cytokine antagonism means that one cytokine inhibits or offsets the effects of the other. The induction of a cytokine cascade results from the action of cytokines on a target cell inducing that cell to produce a variety of cytokines, which in turn interact with additional target cells to produce other cytokines and so forth[253]. However, cytokine production is carefully regulated and generally the secretion of cytokines from activated cells is short-lived to prevent an over-exuberant and persistent immune response.

3.3 Cytokine Structure

Generally, cytokines have a mass of less than 30kDa. X-ray and nuclear magnetic resonance (NMR) studies have generated accurate structures of many cytokines. This combined with gene homology studies have enabled most cytokines to be placed into one of at least six structurally different families (Table 3-1). Structural models of representative members of each family are shown in Figure 3-1.

Table 3-1. Structural families of cytokines[249]

Family	Members	Receptor type
Haematopoietins	IL-2, IL-3, **IL-4**, IL-5,	Cytokine receptor class I
(4 α-helical bundles)	IL-6, IL-7, IL-9, IL-13,	
	G-CSF, GM-CSF, OSM	
	IL-10, IFN-α, IFN-β, IFN-γ	Cytokine receptor class II
	M-CSF	Tyrosine kinase
EGF (β-sheet)	EGF, **TGF-α**	Tyrosine kinase
β-Trefoil	FGF-α, FGF-β,	Split tyrosine kinase
	IL-1α, **IL-1β**, IL-1Rα	IL-1 receptor
TNF	**TNF-α**, TNF-β	NGF receptor
Cysteine knot	NGF	NGF receptor
	TGF-β1, TGF-β2, TGF-β3	Serine/threonine kinase
	PDGF, VEGF	Tyrosine kinase
Chemokines (triple	**IL-8**, MIP-1α, MIP-1β,	Rhodopsin superfamily
stranded, anti-parallel β-	MIP-2, I-309, MCP-1,	
sheet in greek key motif)	MCP-2, MCP-3, γIP-10	

The three-dimensional structures of the cytokines in bold type are shown in Figure 3-1. *IL*, interleukin; *G-CSF*, granulocyte colony stimulating factor; *GM-CSF*, granulocyte/macrophage colony stimulating factor; *OSM*, oncostatin M; *IFN*, interferon; *M-CSF*, macrophage colony stimulating factor; *EGF*, epidermal growth factor; *TGF*, transforming growth factor; *FGF*, fibroblast growth factor; *IL-1Rα*, interleukin-1 receptor antagonist; *TNF*, tumour necrosis factor; *NGF*, nerve growth factor; *PDGF*, platelet derived growth factor; *VEGF*, vascular endothelial growth factor; *MIP*, macrophage inflammatory protein; *MCP*, monocyte chemoattractant protein; *γ-IP*, γ-interferon inducible protein.

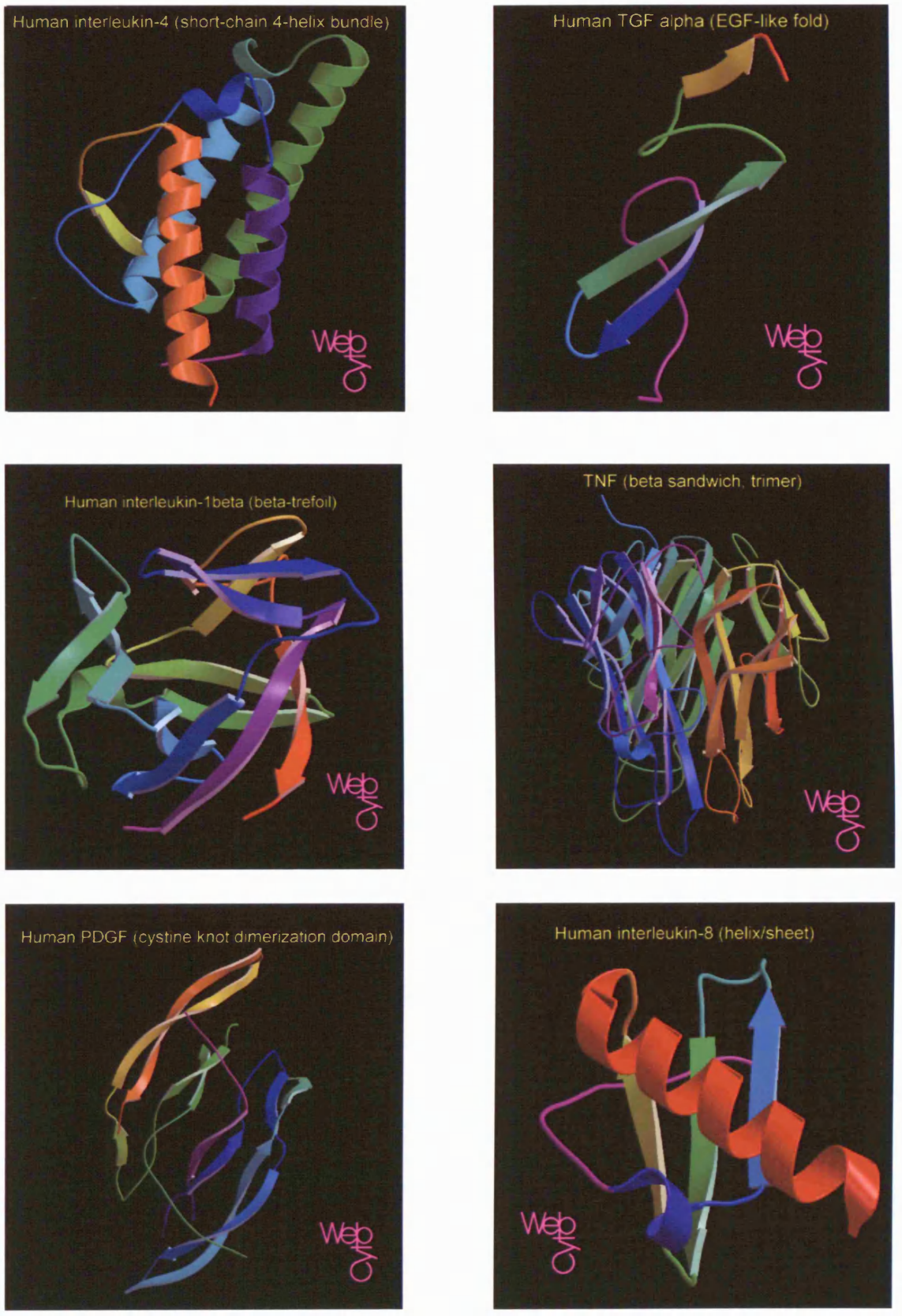

Figure 3-1. Three-dimensional structures of cytokines which are representative of the main structural families[254].

3.4 Cytokine Function

Many cell types can secrete cytokines, however the main producers are T_H-lymphocytes and macrophages. In broad terms, cytokines can interact with a network of immune and inflammatory cells to develop cellular and humoral responses, induce inflammatory responses, regulate haematopoiesis, control cellular proliferation and differentiation and promote wound healing. The form of biological activity that is induced depends on the individual cytokine and the target cell. Cytokines act in a non-antigen specific manner and will bind to any cell that expresses its specific receptor. *In vivo* cytokines rarely act alone as a target cell is usually exposed to a multitude of cytokines and the combined effect can vary greatly from the expected effect of the individual cytokines. Additionally, cytokines may induce a cascade of cytokine activities in which the cytokines produced at a later step may override the influence of the earlier cytokines on the immune response. With the non-specific nature of cytokines the specificity of the immune response is maintained by the inducible expression of cytokine receptors on the target cells, or by a requirement for the direct interaction between the cytokine-producing cell and the target cell to induce cytokine secretion.

Whilst it is beyond the scope of this thesis to examine in detail the role of each cytokine within the immune system the following sections aim to give a brief overview.

3.4.1 Role of Cytokines in Regulating the Immune Response

The $CD4^+$ T_H-lymphocyte is central to cytokine-mediated responses (Figure 3-2). The secretion of IL-1, accompanying the presentation of antigen by an APC to a resting T_H-lymphocyte, results in T_H-lymphocyte activation. The activated T_H-lymphocyte secretes IL-2 and up-regulates the expression of the IL-2 high affinity receptor. The autocrine binding of IL-2 to its receptor induces cell proliferation resulting in clonal expansion[255]. This expansion can be inhibited by the actions of TGF-β[256]. Activated T_H-lymphocytes secrete IL-4 which can cause an up-regulation of MHC II on resting B-lymphocytes and allows these B-lymphocytes to act as APCs to T-lymphocytes. This, in the presence of an antigen, results in further activation of T_H-lymphocytes.

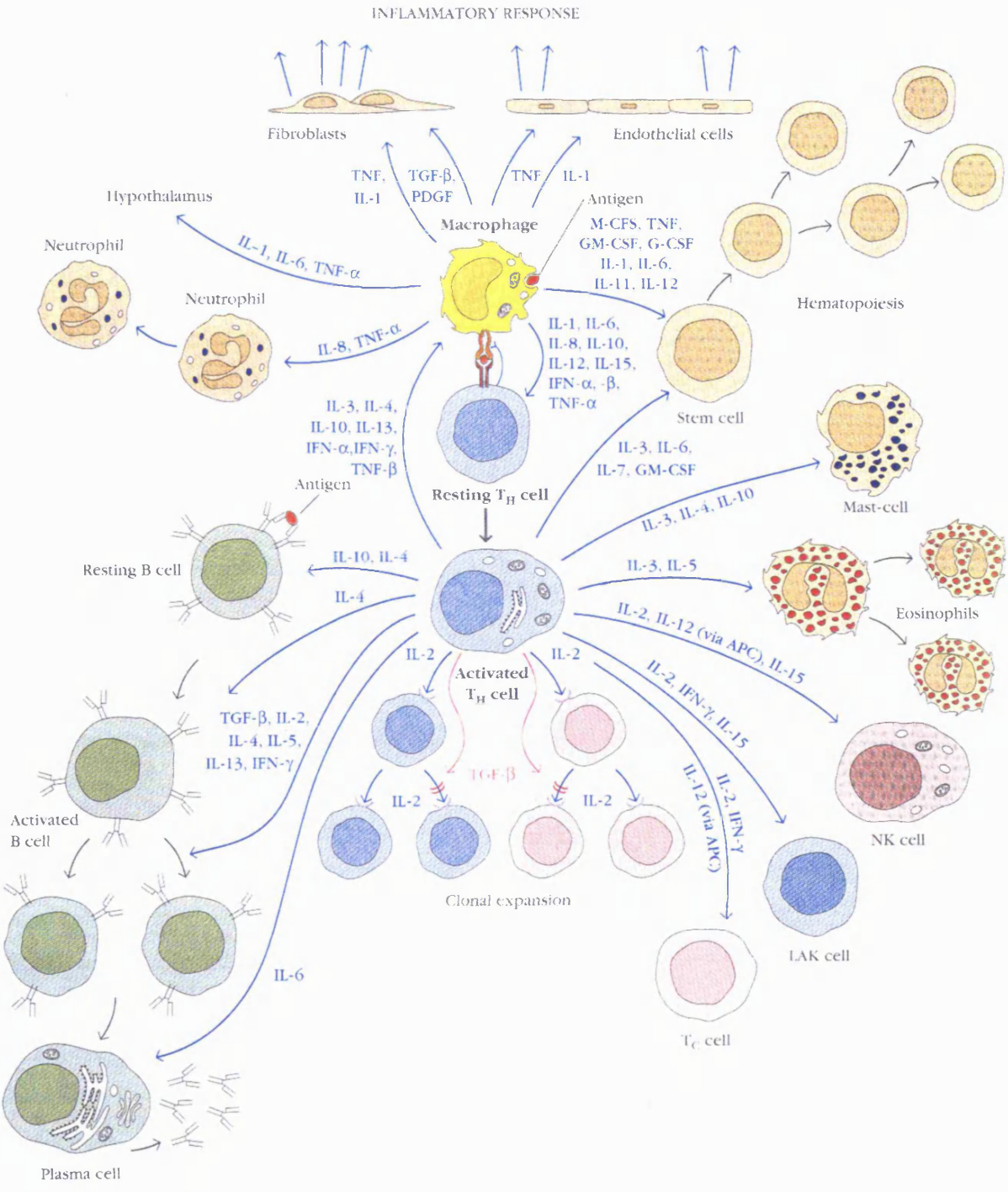

Figure 3-2. The role of cytokines in the regulation of the immune response[253]. See text, *3.4.1 Role of Cytokines in Regulating the Immune Response*, for details. Blue arrows indicate a stimulatory cytokine pathway. Red arrows indicate an inhibitory pathway.

IL-4 also causes the activation of antigen-primed B-lymphocytes and, along with IL-2, IL-5, IL-13, TGF-β and interferon-γ (IFN- γ), stimulates the proliferation, class switching and differentiation of activated B-lymphocytes[257, 258]. IL-6 is involved in the terminal differentiation of activated B-lymphocytes into antibody secreting-plasma cells[259].

Macrophage activity can be enhanced by a range of cytokines secreted by activated T_H-lymphocytes. IFN-γ is a potent activator of macrophage activity[260], IL-4 up-regulates MHC II[261], enhancing the macrophage's ability to act as an APC, and increases phagocytic activity. IL-10 and IL-13 inhibit macrophage secretion of cytokines[262-264]. The activated macrophage can produce IL-8[265], which chemotactically attracts neutrophils into the inflamed tissue and results in neutrophil activation. Neutrophils are phagocytic cells involved in the digestion and elimination of bacteria. A number of cytokines are involved in the inflammatory process and will be discussed in more detail in *3.4.3 Role of Cytokines in the Inflammatory Response.*

A multitude of growth factors and cytokines produced by both the activated T-lymphocytes and macrophages are involved in haematopoiesis, the formation of blood cells. IL-3 and granulocyte/macrophage colony stimulating factor (GM-CSF) stimulate stem cells from which all blood cell lineages are derived and cooperate with other cytokines in the development of the different lineages. The leukocyte progenitor cells in the bone marrow give rise to macrophage and granulocyte lineages in response to macrophage colony stimulating factor (M-CSF) and granulocyte colony stimulating factor (G-CSF), respectively[266].

3.4.2 Role of Cytokines in the T_{H1} and T_{H2} Response

Within the T_H-lymphocyte population exists two subsets: the T_{H1}-lymphocyte and the T_{H2}-lymphocyte[267]. These subsets vary in the panel of cytokines they secrete and therefore the form of immune response they control. The T_{H1}-lymphocytes stimulate cell-mediated immunity by producing IL-2 and IFN-γ, thereby stimulating cytotoxic T-lymphocytes (T_C-lymphocytes) and macrophages. The T_{H2}-lymphocyte produces IL-4 and IL-10 which stimulate B-lymphocytes and antibody production leading to a humoral response[267, 268]. These two subsets are thought to arise from a common precursor. Macrophage derived IL-12 stimulates T_{H1}-lymphocyte development[269], whereas IL-4, derived from T_{H2}-lymphocytes, natural killer cells and mast cells, stimulates T_{H2}-lymphocyte development.

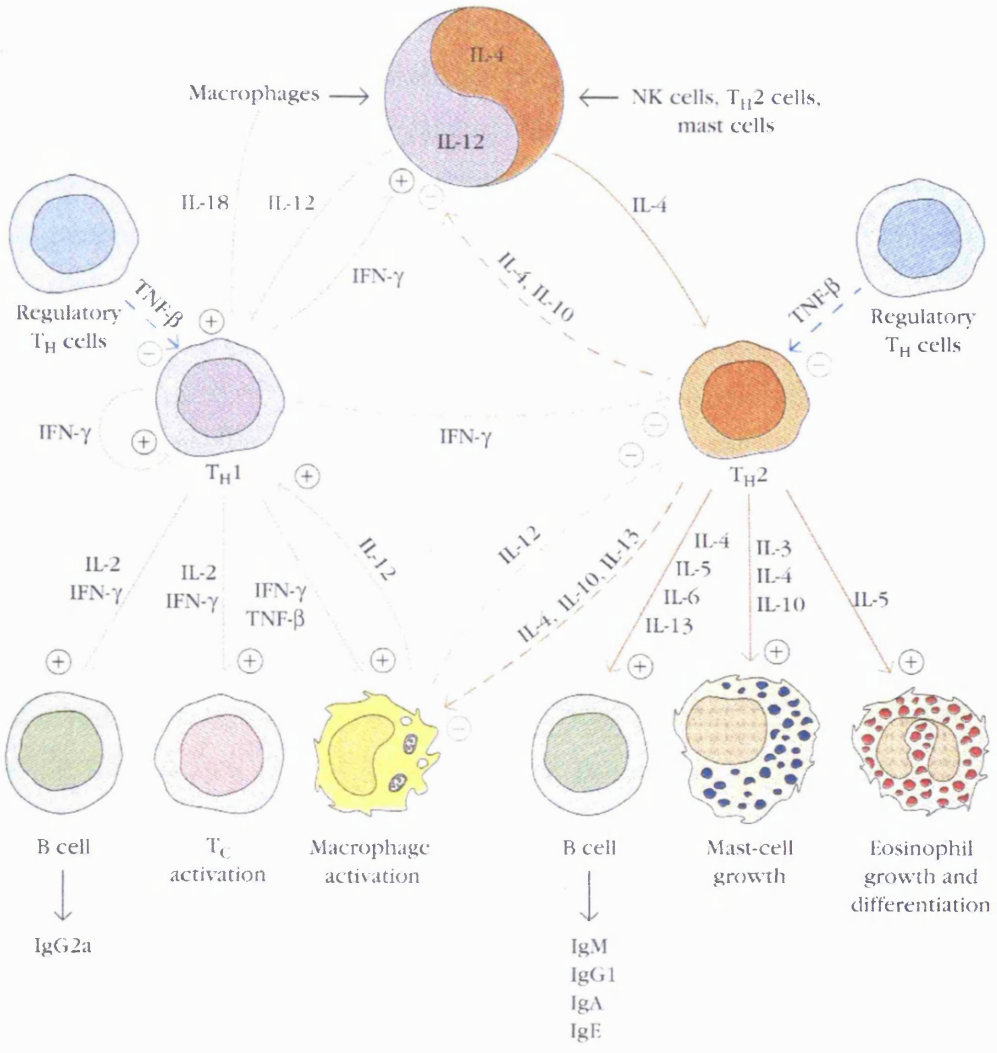

Figure 3-3. The regulation of T_H subsets by cytokines[253]. See text, *3.4.2 Role of Cytokines in the T_{H1} and T_{H2} Response,* for details. Solid arrows indicate stimulatory effects (+), dashed arrows indicate inhibitory effects (-). Purple arrows indicate the cytokines of the T_{H1} pathway; orange, the T_{H2} pathway.

T_{H1}- and T_{H2}-lymphocytes are mutually inhibitory through the cytokines that they produce: IFN-γ inhibits the proliferation of T_{H2}-lymphocytes[270], whereas IL-10 inhibits the proliferation of T_{H1}-lymphocytes[268]. The action of IL-10 is achieved through its suppression of cytokine production by macrophages and therefore it indirectly affects T_{H1}-lymphocyte proliferation.

3.4.3 Role of Cytokines in the Inflammatory Response

Physical trauma induces a cascade of events known as the inflammatory response. An acute inflammatory response provides early protection from infection, lasts a short while and is usually accompanied by a systemic response. Neutrophils, attracted by IL-8, migrate to the site of infection to phagocytose invading pathogens, release cytokines and other mediators. Amongst the cytokines released are macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, chemokines that attract macrophages to the site of inflammation[271]. The activated macrophages result in the release of more cytokines and increased phagocytosis. The activated macrophages secrete the cytokines IL-1, IL-6 and TNF-α and these account for many of the localised and systemic changes observed in the acute inflammatory response[253]. All three cytokines act locally to induce coagulation and increase vascular permeability. TNF-α and IL-1 increase adhesion molecule expression on vascular endothelial cells[272] and also induce macrophages and endothelial cells to secrete chemokines[273], which results in an increase in extravasation of leukocytes into the vessel wall. Additionally, IFN-γ and TNF-α increase the phagocytic activity of macrophages and neutrophils and encourage extracellular release of lytic enzymes[260, 274]. The inflammatory response provides a first line of defence against pathogens, however the duration and intensity needs to be regulated to avoid unnecessary tissue damage. TGF-β is involved in limiting the inflammatory response and aiding wound repair[275, 276].

Chronic inflammation develops due to the persistence of an antigen and results in substantial tissue damage. Cytokines produced by chronically activated macrophages stimulate fibroblast proliferation, collagen production and fibrosis[277, 278]. Two principal cytokines involved in the development of chronic inflammation are IFN-γ and TNF-α. In chronic inflammation the accumulation of activated macrophages is responsible for much of the tissue damage through release of lytic enzymes, ROS and nitrogen intermediates[279]. Activation of macrophages by IFN-γ induces TNF-α expression. Synergistically IFN-γ and TNF-α induce a chronic inflammatory response through the over-expression of adhesion molecules, resulting in an increase in inflammatory cell infiltration, and MHC I expression, which presents antigen to T_C-lymphocytes[280, 281].

3.5 Cytokines and Abdominal Aortic Aneurysms

As key regulators of the immune response a discrepancy in the cytokine profile may affect the homeostatic balance of the local system and result in tissue damage. Several pathophysiological disease states result from inappropriate cytokine expression, such as rheumatoid arthritis[282-284], inflammatory bowel disease[285-287], bacterial septic shock[288, 289], various cancers[290-292] and atherosclerosis[293-295]. With the presence of a large inflammatory infiltrate within the AAA wall several studies have investigated the expression of various candidate cytokines within the wall and plasma of patients with AAAs. However, with variations in experimental techniques, stage of aneurysm formation and type of control groups used many of the studies to date are contradictory. The main cytokines that have been studied can be grouped on the basis of their function into the four groups; pro-inflammatory, anti-inflammatory, chemokines and growth factors.

3.5.1 Pro-inflammatory Cytokines

Pro-inflammatory cytokines generally promote further cytokine secretion, cause inflammatory cell and lymphocyte activation, increased proteolysis and tissue degradation. The main inflammatory cytokines which have been investigated within the AAA are IL-1, TNF-α and IL-6. IL-1 exists in two forms, the non-secreted form IL-1α and the secreted form IL-1β. Both IL-1α and IL-1β bind to the same receptor and result in T-lymphocyte activation, B-lymphocyte maturation and clonal expansion, and increased adhesion molecule expression on vascular endothelial cells. IL-1β as the secretory form has been extensively studied. Several studies on IL-1β expression in the AAA wall have found it to be increased[200, 296], not significantly different[82], or lower in the AAA compared to controls[84].

TNF-α is cytotoxic, induces chronic inflammation and secretion of cytokines. Several studies have described the up-regulation of TNF-α within the AAA compared to controls[82, 200, 297], although this is conflicted by reports that TNF-α is not differentially expressed within the AAA[296] or that it is lower in the AAA wall[84]. TNF-α is present in soluble and membrane bound forms, like IL-1, and the variation in the reports may reflect the various methods used to extract the protein. A mRNA study of TNF-α expression found that it was higher in AAA samples than in AOD[82]. Analysis of the soluble and membrane bound forms showed that the membrane bound form was present within AAA samples but was

absent in AOD[200]. This would account for the observed total increase in TNF-α in AAA samples. However, another study reported a decrease in the membrane bound form in AAAs compared to AOD[84].

IL-6 is a pleiotropic cytokine secreted by both lymphoid and non-lymphoid cells which regulates B- and T-lymphocyte function, haematopoiesis and acute phase reactions. IL-6 has a polymorphism in its promoter region (-174 G>C) which can result in lower IL-6 plasma concentrations in patients with a GG genotype[227]. However, the study found that in small aneurysms the frequency of the C allele was similar to that of the normal population[227]. Likewise, Bown *et. al.* did not find an association between this polymorphism and AAAs[228]. Several studies have measured the expression of IL-6 in plasma and in the aneurysmal wall of patients with AAAs. Overall, the expression of IL-6 was found to be raised in aneurysm patients[298-300].

3.5.2 Anti-inflammatory Cytokines

The overall inflammatory response depends on the balance of pro- versus anti-inflammatory cytokines. Anti-inflammatory cytokines limit the actions of the pro-inflammatory cytokines, partly through the inhibitory effect of IL-10 on T_{H1}-lymphocytes and the resulting attenuation of the cell-mediated immune response. The main anti-inflammatory cytokines studied within the aneurysm are IL-10 and IL-4. It has been debated if a T_{H1} (pro-inflammatory) or T_{H2} (anti-inflammatory) immune response dominates within the AAA wall, as defined by the panel of cytokines produced by the T_H-lymphocytes[301]. Schonbeck *et. al.* described the increased expression of T_{H2} cytokines IL-4, IL-5 and IL-10 within the AAA compared to control aorta, with only low levels of T_{H1} cytokines IL-2 and IL-15[302]. Davis *et. al.* found that AAAs expressed higher levels of IL-10 compared to AOD and that the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were significantly lower in AAAs than in AOD[84]. However, these findings on pro-inflammatory cytokines are contradicted by several studies, as discussed in *3.5.1 Pro-inflammatory Cytokines*. Additionally, Galle *et. al.* found that the infiltrating $CD4^+$ T-lymphocytes were a source of IFN-γ, a T_{H1} cytokine, but not IL-4[161]. Szekanecz *et. al.* also described the presence of pro-inflammatory cytokines IL-6 and IFN-γ in the AAA wall, but not IL-4[299].

The anti-inflammatory cytokine IL-4 stimulates the production of a variety of ECM proteins[303], whilst blocking MMP-1 and MMP-9 release from alveolar macrophages without altering the secretion of TIMPs[304]. IL-10 is a potent inhibitor of pro-inflammatory cytokine secretion by macrophages[305] and can regulate MMP/TIMP expression[201]. Considering the actions of IL-4 and IL-10 it could be hypothesised that the anti-inflammatory cytokines would provide a protective mechanism against AAA formation. Whilst it is unclear whether IL-4 is raised in the aneurysm or not, if these anti-inflammatory cytokines are elevated within the aneurysm wall this may reflect a homeostatic attempt to reduce inflammation within the wall and restrain the ongoing aneurysmal degeneration. However, Schonbeck *et. al.* suggested that the T_{H2} response may actually drive aneurysm formation[302]. Using a murine allograft AAA model one study demonstrated that mice deficient in the IFN-γ receptor developed AAAs whilst the wildtype recipients developed intimal hyperplasia. Furthermore, treatment of the IFN-γ receptor-deficient mice with an anti-IL-4 antibody prevented AAA formation and reduced collagenolytic and elastolytic activity. They suggested that a skewing of the immune response to that of a T_{H2} response actually promotes AAA formation[306].

The dominance of the T_{H2} response in AAA pathogenesis is contradicted by a $CaCl_2$ induced-AAA murine model where $CD4^{-/-}$ and IFN-$\gamma^{-/-}$ mice were both protected from AAA formation, but upon administration of IFN-γ aneurysm development was partially reconstituted[164], suggesting T_{H1} response involvement. As AAA models these systems do not truly represent the natural progression of human AAAs and currently no consensus has been reached on whether a T_{H1}/T_{H2} response dominates.

3.5.3 Chemokines

Directional movement of inflammatory cells into injured tissue is often induced by chemotactic molecules. The chemokines represent a superfamily of cytokines which function as potent chemoattractants and activators of specific leukocytes. They also have a wide range of effects on other cells such as fibroblasts and SMC, and can affect angiogenesis and haematopoiesis. Chemokines are grouped into subfamilies based on the amino acid sequence of a cysteine motif; CXC, CC, C and CX3C[307].

The CXC chemokines contain one amino acid between the two amino-terminal cysteine residues and can be further subdivided into ELR^+ and ELR^- CXC chemokines, depending

on the presence of the glutamate-leucine-arginine (ELR) motif preceding the first two cysteines. IL-8, growth-regulated oncogene-α (GRO-α), epithelial neutrophil activating peptide-78 (ENA-78) and other ELR^+ CXC chemokines predominately act as chemoattractants and activators for neutrophils. Members of the ELR^- subfamily, for example monokine-induced by IFN-γ (MIG), lack the ELR sequence and mainly activate lymphocytes[252].

The two conserved cysteine residues are adjacent in the CC chemokine family and members of this family act on a wider variety of target cells, including eosinophils, basophils and lymphocytes[308]. The CC family includes MIP–1α, MIP-1β, regulated upon activation, normal T-cell expressed and secreted (RANTES), MCP–1 and MCP-2. The CC chemokines are either chemoattractants or activators. For example, RANTES and MCP-1 both target basophils but RANTES induces chemotaxis whilst MCP-1 induces histamine release[309].

Chemokines have not been extensively studied within the AAA wall. AAA tissue has been shown to produce IL-8 and MCP-1[310], but the remainder of the chemokine family have been relatively overlooked. Plasma levels of IL-8 have been associated with increasing AAA diameter[311]. The potential importance of chemokines in AAA development was highlighted by one study where MCP-1 and RANTES were detected during the early stages of AAA development in an elastase-induced AAA model and before the onset of chronic inflammation[312]. It may be that subsequent to an initial injury chemokine expression results in the infiltration of inflammatory cells which results in chronic inflammation and increased proteolysis. However, the role of chemokines in AAA development is complex and another study found that a deletion polymorphism (Δ32) in the CCR5 gene, which in homozygotes results in a lack of CCR5 on leukocytes, was associated with AAA incidence[313]. CCR5 is a receptor that is found on leukocytes and vascular SMCs and binds MIP-1β and RANTES[314]. This may suggest that the chemokine signal through CCR5 is required to maintain wall homeostasis and prevent AAA formation[313].

3.5.4 Growth Factors

Various growth factors, the haematopoietic cytokines, are required for proliferation, differentiation and maturation of haematopoietic cells. Historically, growth factors were identified by their stimulatory properties towards the formation of haematopoietic cell colonies in bone-marrow cell cultures. This led to the discovery of a family of colony stimulating factors (CSF), responsible for the formation of distinct haematopoietic cell lines. The members of the CSF family are: multilineage CSF (otherwise known as IL-3), M-CSF, G-CSF and GM-CSF. Other growth factors promoting cellular proliferation include platelet derived growth factor (PDGF) and vascular endothelial derived growth factor (VEGF). PDGF is a mitogen for connective tissue cells and glial cells. It promotes wound healing and has some chemoattractant properties. VEGF is related to the PDGF family of growth factors. It is a mitogen for endothelial cells, activates and is a chemoattractant for monocytes, enhances blood vessel permeability and is a pro-coagulant. TGF-β is a pleiotropic cytokine involved in tissue remodelling, wound repair and haematopoiesis. It is an anti-inflammatory cytokine, which despite its name inhibits cellular growth, including macrophages and lymphocytes. As an anti-inflammatory cytokine it suppresses cytokine production, MHC II expression and promotes wound healing through the induced secretion of ECM components.

Very few growth factors have been studied within the AAA wall. The development of AAAs may be related to increased angiogenesis within the AAA wall, the degree of which correlates with the extent of the inflammatory infiltrate[315] and is an ongoing process in the mature AAA[316]. The relationship between angiogenic factors and angiogenesis is uncertain. One study showed that VEGF was more highly expressed within AAAs compared to AOD and was associated with the extent of neovascularisation[317]. This is also supported by a study which investigated the effects of high and low blood flow, and therefore wall sheer stress, on AAA development and neovascularisation. Low flow promoted aneurysmal changes to the aortic wall and the experimental AAA also contained more adventitial capillaries. The extent of capillary density was associated with the expression of VEGF and PDGF. GM-CSF was also increased under haemodynamic conditions of low AAA blood flow[239]. TGF-β1 expression within the AAA is of interest because of its potential role in wound healing and stabilisation of the AAA. Shteinberg *et. al.* did not find a difference in TGF-β expression between AAAs and AOD[82], however Fukui *et. al.* found that TGF-β1 was significantly higher in the AAA than in AOD[318].

TGF-β1 is expressed by vascular SMCs in the normal arterial wall[319]. It has been suggested that TGF-β1 may provide a therapeutic mechanism for the treatment of AAAs. One study demonstrated that endovascular seeding of an AAA model with vascular SMCs resulted in experimental AAA stabilisation and was associated with a significant increase in TGF-β1 expression[320]. This finding was also supported by a similar study where over-expression of TGF-β1, induced by endovascular gene therapy, in a rat AAA xenograft model resulted in AAA stabilisation, preservation of medial elastin, decreased inflammatory cell infiltration and a decrease in MMPs[321].

3.6 Summary

Cytokines are small extracellular messenger proteins of the immune system. They are secreted by a range of cells and leukocytes and result in an altered cellular response by binding to a receptor on their target cell. This family of proteins regulates the immune response and has roles in cellular and humoral responses, inflammation, haematopoiesis and wound healing. As cytokines are essential in maintaining homeostasis an alteration in the cytokine profile may affect this balance and result in a pro-inflammatory environment leading to tissue damage. Accordingly, several studies have investigated the expression of cytokines within the AAA. Whilst several animal model studies have demonstrated the importance of specific cytokines in experimental AAA formation, studies on cytokine expression within the human AAA wall are highly varied in study design and are contradictory. Despite knowing the potential importance of these macromolecules in AAA formation, to date no comprehensive description of the cytokine profile within the AAA wall has been given.

Chapter Four

Aims & Scope of Thesis

Chapter 4. Aims & Scope of Thesis

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4.1 Rationale of Thesis

AAAs are a common idiopathic disease of elderly males, associated with considerable mortality and a rising annual incidence. At present the only method of treatment is elective prophylactic surgery once the AAA reaches an operable size. However, with the use of screening programmes a larger number of small asymptomatic aneurysms are being identified which are monitored until they reach an operable size. It would be preferable if a pharmacological therapy could be used to treat these small AAAs to reduce or halt their growth. In order to achieve this a more comprehensive understanding of the pathogenesis of AAAs is required.

AAA is a complex multi-factorial disease associated with extensive inflammation of the aneurysm wall. As described in *Chapter 3 Cytokines*, cytokines are extracellular signalling proteins involved in regulating the local immune system and are essential for maintaining the homeostatic balance between protecting against invading pathogens and preventing an over-zealous immune response. In AAAs chronic inflammation results in structural damage to the aortic wall. Animal studies have shown that alterations in cytokine expression affect aneurysm formation[164, 306, 321, 322]. Several human studies have investigated the expression of various cytokines within the aneurysm wall[84, 227, 296, 302], however, the results have been conflicting and with relatively few cytokines studied it is difficult to ascertain a complete picture of the cytokine expression profile of the AAA. Generally, cytokine studies have focused on pro-inflammatory cytokines and have largely ignored the expression of chemokines and growth factors. As the overall effect of cytokines on the inflammation within the aneurysm wall is dependent on the combination of cytokines expressed, a detailed analysis of multiple cytokine expression of the aneurysm was performed.

The AAA is characterised by a prominent inflammatory infiltrate. As cytokines are involved in the regulation of local inflammation the relationship between the cytokines expressed within the aneurysm wall and the inflammatory infiltrate merited further

investigation. In order to extend the knowledge of the involvement of specific cytokines in AAA mural inflammation the localised expression of specific cytokines was examined.

There are multiple possible roles for cytokines within AAA pathogenesis: the induction of SMC apoptosis, recruitment of inflammatory cells into the AAA wall, maintenance of chronic inflammation and activation of inflammatory cells[134, 159, 160, 312]. The characteristic loss of elastin has been attributed to the activities of the gelatinases MMP-2 and MMP-9[153, 173, 177]. The expression of these MMPs is controlled at the transcriptional level by specific cytokines[199-201]. Whilst the effects the pro-inflammatory cytokines IL-1β and TNF-α have on MMP expression within the aneurysm has been examined[199], the main focus on the role of chemokines within the AAA wall has been on their chemoattractant properties. However, chemokines are also involved in inflammatory cell activation and can cause the release of MMPs[323-325]. Cellular migration towards a chemotactic signal within a tissue requires cells to traverse through a network of ECM fibres. Therefore to aid chemotaxis chemokines may not only supply a chemotactic signal but potentially may also up-regulate the expression of MMPs to facility migration. This thesis examines the possibility that specific chemokines found elevated within the aneurysm can up-regulate MMP expression within the AAA wall.

4.2 Aims

The main aim of this thesis is to investigate the cytokine expression profile of the AAA. According to the rationale of the thesis its main objectives are:

1. To determine the cytokine expression profile of the AAA compared to non-aneurysmal aorta.
2. To validate the results of the mass cytokine screening by measuring the expression of specific cytokines using an absolute quantitative technique.
3. To characterise the expression of the elevated chemokines by determining their localisation within the aneurysm wall and to investigate a potential association between these chemokines and the inflammatory infiltrate.
4. To investigate the relationship between chemokines and MMP expression/activation.

4.3 Scope

In order to address these points this thesis describes the characterisation of the cytokine expression profile of the aneurysm and the potential involvement of these cytokines in inflammation and MMP expression/activation.

Patients undergoing elective transperitoneal repair of infrarenal abdominal aortic aneurysm were recruited into the study. AAA wall was obtained from the anterior wall at the point of maximal aortic dilation. Control specimens were obtained from non-aneurysmal abdominal aorta from cadaveric kidney donors and from non-aneurysmal thoracic aorta from patients undergoing aorto-coronary bypass graft operations. With the limited availability of abdominal aorta, thoracic aorta was used to provide an additional control group with similar demographic and clinical features to that of the AAA group.

Multiple complementary laboratory techniques were used to characterise the expression and involvement of cytokines within the AAA:

1. Multiple Cytokine Protein Array

The analysis of the expression of 42 cytokines within the aneurysm and the controls was measured using a 42-cytokine specific antibody-based array. This technique measured simultaneously the relative cytokine expression within each specimen at the protein level.

2. Enzyme Linked Immunosorbent Assays

The array findings were verified using enzyme linked immunosorbent assays (ELISAs) for IL-6, IL-8, MCP-1 and MCP-2. Larger AAA and control groups were used. The ELISAs provided an absolute quantitative measure of the concentration of the cytokine proteins identified by the arrays.

3. Immunohistochemistry

Characterisation of the localised expression of IL-8 and MCP-1 within the aneurysm wall was visualised using immunohistochemistry. Co-localisation studies of these chemokines were performed using serial wall sections stained for T-lymphocytes (CD3, CD4 and CD8), B-lymphocytes (CD20) and macrophages (CD68). The association between these chemokines and the extent of inflammation was examined.

4. *In vitro* explant model

The effect of IL-8 on MMP-2 and MMP-9 expression was assessed using a human AAA explant model treated with various concentrations of IL-8 (including a non-treated control). Changes in the MMP expression and/or activation were measured using standard ELISAs and specific MMP activity assays.

4.4 Null Hypotheses

The null hypotheses tested are:

1. There are no significant differences in the relative expression of any of the 42-cytokines, as detected by the arrays, between the AAA, the abdominal aorta and the thoracic aorta.
2. There are no significant differences in the quantitative expression of IL-8, IL-6, MCP-1 and MCP-2, as measured by ELISAs, between the AAA, the abdominal aorta and the thoracic aorta.
3. There are no significant differences in the cellular composition of the inflammatory infiltrate between AAAs with different grades of inflammation.
4. There is no significant difference in the extent of mural inflammation between AAAs expressing increasing levels of IL-8 or MCP-1.
5. In the event that (4) is disproved: There are no significant differences in the cellular composition of the inflammatory infiltrate between AAAs expressing increasing levels of IL-8 or MCP-1.
6. There are no significant differences in the expression/activation of MMP-2 and MMP-9 between explants treated with IL-8 compared to non-treated controls.

Chapter Five

Materials & Methods

Chapter 5. Materials & Methods

5. Materials and Methods

5.1 Introduction

This chapter describes the techniques used in this thesis. All research was performed in the Vascular Surgery Group, Department of Cardiovascular Sciences, University of Leicester with the exception of the histological work, which was carried out in the Special Histology Laboratory at Leicester Royal Infirmary. Unless otherwise stated all reagents were obtained from Sigma-Aldrich, Poole, UK and all procedures were performed at room temperature (RT). The compositions of the solutions used are given in Appendix I.

5.2 Study Design

Patients undergoing elective open AAA repair for asymptomatic infrarenal AAAs were recruited to this study. Symptomatic or ruptured AAAs were excluded, as were patients with IAAAs or evidence of retroperitoneal fibrosis. Full thickness AAA wall specimens were obtained from the anterior wall at the point of maximal aortic dilation during open AAA repair.

Ideally, infrarenal aorta obtained during surgery from age- and sex-matched patients would have been used as a control group. In the past infrarenal aorta obtained from patients with aortic occlusive disease has been used as an age- and sex-matched control group for AAA studies. However, with advances in surgery the frequency that this operation is performed has been reduced to the extent that insufficient samples would have been collected during the time duration of my research. There are no other suitable surgical procedures that would have enabled the collection of age- and sex-matched infrarenal aorta. To address this limitation control non-aneurysmal aortic specimens were obtained from two sources: cadaveric kidney donors supplied by the UK Human Tissue Bank (Leicester, UK) (non-aneurysmal abdominal aorta), and from patients with symptomatic coronary disease undergoing elective aorto-coronary bypass graft operations (non-aneurysmal thoracic aorta).

Abdominal aorta was obtained from the infrarenal aortic patches from donated kidneys that were unsuitable for transplantation. The abdominal aortic specimens provided a control group which encompassed the anatomical site of AAAs and represented the morphology of the non-aneurysmal abdominal aorta. Furthermore, these specimens would have been under similar systolic pressures, flow conditions and wall stress as the aneurysmal abdominal aortas. However, the age for kidney donation is younger than the age for the development of AAAs and despite every effort to collect from donors at the upper age range for donations, the abdominal aortic specimens were younger than the AAA specimens. With the control abdominal aorta group being younger than the AAA group there may have been changes within the AAA wall which related to the ageing process, prolonged exposure to physical stresses and increased atherosclerosis, which were not accounted for by the younger control group.

To account for this thoracic aorta was used as an additional control group to provide the study with aortic samples with demographic features more closely matched to those of the AAA group. Thoracic aortic specimens were obtained from discarded pieces of thoracic aorta, which were routinely removed by punch biopsies during aorto-coronary bypass graft operations. However, there are several limitations to this control group relating to key differences between thoracic and abdominal aorta. Thoracic aorta has structural differences compared to abdominal aorta, for example it has a higher elastin content and a greater number of vasa vasorum. It was therefore not used as a control in the histology studies described in *Chapter 8. Chemokine Expression and the Inflammatory Infiltrate in the Abdominal Aortic Aneurysm.* Additionally, thoracic aorta is less likely to suffer from hypoxia than the abdominal aorta and the difference in the location means that the local wall stresses differ between the two regions. Whilst the reason is not fully understood, the abdominal aorta is more frequently the site of aneurysm formation than the thoracic aorta. However, despite these differences thoracic aorta provided a control group that was more closely age- and sex-matched to the AAA group and was taken from patients with atherosclerosis. As an alternative to using thoracic aorta as a control group the data could have been age standardised to take into account the differences in age between the control abdominal aorta and the AAA groups. To demonstrate this, the results of the IL-6 assay in *Chapter 7.* were adjusted for age.

Studies were all performed with the approval of the Leicestershire research ethics committee and written consent was obtained from all participants or next of kin.

5.3 Tissue Collection

Upon excision of the specimen it was collected immediately and initially processed in theatre before being transported to the laboratory. The method for collecting the tissue varied depending on the stage of the project. Freshly excised samples were washed briefly in 0.9% sterile saline to remove blood. The majority of the specimens were divided and the sections were either snap frozen or fixed in 10% formalin. Additionally, for the explant project specimens were also collected in Dulbeccos modification of Eagles medium (DMEM) [DMEM (Sigma #D5671), 4mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin (all supplements from Gibco BRL, NY, USA)]. Due to the limited quantity of thoracic aorta obtained from the punch biopsies these samples were only snap frozen.

Once transported back to the laboratory all snap frozen specimens were stored at -80°C for batch analysis. The 10% formalin fixed samples were fixed for 24 hours at RT and embedded in paraffin wax for long-term storage. Samples collected in DMEM were prepared for culturing as described in *5.9 Explant Culture.*

5.4 Aorta Homogenisation

In order to investigate cytokine expression within the aneurysm wall using the techniques of ELISAs and protein arrays the protein content of the wall needed to be extracted through sample homogenisation.

The snap frozen aortic specimens were weighed, 100mg of tissue was diced into 1mm^2 pieces with a scalpel blade and transferred to a dupont tube on ice. 1ml of homogenising buffer per 100mg of wet weight of specimen was used. Two forms of homogenising buffers were used, depending on the application of the homogenised tissue:

For cytokine arrays:

The tissue was homogenised using a 1x cell lysis buffer [supplemented with 1x complete, mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)] provided by the cytokine protein array 3.1 kit (RayBiotech, Inc. Norcross, GA).

For ELISAs and all other applications:

Homogenates were generated using the in-house homogenising buffer [2M urea, 50mM Tris-HCl, 20mM sodium chloride, 3mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Brij 35, pH 7.6 (supplemented with 1x complete, mini protease inhibitor cocktail)].

All homogenising buffers contained protease inhibitors to prevent proteolysis once the tissues were disrupted which would have resulted in the release of intracellular proteases. All specimens were homogenised using an Ultra-turrax T-25 homogeniser (IKA Labortechnik, Staufen, Germany) for 3 minutes at 13,500rpm. The homogenates were sonicated for 3 x 10 seconds, 12μm amplitude, in a Soniprep 150 sonicator (Sanyo, Watford, UK) to ensure that all cellular membranes were disrupted. Cellular debris was removed by centrifugation at 14,000rpm for 1 hour at 4°C, with no brake. The resulting supernatant was retained and its total protein content was determined.

5.5 Total Protein Quantification of Homogenised Tissue

5.5.1 Bicinchoninic Acid Protein Assay

Quantification of the total protein concentration of the homogenates' supernatant was determined using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, USA). This method uses the reduction of cupric cations (Cu^{2+}) to cuprous cations (Cu^{1+}) by proteins in an alkaline environment. The cuprous cations generated chelate with two molecules of BCA to form a purple coloured solution, which absorbs light at 562nm and is linear with increasing protein concentrations over a working range of 20-2,000μg/ml. The protein concentration of an unknown sample can be determined from the generation of a standard curve of light absorbance against known concentrations of bovine serum albumin (BSA).

Duplicate sets of BSA standards at a concentration range of 20-2,000μg/ml were made from serial dilutions of a stock solution (2mg/ml). The BCA working reagent was prepared from 50 parts Reagent A and 1 part Reagent B. 100μl of standard or unknown homogenate were incubated with 2ml of working reagent for 30 minutes at 37°C. 100μl of deionised water was used instead of a protein solution as a blank. After the incubation period the tubes were cooled to RT and the absorbance at 562nm was measured by a spectrophotometer for each tube. The average value of the blank was subtracted away from all readings and the total protein concentration of the unknown homogenates was determined from the constructed standard curve.

5.5.2. Manipulation of Protein Concentration

In order to directly compare the results of each sample from the arrays and ELISAs all homogenates were manipulated to an equal total protein concentration of 250μg/ml for the arrays and 1mg/ml for the ELISAs. Homogenised samples that were too concentrated were diluted down with phosphate buffered saline, pH 7.45 (PBS) to the appropriate concentration. Samples which were too dilute were concentrated using a microcon YM-3 centrifugal filter device (Millipore (UK) Ltd., Watford, UK) with a 3kDa molecular weight cut off to ensure that the small molecular weight chemokines were retained. 500μl of sample was loaded onto the microcon spin column and centrifuged at 13,000rpm until excess liquid was removed. The column containing the concentrated homogenate was placed inverted into a new 1.5ml microcentrifuge tube and centrifuged at 4,000rpm to transfer the concentrate. Samples were stored in single-use aliquots at -80°C.

5.6 Human Cytokine Protein Array

The simultaneous detection of multiple cytokines within aneurysm tissue was achieved using a cytokine protein array 3.1 kit (RayBiotech, Inc.). The array consisted of duplicate spots of immobilised antibodies specific to 42 different human cytokines (Table 5-1), 6 positive detection spots of biotinylated bovine IgG complexed to horseradish peroxidase-streptavidin (HRP-SA) and 6 negative spots of tris buffered saline (TBS) on a nylon membrane. All reagents, unless otherwise stated, were supplied with the cytokine protein array 3.1 kit.

5.6.1 Array

The detection of cytokines within the homogenised tissue worked on a similar basis as an ELISA (Figure 5-1). Non-specific binding of cytokines to the array membranes was blocked using 2ml of 1x blocking buffer per membrane and incubated overnight at 4°C. The blocking buffer was replaced with 1ml of sample per membrane (250μg/ml) and incubated at RT for 2 hours to allow binding between the cytokines within the homogenate and their specific antibodies. The homogenates were removed and the membranes were thoroughly washed 3 times in 1x wash buffer I and 2 times in 1x wash buffer II. Each wash consisted of agitating the membranes in 2ml of the appropriate wash buffer for 5 minutes. The bound cytokines were detected by a cocktail of biotinylated secondary antibodies, each specific to a single cytokine, at a working concentration of 1:250 dilution in 1x blocking buffer. Each membrane was incubated with 1ml of the secondary antibody cocktail for 2 hours at RT. The membranes were washed as previously described and incubated for 1 hour at RT in 2ml of a 1:1000 dilution of HRP-SA to allow the streptavidin to bind to the biotinylated antibodies. The HRP-SA was removed and the membranes were washed as described. 1ml of the detection buffer, containing the horseradish peroxidase's (HRP) substrate, was added per membrane and incubated at RT for 1 minute. Any HRP present on the membranes converted the substrate to a chemiluminescent signal. The membranes were blotted briefly to remove excess detection buffer and exposed to Kodak BioMax ML Film for 5 minutes. Figure 5-1 shows the working principle of the assay. The intensity of the signal developed as spots on the film and was proportional to the quantity of each specific cytokine present within the tissue homogenates.

Table 5-1. Cytokines present on the protein array.

Array Cytokines	
ENA-78	MCP-2
G-CSF	MCP-3
GM-CSF	M-CSF
GRO	MDC
GRO-α	MIG
I-309	MIP-1δ
IL-1α	RANTES
IL-1β	SCF
IL-2	SDF-1
IL-3	TARC
IL-4	TGF-β1
IL-5	TNF-α
IL-6	TNF-β
IL-7	EGF
IL-8	IGF-1
IL-10	Angiogenin
IL-12	OSM
IL-13	Thrombopoietin
IL-15	VEGF
IFN-γ	PDGF-β
MCP-1	Leptin

For full list of abbreviations see *List of Abbreviations*.

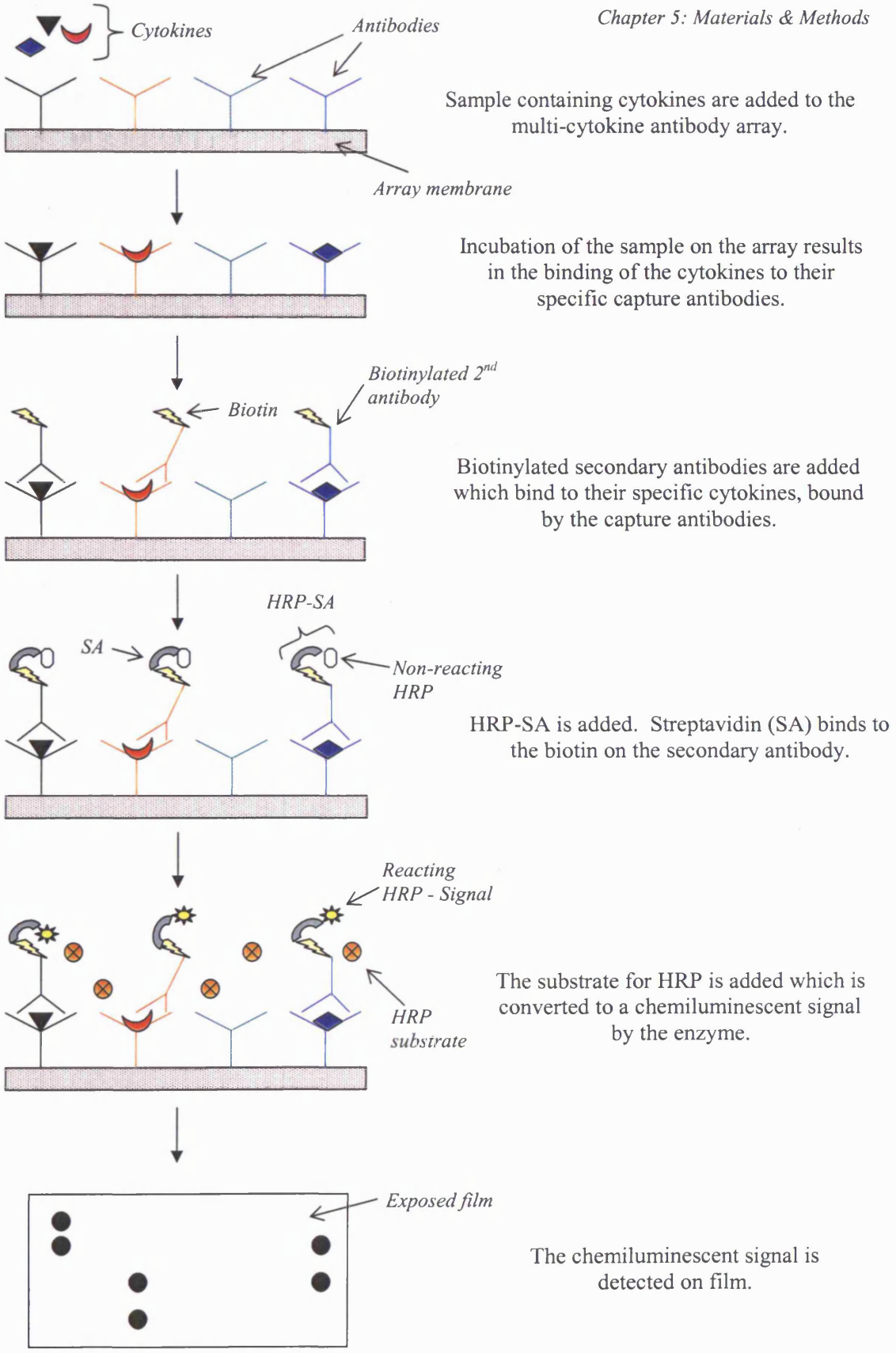


Figure 5-1. Detection of 42 cytokines simultaneously by the cytokine array. A diagram illustrating the experimental stages involved in the detection of cytokines in aortic homogenate using an antibody-based cytokine array.

5.6.2 Spot Densitometry

Relative quantification of the intensity of the cytokine signal from the arrays was determined by spot densitometry using an AlphaImager 1220 analysis and documentation system (Alpha Innotech, Braintree, UK). Images of the array films were captured and the intensity of the spot signals were measured as the sum of the intensity of the pixels under a fixed area. Intensities were corrected for adjacent background. To allow a direct comparison between arrays the cytokine signal intensities within an array were normalised to the array's positive control spot.

5.7 ELISAs

ELISAs enable absolute quantification of specific cytokines through comparison of the optical density (OD) of the unknown specimen to the OD of a standard curve of known concentrations.

5.7.1 Standard ELISAs

The expression of IL-8, MCP-1 and IL-6 in tissue homogenate (BioSource International, Inc., Nivelles, Belgium) and MMP-2 and MMP-9 in culture medium (Amersham Biosciences UK Ltd., Little Chalfont, UK) was measured using commercially available kits according to the manufacturer's protocol. The MMP kits measured the total amount of MMP present (pro-MMP and active).

Whilst the experimental protocols varied between the kits the basic principle of the assays were similar. Each ELISA investigated the expression of one specific protein within a number of samples. A known concentration range was made from serial dilutions of the standard protein at a fixed concentration. Positive controls were either supplied with the ELISA or purchased separately from the manufacturer. Negative controls of the homogenate buffer or culture medium were used to determine the background light absorbance of the solutions. Pre-coated 96-microtitre plates, coated with capture antibody specific to the protein under investigation, were incubated with the samples or standards for a fixed time period to allow binding between the antibodies and the specific protein within the samples/standards. Each sample/standard was measured in duplicate. The samples/standards were aspirated off and the wells were washed numerous times. A

secondary detection antibody, which recognised a second epitope on the protein, was incubated in the wells for a fixed time period to allow binding between antibody and protein. The unbound antibody was removed and the wells were washed thoroughly. In some assays the detection antibody was directly conjugated to HRP. Other assays used a biotinylated secondary antibody which was detected by incubating the wells with HRP-SA for 30 minutes, which allowed the streptavidin to bind to the biotin, and then the wells were washed to remove any unbound HRP-SA. In all assays the amount of immobilised peroxidase in each well was directly proportional to the amount of cytokine captured within each well and was measured by a colorimetric reaction between the HRP and its chromogen substrate (tetramethylbenzidine). In the presence of peroxidase this colourless substrate turned blue. This reaction was stopped by the addition of 1M sulphuric acid resulting in a yellow colour change (Figure 5-2) which was read at 450nm using a microtitre plate spectrophotometer. All wells were background corrected and a standard curve was constructed from the OD of the standards of a known concentration. The concentrations of the unknown samples were interpolated from the standard curve. More detailed information on the separate protocols used is provided in Appendix II.

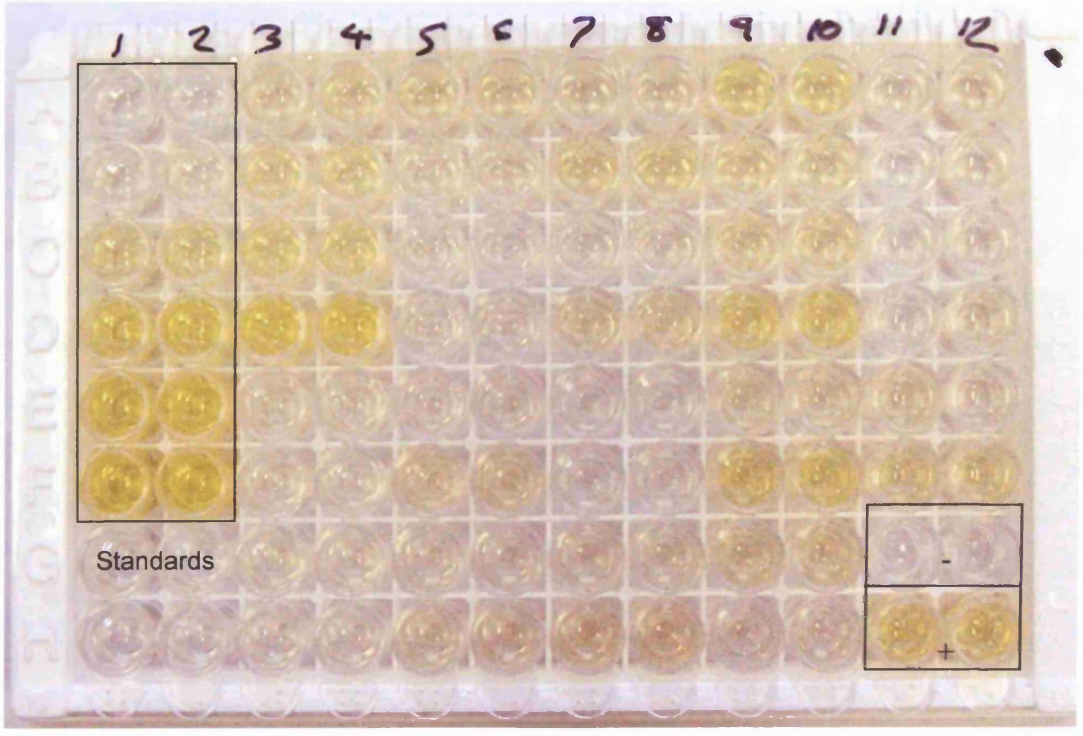


Figure 5-2. Example of an ELISA plate. An ELISA provides an absolute quantified measure of the expression of one specific cytokine. The concentrations of the unknown samples (rows 3-12) are determined from a standard curve constructed from the OD values of the standards versus their concentration.

5.7.2 MMP Activity ELISAs

Whilst the previous MMP ELISAs gave a measure of the total MMP expression within the culture medium, in order to measure the level of endogenous active MMP an additional activity assay was done for MMP-2 and MMP-9. A working concentration range of standards was generated from serial dilutions of the stock pro-MMP standard. 100μl of standards and samples were pipetted into a pre-coated microtitre plate, coated with a capture antibody specific to the MMP under investigation, and incubated overnight at 4°C. The wells containing the captured MMP were washed four times with wash buffer to remove the rest of the sample. 50μl of p-Aminophenylmercuric Acetate (APMA), an activator of pro-MMPs, was added to the pro-MMP standards, whilst 50μl of assay buffer was added to the samples. The activator was not added to the samples in order to measure their endogenous MMP activity level.

For the MMP-2 activity assay 50μl of detection reagent was added immediately to all the wells and the plate was read at 405nm to obtain a time zero absorbance value ($Abs_{t=0}$). The detection reagent contains an enzyme in its inactivated state and its substrate. In the presence of activated MMPs this pro-enzyme is proportionally activated and catalyses the conversion of its colourless substrate to a coloured compound. The conversion of the detection reagent was left to incubate for 3 hours at 37°C and a final absorbance reading at 405nm was obtained ($Abs_{t=final}$).

Following the addition of APMA and assay buffer in the MMP-9 activity assay the plate was incubated for 1.5 hours at 37°C to allow the conversion of the pro-MMP-9 standards to activated MMP-9. 50μl of the detection reagent was pipetted into the wells and the plates were read at 405nm to obtain a time zero absorbance value ($Abs_{t=0}$). The reaction was left to incubate for 2 hours at 37°C and a final absorbance reading at 405nm was obtained ($Abs_{t=final}$).

Calculation of Results

The MMP activity is directly proportional to the generation of colour through the cleavage of a peptide substrate. Therefore the enzymatic activity can be measured through the rate of change of absorbance at 405nm and can be described by the following equation:

$$\frac{Abs_{t=final} - Abs_{t=0}}{h^2}$$

Where h is the incubation time with the detection reagent in hours. The absorbance change is linear with respect to the square of the incubation time.

The concentration of the active MMP in the unknown samples was determined from a standard curve of $\delta Absorbance_{405nm}/h^2$ (y axis) against known concentration of standards, ng/ml (x axis). The unknown concentrations were interpolated from the graph.

5.7.3 MCP-2 ELISA

A ready-to-use ELISA kit was not commercially available and therefore for the detection of MCP-2 a human MCP-2 DuoSet ELISA development system (R&D Systems, (Abingdon, UK) [containing anti-MCP-2 capture and detection antibodies, MCP-2 standards and SA-HRP] was optimised. The final protocol is described below.

A 96-well microplate was coated with 100μl per well of 5μg/ml anti-MCP-2 capture antibody, sealed and incubated at 37°C for two hours. The old solution was removed and the plate was washed three times with wash buffer [0.05% Tween® 20 in PBS, pH 7.2]. Non-specific binding was blocked by adding 300μl of reagent diluent [1% BSA in PBS, pH 7.2, 0.2μm filtered] to the wells and incubating for two hours at RT. The reagent diluent was removed and the wells were washed as before.

The range of standards recommended by the assay ranged from 62.5pg/ml to 4000pg/ml (2-fold dilution). As will be discussed in *Chapter 7.3.4 Monocyte Chemoattractant Protein-2*, the assay was optimised to increase its sensitivity. Validation of the standard curve demonstrated that a range of 31.25pg/ml to 250pg/ml could be used. Standards were diluted down 2-fold in reagent diluent. 100μl of standard, samples and controls [consisting

of a negative control of homogenising buffer and a positive control of recombinant MCP-2 (PeproTech EC, London, UK)] were added to the appropriate wells and were incubated for two hours at RT. For each incubation stage the plates were agitated to increase the sensitivity of the assay. Wells were washed as before. 100μl of the detection antibody at a concentration of 50ng/ml was added to the wells and incubated for 2 hours at RT. Wells were washed as before. 100μl of a 1/50 dilution of the SA-HRP solution was added to each well and incubated for 20 minutes at RT in the dark. Wells were washed as before. 100μl of a substrate solution [1:1 mixture of reagent A (hydrogen peroxide) and reagent B (tetramethylbenzidine) (R&D Systems)] was added to each well and incubated for 20 minutes at RT in the dark. 50μl of stop solution [2N sulphuric acid] was added to the reaction and mixed. The plates were read at 450nm. The data were analysed as described for the standard ELISAs.

5.8 Histology

The structural characteristics, the composition of the inflammatory infiltrate and the localised expression of specific cytokines were studied through a series of specific stains and immunohistochemistry.

5.8.1 Tissue Sectioning for Histology

Paraffin-embedded aortic tissue was used as opposed to frozen sections as the structural integrity of the tissue is better maintained. As describe in *5.3 Tissue Collection*, the aortic tissue had been fixed in 10% formalin prior to paraffin embedding. Tissue sections were sliced at 5μm onto silane coated slides and dried at 37°C overnight.

5.8.2 Haematoxylin and Eosin Staining

General staining of the inflammatory infiltrate and tissue was achieved through haematoxylin and eosin (H&E) staining, with the negatively charged nuclei staining blue with the positively charged haematoxylin. Collagen and elastin being positively charged proteins attracted the negatively charged eosin and stained pink. The mounted tissue sections were dewaxed by passing sequentially through three washes of xylene, then rehydrated by two washes in 99% industrial methylated spirit (IMS), one wash in 95% IMS and finally washed in water. All washes were done for 1 minute. This process is

referred to as "taking to water". The slides were immersed in Mayer's haematoxylin for 5 minutes and the unbound stain was washed off in running water. Slides were then transferred to a bath of 0.5% eosin for 1 minute and excess stain was washed off in running water. The sections were dehydrated by 1 minute immersions in one bath of 95% IMS and two baths of 99% IMS. The slides were finally immersed in three successive baths of xylene and cover-slipped using DPX mounting media (RA Lamb, Eastbourne, UK).

5.8.3 Elastin van Gieson Staining

Elastin van Gieson (EVG) staining was used to assess the extent of elastin loss from AAA specimens and stains elastin black, collagen red and SMCs yellow. Slides were taken to water as described for H&E staining. Sections were immersed for 5 minutes in 0.25% potassium permanganate, followed by submersion in 0.1% oxalic acid for a further 5 minutes. Excess stain was washed off in running water. Slides were washed in 95% ethanol prior to staining with Miller's elastin stain (RA Lamb) for 45 minutes. Slides were washed in 95% ethanol and rinsed off in water. The slides were stained in van Gieson counterstain (RA Lamb) for 2 minutes. The slides were washed in water and the sections were dehydrated, xylene treated and coverslipped as describe for H&E staining.

5.8.4 Immunohistochemistry

Immunohistochemistry was used to identify specific cell types and the localised expression of certain cytokines. The inflammatory infiltrate was characterised through the use of specific primary antibodies against CD markers uniquely expressed on specific cell types. The regional expression of IL-8 and MCP-1 within the AAA wall was visualised using specific antibodies against these cytokines.

<u>Characterisation of the Inflammatory Infiltrate</u>

Formalin fixation can lead to masking of antigens and prevent their exposure to antibodies. In order to counter this various antigen retrieval methods can be used to improve antigen detection by increasing accessibility to the antigen. The optimum antigen retrieval methods and primary antibody concentrations used for each specific staining are shown in Table 5-2. A ChemMate Envision detection kit (DakoCytomation, Ely, UK) was used for detecting the binding of the primary antibodies in accordance with the manufacturer's instructions.

Table 5-2. Primary antibodies used to stain the inflammatory infiltrate.

Immune cell type	Cell-specific CD marker	Primary antibody	Primary antibody dilution factor	Antigen retrieval method
T-cell	CD3	Polyclonal rabbit anti-human CD3*	1:400	Pressure cooker
T_H-cell	CD4	Mouse anti-human CD4, clone 1F6**	1:25	Pressure cooker
T_C-cell	CD8	Mouse anti-human CD8, clone 1A5**	1:200	Pressure cooker
B-cell	CD20	Mouse anti-human CD20cy, clone L26*	1:1000	Citrate buffer, pH 6.0 Microwave
Macrophage	CD68	Mouse anti-human CD68, clone PG-M1*	1:150	Trypsin

The primary antibodies were manufactured by * DakoCytomation or ** Novocastra Laboratories, Newcastle upon Tyne, UK.

As before the slides were taken to water by three xylene washes, then rehydrated by two washes in 99% IMS, 1 wash in 95% IMS and then placed in water. All washes were done for 1 minute. The appropriate antigen retrieval method for the desired staining was used (Table 5-2 & Appendix II). Following antigen retrieval, slides were washed twice in PBS, 5 minutes per wash. 100µl of the appropriately diluted primary antibody was incubated on each slide for 1 hour at RT. PBS was used in place of the antibody as a negative control. Tonsil was used as a positive control. The primary antibody was removed by washing in PBS as described previously. Endogenous peroxidase activity was blocked by incubation with 100µl per slide of a peroxidase blocking solution for 10 minutes at RT. The slides were washed twice in PBS. The secondary antibody was added to the slides in the form of 2-3 drops of the ChemMate Envision complex. This consisted of a mix of secondary anti-mouse and anti-rabbit antibodies complexed to HRP. Slides were incubated for 30 minutes

at RT. The slides were washed in PBS as before and 100µl per slide of the chromogenic solution 3,3'-diaminobenzidine (DAB) was incubated on the slides for 10 minutes. The peroxidase converts DAB to a brown coloured product thereby staining the specific cell types. The DAB was washed off in water for 5 minutes. The tissue samples were counterstained by immersion in haematoxylin dye for 15 seconds. The slides were washed for 2 minutes in water and then dehydrated by 1 minute immersions, once in 95% IMS and twice in 99% IMS. The slides were finally immersed in three successive baths of xylene and coverslipped. The slides were viewed under the light microscope.

Detection of IL-8 and MCP-1

Optimisation of the staining for these cytokines resulted in different detection systems being used. Monoclonal primary antibodies and isotype controls were supplied by R&D Systems.

IL-8 was detected using the R&D Systems' cell and tissue staining kit. Slides were taken to water and treated with the high pH microwaving antigen retrieval method (Appendix II). Slides were washed in PBS for 2 minutes. Endogenous peroxidase activity within the tissue was quenched by incubating the sections with a peroxidase blocking reagent for 5 minutes. Samples were rinsed in PBS and washed in a PBS bath for 5 minutes. Non-specific binding of the antibody was blocked by incubating the slides with serum blocking reagent G for 15 minutes. Excess blocking reagent was wiped off. An avidin blocking reagent was added and the slides were incubated for 15 minutes. Slides were rinsed in PBS and excess PBS was wiped off. A biotin blocking reagent was incubated on the slides for 15 minutes. Slides were rinsed in PBS and wiped to remove excess PBS. 100µl of a monoclonal anti-human IL-8 antibody of mouse origin was incubated on the slides for 1 hour at a working concentration of 30µg/ml. A mouse IgG_1 isotype antibody was used as a negative control. Samples were rinsed with PBS and washed three times in PBS for 15 minute per wash. The primary antibodies were detected by incubating the slides with an anti-mouse biotinylated secondary antibody for 1 hour. Any unbound secondary antibody was removed by rinsing the slides in PBS and washing three times in PBS for 15 minutes per wash. Samples were incubated for 30 minutes with high sensitivity HRP-SA to allow binding between the biotinylated antibodies and the streptavidin. Slides were rinsed to remove unbound SA-HRP and washed three times in PBS for 2 minutes per wash. The antibody/SA-HRP complexes were detected using a 1:10 DAB solution, which was added

to the slides and incubated for 10 minutes. Slides were rinsed in distilled water and washed for 5 minutes in tap water. To counterstain the sections the slides were immersed in Mayer's haematoxylin for 10 seconds and washed in running water for 2 minutes. Slides were taken through progressive concentrations of alcohol and xylene before being coverslipped.

MCP-1 was detected using the ChemMate Envision protocol as described previously. Briefly, slides were taken to water and the trypsin antigen retrieval method was used (Appendix II). The primary antibody used was a monoclonal anti-human MCP-1 antibody of mouse origin, which was used at a working concentration of 20μg/ml. A mouse IgG_{2B} isotype was used as a negative control for non-specific binding of the antibody.

5.9 Explant Culture

The effect of IL-8 on the expression and activation of MMP-2 and MMP-9 within the AAA was measured using an *in vitro* AAA explant system treated with various concentrations of IL-8.

5.9.1 Culture Conditions

AAA specimens were collected from theatre in supplemented DMEM as described in *5.3 Tissue Collection.* The tissue was processed within 1 hour under sterile conditions. AAAs were washed in culture medium to remove blood and any fatty tissue was removed. Four 8mm explant biopsies of the whole thickness of the aortic wall were taken from adjacent sites and each explant was placed into one well of a 6-well plate (tissue culture grade - Nunc A/S, Roskilde, Denmark), luminal side up. 5ml of culture medium [DMEM, 4mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin] was added to each well to ensure that each explant was bathed in culture medium. Plates were incubated at 37°C in an atmosphere of 95% air: 5% carbon dioxide. The medium was changed after 24 hours and discarded (day 0).

5.9.2 IL-8 Treatment

After the first medium change (day 0), fresh untreated medium was added and the explants were incubated for a further 24 hours. After this pre-treatment period the medium was

replaced with fresh medium supplemented with IL-8 at various concentrations. An aliquot of the pre-treatment medium was taken from each explant, centrifuged (13,000rpm for 1 minute) to remove debris and stored at -80°C (day 1). Three out of the four explants were treated with IL-8 concentrations of 1ng/ml, 10ng/ml, or 100ng/ml. The fourth explant was treated with an equivalent volume of PBS (non-treated control).

Subsequently every 48 hours the medium was replaced with fresh cytokine supplemented medium. The explants were kept in culture for 7 days. At day 7 aliquots of the medium from the four explants was collected and stored at -80°C. MMP expression and activity was measured as described in *5.7 ELISAs*.

5.10 Statistical Analysis

All analyses were performed with SPSS, version 12.0 software. The Kruskal-Wallis test was used to determine if a significant difference existed between the medians of three populations. In the event that there was a significant difference, multiple Mann-Whitney *U* tests were performed on each paired combination to identify which population medians were significantly different. Multiple comparisons were corrected for, as described in *Chapter 6 and 7.* An age-adjusted analysis of IL-6 expression between aortic groups was performed by logging IL-6 concentration and using a generalised linear model to compare the mean log IL-6 expression between groups, adjusted for age. A Chi-square test was used to determine an association between two factors, as described in *Chapter 8.* Unless otherwise stated a P value of $<.05$ was used to determine significance.

Chapter Six

The Cytokine Expression Profile of the Abdominal Aortic Aneurysm

Chapter 6. The Cytokine Expression Profile of the Abdominal Aortic Aneurysm

6. The Cytokine Expression Profile of the Abdominal Aortic Aneurysm

6.1 Introduction

The chronic inflammatory nature of the AAA wall is a potentially cytokine-enriched environment. Specific cytokines have been described within the AAA wall, including IL-1β, IL-6 and TNF-α[82, 200, 227, 296]. However, many reports of cytokine expression within the AAA wall are contradictory with some reports suggesting the expression of a T_{H1} response[161, 164, 299], whilst others support the dominant expression of a T_{H2} response[84, 302, 306]. A review of the literature surrounding cytokine expression within the AAA wall is given in *Chapter 3.5 Cytokines and Abdominal Aortic Aneurysms.* With the conflicting studies it has not been possible to define a definite panel of cytokines which are differentially expressed within the AAA. Therefore the role cytokines may have in the development of the AAA remains unclear.

From current research it is difficult to obtain an overview of cytokine expression within the AAA because it is impossible to compare across the studies due to the variations in experimental techniques used, the stage of aneurysm formation of the specimens and the control groups used. Previous studies have used techniques that have only allowed the study of a small repertoire of cytokines, such as ELISAs, western blotting, reverse transcriptase polymerase chain reaction (RT-PCR). However, these techniques require the selection of candidate cytokines by the investigator and can miss novel, unanticipated changes in the cytokine profile. An alternative approach, which addresses the limitations of the previous techniques, is through the use of an array allowing the simultaneous screening of multiple cytokines within a sample. The additional advantage is that by simultaneously screening multiple cytokines the array can reveal potentially synergistic or antagonistic relationships between cytokines and identify cases where cytokine redundancy may mean that the absence of one cytokine is covered by the expression of another.

This investigation describes the first protein array study used to screen for the expression of multiple cytokines within the AAA. The aim of this chapter is to investigate the cytokine expression profile of the AAA wall using a protein array specific to 42 cytokines

and compare this to control tissue in order to identify a cytokine profile specific to the AAA wall.

The null hypothesis is that there are no significant differences in the expression of any of the 42-cytokines examined between the AAA, the abdominal aorta and the thoracic aorta.

6.2 Methods

Specimens from the infrarenal aorta were obtained from patients undergoing open AAA repair (n = 10). Two non-aneurysmal control groups were used, as discussed in *5.2 Study Design*, abdominal aorta from cadaveric kidney donors (n = 9) and thoracic aorta obtained from patients undergoing aorto-coronary bypass graft operations (n = 12). The characteristics of the three groups are shown in Table 6-1. All tissue was snap frozen in liquid nitrogen upon collection and stored at –80°C for batch analysis.

Table 6-1. Demographics of the study population.

Risk Factor	AAA (n = 10)	Abdominal aorta* (n = 9)	Thoracic aorta (n = 12)
Median age (range) / years	73 (67-81)	55 (44-74)	66 (57-69)
Male sex, n (%)	10 (100)	6 (67)	11 (92)
Smoking history, n (%)	8 (80)	8 (89)	9 (75)
Hypertension, n (%)	7 (70)	-	9 (75)
Myocardial infarction, n (%)	5 (50)	-	7 (58)
Angina, n (%)	5 (50)	-	9 (75)
Cerebrovascular accident, n (%)	2 (20)	-	0 (0)
Diabetes, n (%)	0 (0)	-	2 (17)
Median AAA diameter (range) /mm	75 (56-93)	n/a	n/a

* Only a brief medical history was supplied with the abdominal aorta from kidney donors.

As described in *Chapter 5.4-5.6*, all specimens were homogenised and manipulated to a total protein concentration of 250μg/ml. Each homogenised sample was incubated on a 42-cytokine array to simultaneously detect the level of expression of 42 cytokines within the aortic sample. The cytokine array 3.1 was selected for this study because it consisted of a broad range of cytokines involved in the regulation of inflammatory responses.

The chemiluminescent signal from the captured cytokines was detected on film as a spot. The intensity of the signal was proportional to the amount of cytokine captured. Spot densitometry measured the intensity of the spots, which were corrected for adjacent background. The signal intensity value for each cytokine was determined from the mean value of the duplicate spots. To allow a direct comparison between arrays each signal intensity value was normalised to the membrane's positive control. The positive controls consisted of six spots of a fixed quantity of HRP-SA on each membrane and the mean positive signal intensity value was used for normalisation. As the signal from the positive spots should have been constant between the arrays, any changes in the detection signal resulting from inter-assay variation at the detection step were removed by normalising the data. The negative array spots consisted of spots of PBS minus capture antibody. For each aortic group the overall expression of each specific cytokine was expressed as the median signal intensity value (interquartile range).

For each cytokine the data from the three aortic groups were compared using the Kruskal-Wallis test and further analysed by a series of Mann-Whitney *U* tests. With the number of statistical tests required to analyse the expression of 42 cytokines in three aortic groups, a P value of $<.01$ was used to determine significance. The more stringent P value was used in order to compensate for multiple comparisons, based on the advice from the Medical Statistics Group, University of Leicester.

6.3 Results

The cytokines within each sample were captured on the arrays and the resulting chemiluminescent signal was detected by exposing the arrays to film. Figure 6-1 shows cytokine protein arrays representative of each aortic group.

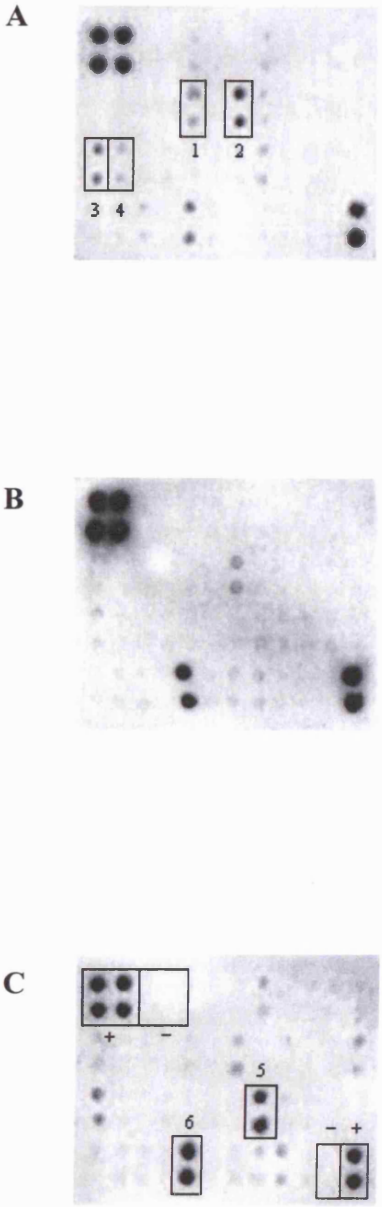


Figure 6-1. Representative cytokine arrays. Arrays representative of (A) the AAA group and the controls, (B) abdominal aorta and (C) thoracic aorta are shown. Arrays consisted of 6 internal positive control spots (+), 6 negative spots (-) and 42 specific anti-human cytokine antibodies spotted in duplicate. The AAA array shows, as an example, the position of several cytokines on the arrays (in duplicate); IL-6 (1), IL-8 (2), MCP-1 (3) and MCP-2 (4). The thoracic aorta array shows the position of RANTES (5) and angiogenin (6).

6.3.1 Aortic Cytokine Profiles

The intensity of the spots was quantified by spot densitometry, as described previously, and the resulting median signal intensity values and interquartile ranges for all 42 cytokines for the three groups are shown in Table 6-2*A* and 6-2*B*.

AAA Profile

Figure 6-2 shows the cytokine profile of the AAA. Out of the ten most highly expressed cytokines in the AAA group six were chemokines, the remainder consisted of one pro-inflammatory cytokine, one anti-inflammatory cytokine and two growth factors.

IL-8 was the most highly expressed cytokine within the AAA group [median signal intensity value (interquartile range) 0.68 (0.49-0.80)]. Other cytokines which were highly expressed within the aneurysm, in descending order of median expression, were: angiogenin [0.50 (0.41-0.60)], MCP-1 [0.41 (0.20-0.48)], RANTES [0.18 (0.11-0.23)], IL-6 [0.15 (0.08-0.23)], MCP-2 [0.14 (0.13-0.18)], IL-10 [0.12 (0.04-0.14)], GRO [0.10 (0.09-0.14)], EGF [0.09 (0.08-0.17)] and ENA-78 [0.09 (0.00-0.12)] (Figure 6-2, Tables 6-2*A* & *B*).

Abdominal Aorta Profile

The most highly expressed cytokines in the abdominal and thoracic aorta were predominately chemokines and growth factors. The most highly expressed cytokine in the abdominal aorta was angiogenin [0.82 (0.17-0.98)], followed, in descending order, by IL-8 [0.16 (0.14-0.31)], thrombopoietin [0.04 (0.00-0.06)], EGF [0.03 (0.02-0.07)], VEGF [0.03 (0.01-0.09)] & MCP-1 [0.03 (0.00-0.25)] (Table 6-2*A* & *B*).

Thoracic Aorta Profile

Angiogenin was also the most highly expressed cytokine in the thoracic aorta [0.93 (0.67-1.24)], followed by RANTES [0.34 (0.22-0.83)], IL-8 [0.24 (0.16-0.39)], GRO [0.11 (0.03-0.29)], TNF-β [0.10 (0.03-0.20)], VEGF [0.10 (0.06-0.29)], MCP-1 [0.09 (0.03-0.28)], thrombopoietin [0.09 (0.06-0.15)], IL-1α [0.08 (0.04-0.10)], EGF [0.07 (0.02-0.13)] and M-CSF [0.07 (0.04-0.16)] (Table 6-2*A* & *B*).

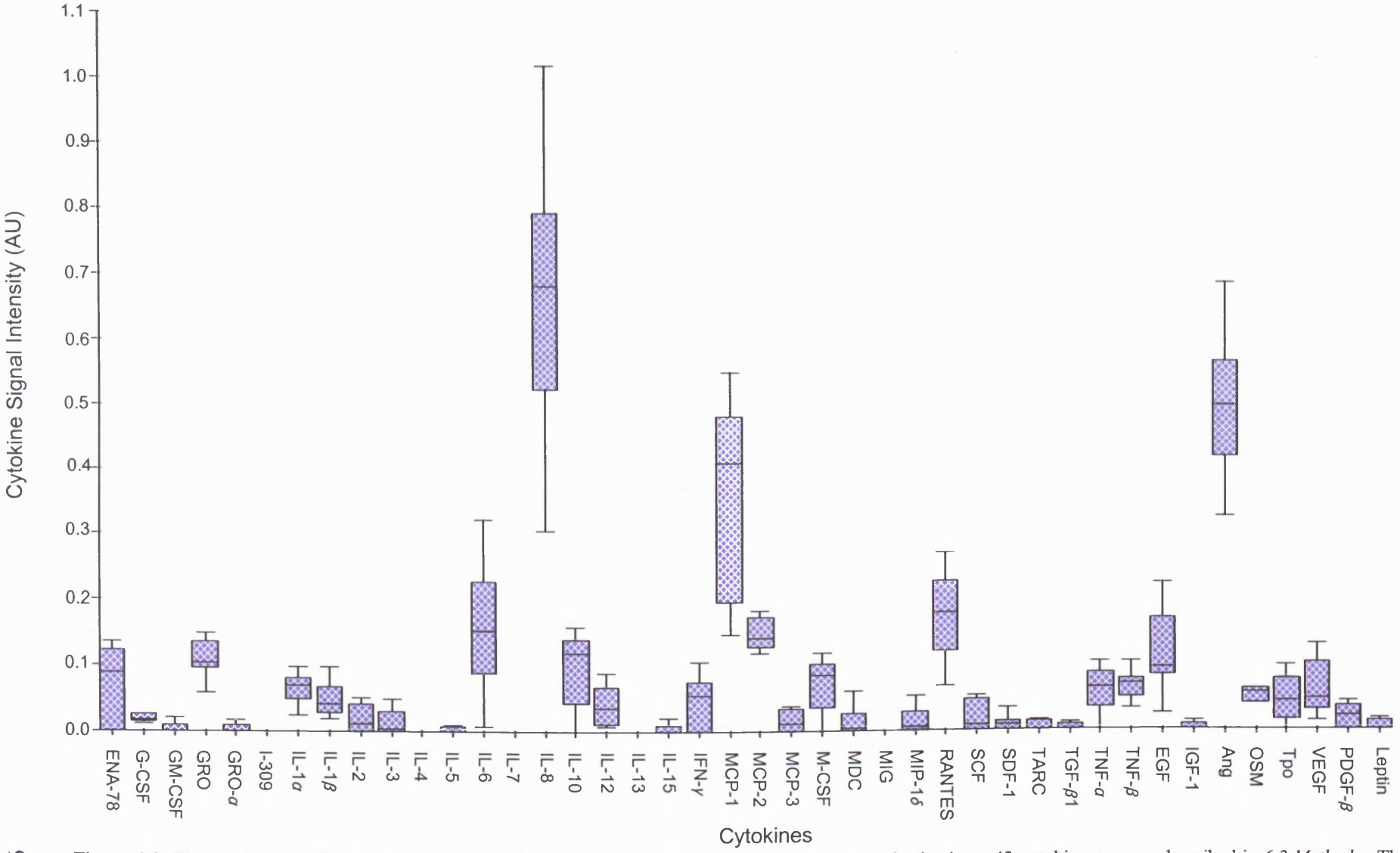


Figure 6-2. The cytokine profile of the AAA. The cytokine profile of 10 AAA specimens was determined using a 42-cytokine array as described in *6.2 Methods*. The boxplots present the median signal intensity value (internal bar) and interquartile range (box) for each cytokine. The range is shown as whiskers above and below the boxes. *Ang*, angiogenin; *Tpo*, thrombopoietin.

Table 6-2*A*. Median signal intensity values for those cytokines which were significantly different between at least two groups, as determined by a Kruskal-Wallis test.

Cytokines	Median signal intensity value (interquartile range)			Kruskal-Wallis P value
	AAA (n = 10)	Abdominal aorta (n = 9)	Thoracic aorta (n = 12)	
Significant results ($P < .01$)				
ENA-78	0.09 (0.00-0.12)	0.00 (0.00-0.00)	0.00 (0.00-0.04)	.006
GRO	0.10 (0.09-0.14)	0.00 (0.00-0.01)	0.11 (0.03-0.29)	<.001
IL-1α	0.07 (0.04-0.09)	0.00 (0.00-0.00)	0.08 (0.04-0.10)	.001
IL-1β	0.04 (0.03-0.07)	0.00 (0.00-0.00)	0.01 (0.00-0.04)	<.001
IL-6	0.15 (0.08-0.23)	0.01 (0.00-0.02)	0.00 (0.00-0.01)	<.001
IL-8	0.68 (0.49-0.80)	0.16 (0.14-0.31)	0.24 (0.16-0.39)	<.001
IL-10	0.12 (0.04-0.14)	0.00 (0.00-0.00)	0.05 (0.01-0.08)	.004
IL-15	0.00 (0.00-0.01)	0.00 (0.00-0.01)	0.06 (0.01-0.14)	.003
MCP-1	0.41 (0.20-0.48)	0.03 (0.00-0.25)	0.09 (0.03-0.28)	.003
MCP-2	0.14 (0.13-0.18)	0.00 (0.00-0.05)	0.07 (0.00-0.13)	<.001
M-CSF	0.08 (0.04-0.10)	0.01 (0.00-0.02)	0.07 (0.04-0.16)	.001
RANTES	0.18 (0.11-0.23)	0.02 (0.00-0.05)	0.34 (0.22-0.83)	<.001
TGF-β1	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.04 (0.00-0.06)	.002
TNF-α	0.06 (0.03-0.09)	0.00 (0.00-0.01)	0.02 (0.01-0.11)	.004
TNF-β	0.07 (0.04-0.08)	0.02 (0.01-0.04)	0.10 (0.03-0.20)	.005
Thrombopoietin	0.04 (0.01-0.08)	0.04 (0.00-0.06)	0.09 (0.06-0.15)	.004
PDGF-β	0.02 (0.00-0.04)	0.00 (0.00-0.00)	0.05 (0.01-0.25)	.005

Table 6-2*B*. Median signal intensity values for those cytokines which were not significantly different between groups, as determined by a Kruskal-Wallis test.

Cytokines	Median signal intensity value (interquartile range)			Kruskal-Wallis P value
	AAA (n = 10)	Abdominal aorta (n = 9)	Thoracic aorta (n = 12)	
Non-significant results (P > .01)				
G-CSF	0.02 (0.01-0.03)	0.00 (0.00-0.00)	0.01 (0.00-0.03)	.012
IL-12	0.04 (0.01-0.07)	0.00 (0.00-0.04)	0.05 (0.00-0.07)	.046
IFN-γ	0.06 (0.00-0.08)	0.00 (0.00-0.00)	0.03 (0.00-0.30)	.014
EGF	0.09 (0.08-0.17)	0.03 (0.02-0.07)	0.07 (0.02-0.13)	.045
Angiogenin	0.50 (0.41-0.60)	0.82 (0.17-0.98)	0.93 (0.67-1.24)	.021
OSM	0.06 (0.03-0.07)	0.01 (0.00-0.02)	0.07 (0.01-0.11)	.020
VEGF	0.05 (0.03-0.10)	0.03 (0.01-0.09)	0.10 (0.06-0.29)	.031
Leptin	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.02 (0.00-0.14)	.013
GM-CSF	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	.220
GRO-α	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.00 (0.00-0.05)	.085
I-309	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	.652
IL-2	0.01 (0.00-0.04)	0.00 (0.00-0.01)	0.03 (0.00-0.09)	.141
IL-3	0.00 (0.00-0.03)	0.00 (0.00-0.00)	0.01 (0.00-0.04)	.183
IL-4	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.02)	.232
IL-5	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	.161
IL-7	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	1.00
IL-13	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	.981
MCP-3	0.01 (0.00-0.03)	0.00 (0.00-0.03)	0.02 (0.00-0.07)	.501
MDC	0.00 (0.00-0.03)	0.01 (0.00-0.02)	0.02 (0.01-0.03)	.235
MIG	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.05)	.133
MIP-1δ	0.01 (0.00-0.03)	0.00 (0.00-0.02)	0.00 (0.00-0.03)	.576
SCF	0.01 (0.00-0.05)	0.00 (0.00-0.02)	0.03 (0.00-0.06)	.261
SDF-1	0.01 (0.00-0.02)	0.01 (0.00-0.02)	0.01 (0.00-0.08)	.913
TARC	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.00 (0.00-0.01)	.485
IGF-1	0.00 (0.00-0.01)	0.00 (0.00-0.00)	0.00 (0.00-0.04)	.168

6.3.2 Differential Cytokine Expression

In addition to the median signal intensity values and interquartile ranges for all 42 cytokines, Tables 6-2*A* & *B* show the results of the Kruskal-Wallis analysis for each cytokine. Table 6-2*A* displays the cytokines which were found to be significantly different between at least two of the groups ($P < .01$). Table 6-2*B* displays those cytokines that were not significantly different between any of the groups.

The results of the Kruskal-Wallis analysis of the signal intensity data from the three aortic groups revealed that 17/42 cytokines were significantly different between at least two aortic groups ($P < .01$) (Table 6-2*A*).

Post-hoc analysis of the expression of each cytokine was done using multiple Mann-Whitney tests to identify which combination(s) of aortic group pairs were significantly different from each other ($P < .01$). The results of the Mann-Whitney analyses are shown in Table 6-3*A* & *B*. Table 6-3*A* displays those cytokines which were found to be significantly different between at least one group pair. Table 6-3*B* displays those cytokines that were not significantly different between any of the group pairs. Slight discrepancies are apparent between the results of the Kruskal-Wallis analyses and the Mann-Whitney tests. For example, G-CSF is not significantly different between any of the aortic groups when compared by the Kruskal-Wallis test ($P = .012$). However, when the AAA and abdominal aorta groups are compared by a Mann-Whitney analysis these groups are significantly different ($P = .003$). These discrepancies may arise as a Mann-Whitney test cannot analyse data from all three groups within the same test, unlike the Kruskal-Wallis test, and with differences in their calculations the Mann-Whitney test is not guaranteed to give results consistent with the Kruskal-Wallis test[326]. Additionally, it may be that by using a P value of $< .01$ the results are at an increased risk of false negative results.

From the Mann-Whitney analyses significant differences were found in 15/42 cytokines between the AAA and the abdominal aorta; 8/42 cytokines in the AAA compared to the thoracic aorta and 11/42 cytokines in the abdominal aorta compared to the thoracic aorta (Table 6-*3A* and Figures 6-3 to 6-8).

Table 6-3*A*. Post-hoc analysis of cytokine expression by paired groups using a Mann-Whitney *U* test – Cytokines that displayed at least one significant difference between aortic groups (P < .01).

Cytokines	AAA vs. Abdominal aorta (P value)	AAA vs. Thoracic aorta (P value)	Abdominal vs. Thoracic aorta (P value)
ENA-78	**.006**	.020	.091
G-CSF	**.003**	.261	.055
GRO	**<.001**	.843	**<.001**
IL-1α	**.001**	.575	**.001**
IL-1β	**<.001**	.024	.018
IL-6	**.001**	**<.001**	.215
IL-8	**.001**	**.001**	.201
IL-10	**.002**	.120	.018
IL-15	.792	**.005**	**.005**
IFN-γ	.012	.665	**.008**
MCP-1	**.003**	**.004**	.568
MCP-2	**<.001**	**.005**	.125
M-CSF	**.004**	.598	**.001**
RANTES	**.001**	**.008**	**<.001**
TGF-β1	.360	**.006**	**.003**
TNF-α	**.002**	.303	**.006**
TNF-β	**.002**	.155	.010
Angiogenin	.624	**.005**	.102
OSM	**.007**	.741	.025
Thrombopoietin	.362	.010	**.003**
PDGF-β	.122	.039	**.003**
Leptin	.083	.078	**.008**

P values are unadjusted, due to multiple testing a P value of <.01 is considered significant. Significant P values are shown in bold.

Table 6-3*B*. Post-hoc analysis of cytokine expression by paired groups using a Mann-Whitney *U* test – Cytokines that were not significantly different between aortic groups (P <. 01).

Cytokines	AAA vs. Abdominal aorta (P value)	AAA vs. Thoracic aorta (P value)	Abdominal vs. Thoracic aorta (P value)
IL-12	.017	.947	.044
EGF	.011	.166	.271
VEGF	.462	.056	.016
GM-CSF	.083	.473	.209
GRO-α	.083	.379	.032
I-309	.343	.947	.386
IL-2	.251	.311	.060
IL-3	.120	.725	.088
IL-4	.878	.151	.216
IL-5	.083	.260	.386
IL-7	1.00	1.00	1.00
IL-13	1.00	.843	.889
MCP-3	.546	.682	.215
MDC	1.00	.180	.131
MIG	.909	.126	.088
MIP-1δ	.251	.672	.626
SCF	.317	.511	.107
SDF-1	.967	.838	.604
TARC	.235	.760	.373
IGF-1	.083	.811	.062

The comparative cytokine expression profiles of the AAA, abdominal aorta and thoracic aorta are shown in Figures 6-3 to 6-8. The cytokines have been grouped on the basis of their main function, due to the pleiotropic nature of cytokines several of the cytokines could have fitted into more than one category.

CXC Chemokines

Figure 6-3 shows the expression of the chemokines on the array which belong to the CXC subfamily. Interestingly, IL-8 was significantly higher in the AAA than in the abdominal aorta (4-fold, P = .001) and in the thoracic aorta (3-fold, P = .001).

The CXC chemokines ENA-78 and GRO were significantly higher in the AAA than in the abdominal aorta, where they were not expressed (P = .006 & P < .001, respectively). However, there was no significant difference in the expression of these chemokines between the AAA and the thoracic aorta.

There were no significant differences in the expression of GRO-α, MIG and SDF-1 between any of the aortic groups and these cytokines were negligibly expressed. The only significant difference in CXC chemokine expression between the abdominal aorta and thoracic aorta was seen in GRO (P < .001), which was expressed in the thoracic aorta but not in the abdominal aorta.

CC Chemokines

The CC chemokines on the array are shown in Figure 6-4. Out of the CC chemokines only MCP-1 and MCP-2 were significantly higher in the AAA group than in the abdominal aorta (14-fold, P = .003 & P < .001, respectively) and the thoracic aorta (5-fold, P = .004 & 2-fold, P = .005, respectively). MCP-2 was not expressed in abdominal aorta.

RANTES was also significantly elevated in the AAA compared to abdominal aorta (9-fold, P = .001) but was significantly lower in the AAA than in the thoracic aorta (2-fold, P = .008). There were no significant differences between the AAA group and the abdominal aorta or thoracic aorta for I-309, MCP-3, MDC, MIP-1δ and TARC.

RANTES was also significantly higher in the thoracic aorta than in the abdominal aorta (17-fold, P < .001). The remainder of the CC chemokines were not significantly different between the abdominal aorta and thoracic aorta.

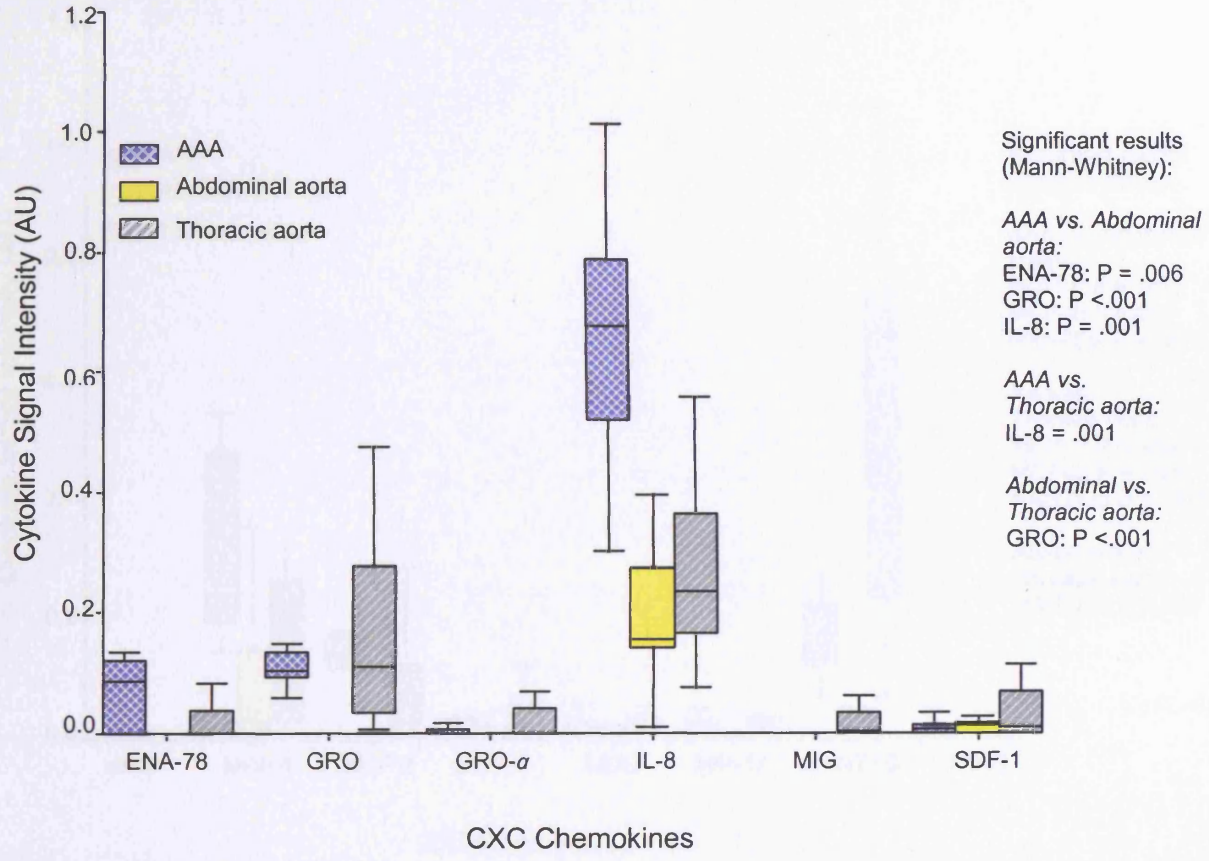


Figure 6-3. Differential expression of CXC chemokines in the AAA. The figure displays the expression of CXC chemokines in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each chemokine. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each chemokine. The significant results (P < .01) are displayed on the graph.

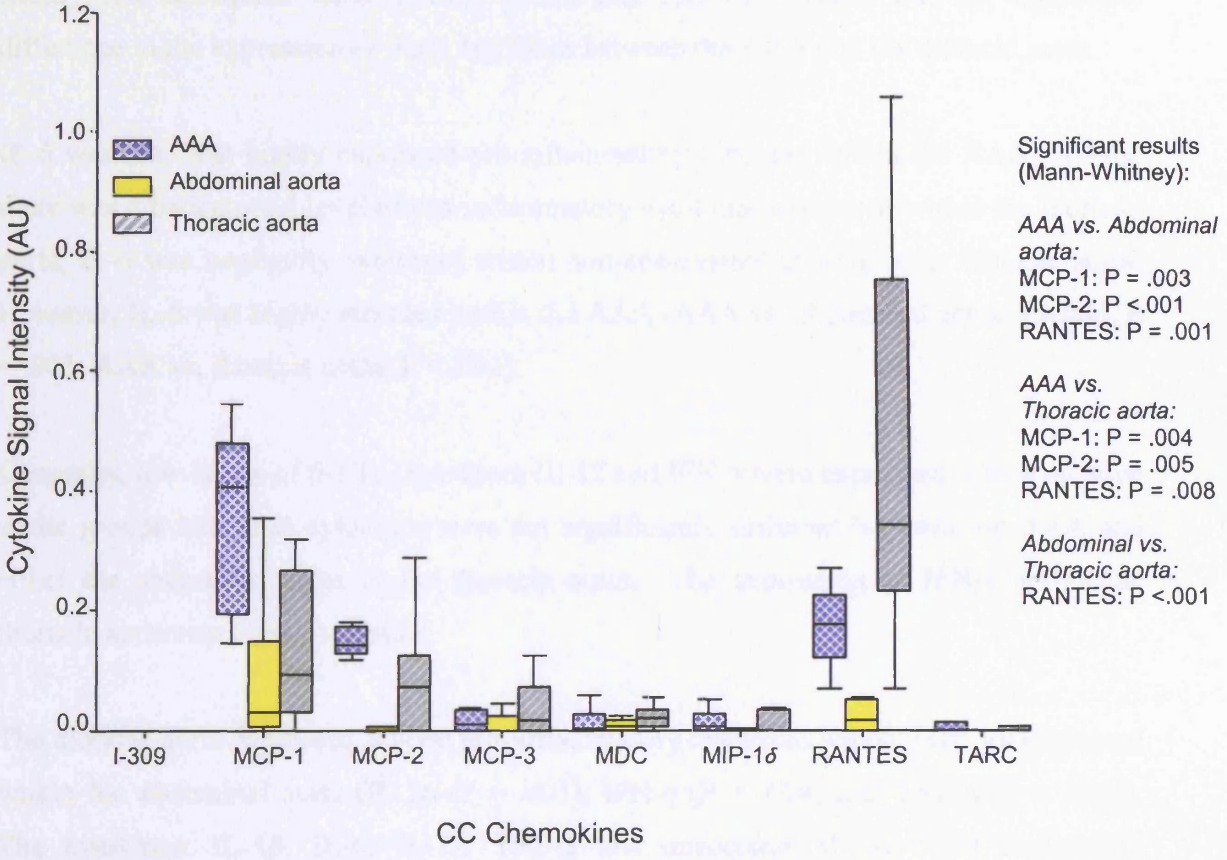


Figure 6-4. Differential expression of CC chemokines in the AAA. The figure displays the expression of CC chemokines in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each chemokine. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each chemokine. The significant results (P < .01) are displayed on the graph.

Pro-inflammatory Cytokines

The pro-inflammatory cytokines in Figure 6-5 showed a significant increase in IL-1α (P = .001), IL-1β (P < .001), TNF-α (P = .002), TNF-β (3.5-fold, P = .002) and oncostatin M (6-fold, P = .007) in the AAA compared to the abdominal aorta. Several pro-inflammatory cytokines were expressed in the AAA which were not present in the non-aneurysmal abdominal aorta (IL-1α, IL-1β and TNF-α). There was no significant difference in the expression of these cytokines between the AAA and the thoracic aorta.

IL-6 was the most highly expressed pro-inflammatory cytokine within the AAA. Whilst there was a background level of pro-inflammatory cytokines expressed within the thoracic aorta, IL-6 was negligibly expressed within non-aneurysmal abdominal or thoracic aorta. However, IL-6 was highly elevated within the AAA (AAA vs. abdominal aorta: 15-fold, P = .001; AAA vs. thoracic aorta, P < .001).

Generally, low levels of the T_{H1} cytokines IL-12 and IFN-γ were expressed within all three aortic groups but these cytokines were not significantly different between the AAA and either the abdominal aorta or the thoracic aorta. The expression of IFN-γ within the thoracic aorta was highly variable.

The thoracic aorta expressed several pro-inflammatory cytokines which were not expressed within the abdominal aorta (IL-1α (P = .001), IFN-γ (P = .008) and TNF-α (P = .006)). The cytokines: IL-1β, IL-6, IL-12, TNF-β and oncostatin M were not statistically significantly different between abdominal aorta and thoracic aorta.

Anti-inflammatory Cytokines

Figure 6-6 shows the anti-inflammatory cytokines. IL-4 and IL-13 were negligibly expressed in all aortic groups. The T_{H2} anti-inflammatory cytokine IL-10 was significantly higher in the AAA compared to the abdominal aorta where it was not expressed (P = .002), however, this did not extend to a comparison of IL-10 expression in the AAA versus thoracic aorta. There was no significant difference in the expression of IL-10 between the abdominal or thoracic aorta.

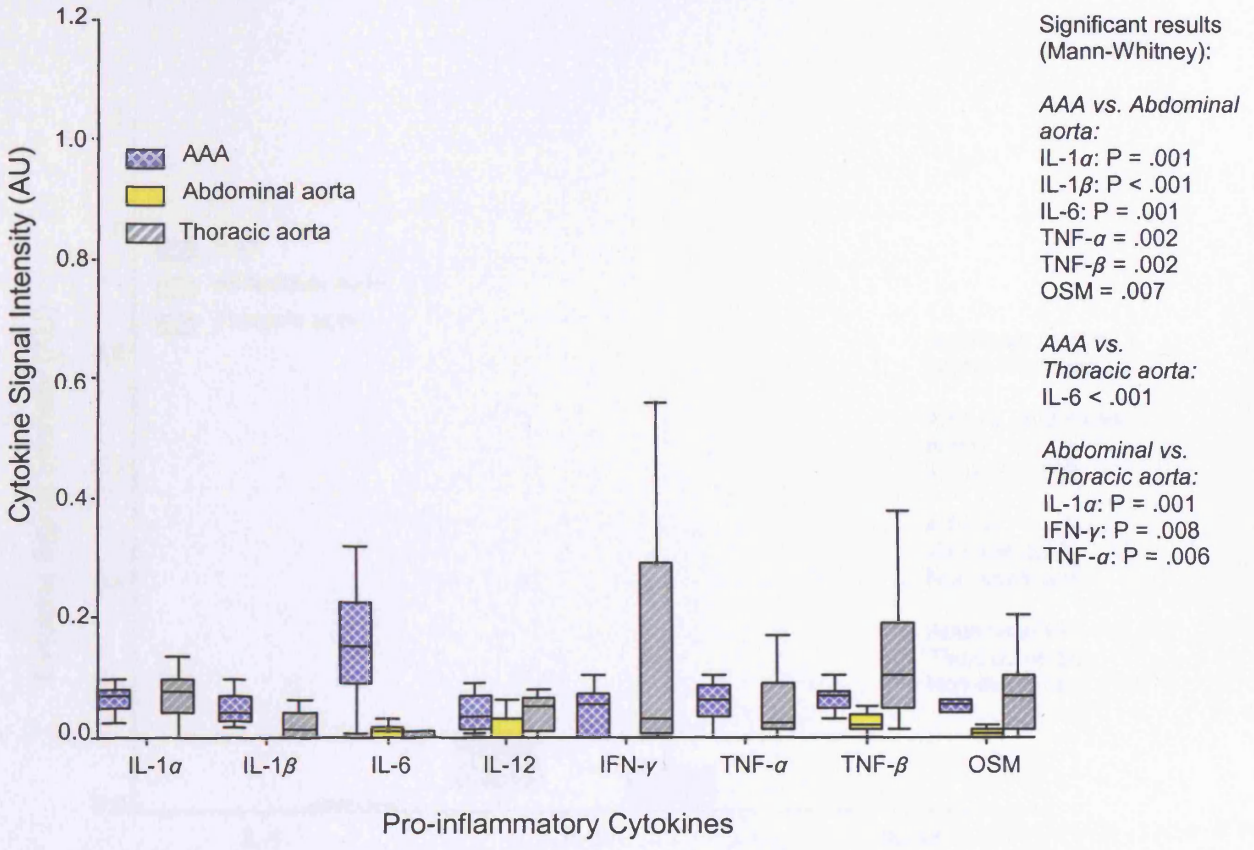


Figure 6-5. Differential expression of pro-inflammatory cytokines in the AAA. The figure displays the expression of pro-inflammatory cytokines in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each cytokine. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each cytokine. The significant results (P < .01) are displayed on the graph.

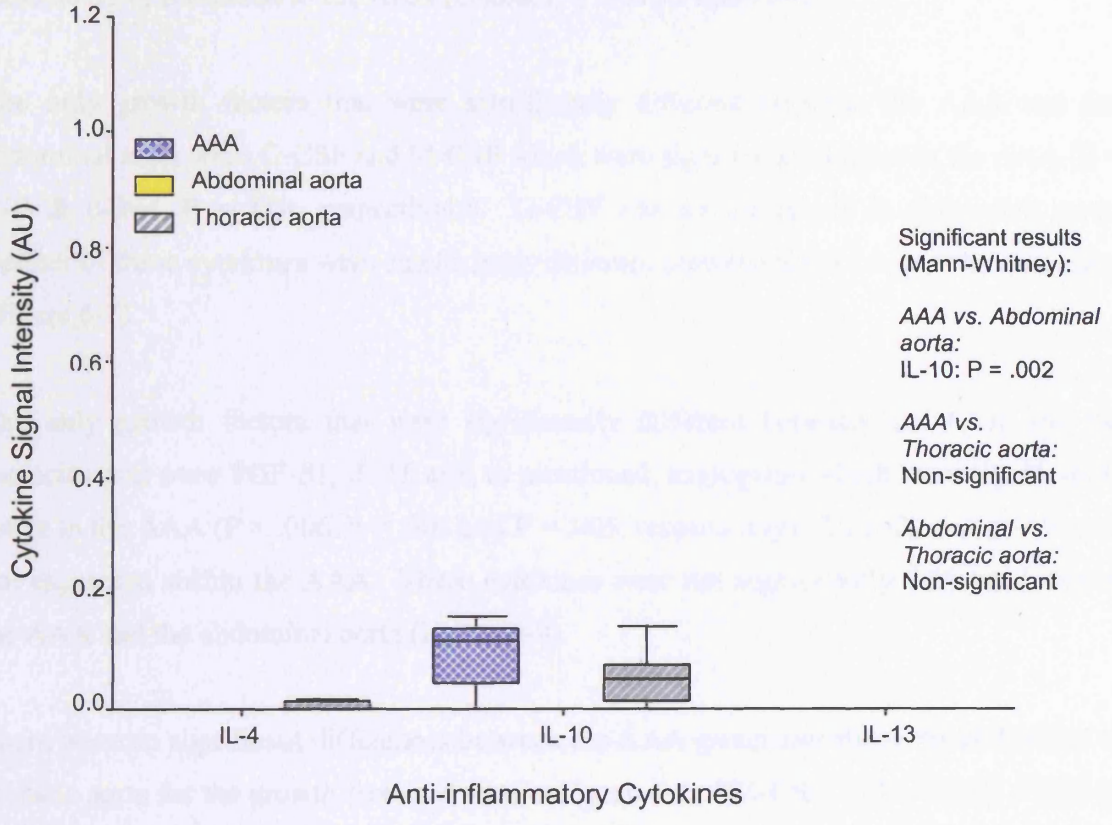


Figure 6-6. Differential expression of anti-inflammatory cytokines in the AAA. The figure displays the expression of anti-inflammatory cytokines in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each cytokine. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each cytokine. The significant results ($P < .01$) are displayed on the graph.

Growth Factors

The remainder of the cytokines on the arrays were growth factors, as shown in Figure 6-7 and 6-8. Out of the growth factors angiogenin was the mostly highly expressed growth factor in aortic tissue. Whilst there was no significant difference in the expression of angiogenin between the AAA and abdominal aorta, it was significantly higher in the thoracic aorta compared to the AAA (2-fold, P = .005) (Figure 6-8).

The only growth factors that were significantly different between the AAA and the abdominal aorta were G-CSF and M-CSF which were significantly higher in the AAA (P = .003 & 8-fold, P = .004, respectively). G-CSF was not expressed in abdominal aorta. Neither of these cytokines were significantly different between the AAA and thoracic aorta (Figure 6-7).

The only growth factors that were significantly different between the AAA and the thoracic aorta were TGF-β1, IL-15 and, as mentioned, angiogenin which were significantly lower in the AAA (P = .006, P = .005 and P = .005, respectively). TGF-β1 and IL-15 were not expressed within the AAA. These cytokines were not significantly different between the AAA and the abdominal aorta (Figure 6-8).

There were no significant differences between the AAA group and either the abdominal or thoracic aorta for the growth factors shown in Figure 6-7: GM-CSF, SCF, VEGF, PDGF-β, EGF and IGF-1. As shown in Figure 6-8, there were also no significant differences between the AAA group and either the abdominal or thoracic aorta for leptin, thrombopoietin and the interleukin family: IL-2, IL-3, IL-5 and IL-7.

A comparison of abdominal aorta and thoracic aorta revealed a regional difference in growth factor expression. The growth factors M-CSF, PDGF-β were significantly higher in the thoracic aorta compared to the abdominal aorta (7-fold, P = .001 and P = .003, respectively) (Figure 6-7); as were the growth factors TGF-β1, leptin, IL-15 and thrombopoietin (P = .003; P = .008; P = .005; 2-fold, P = .003) (Figure 6-8). PDGF-β, TGF-β1, leptin and IL-15 were not expressed in abdominal aorta. G-CSF, GM-CSF, SCF, VEGF, EGF, IGF-1, IL-2, IL-3, IL-5, IL-7 and angiogenin were not significantly different between the abdominal and thoracic aorta.

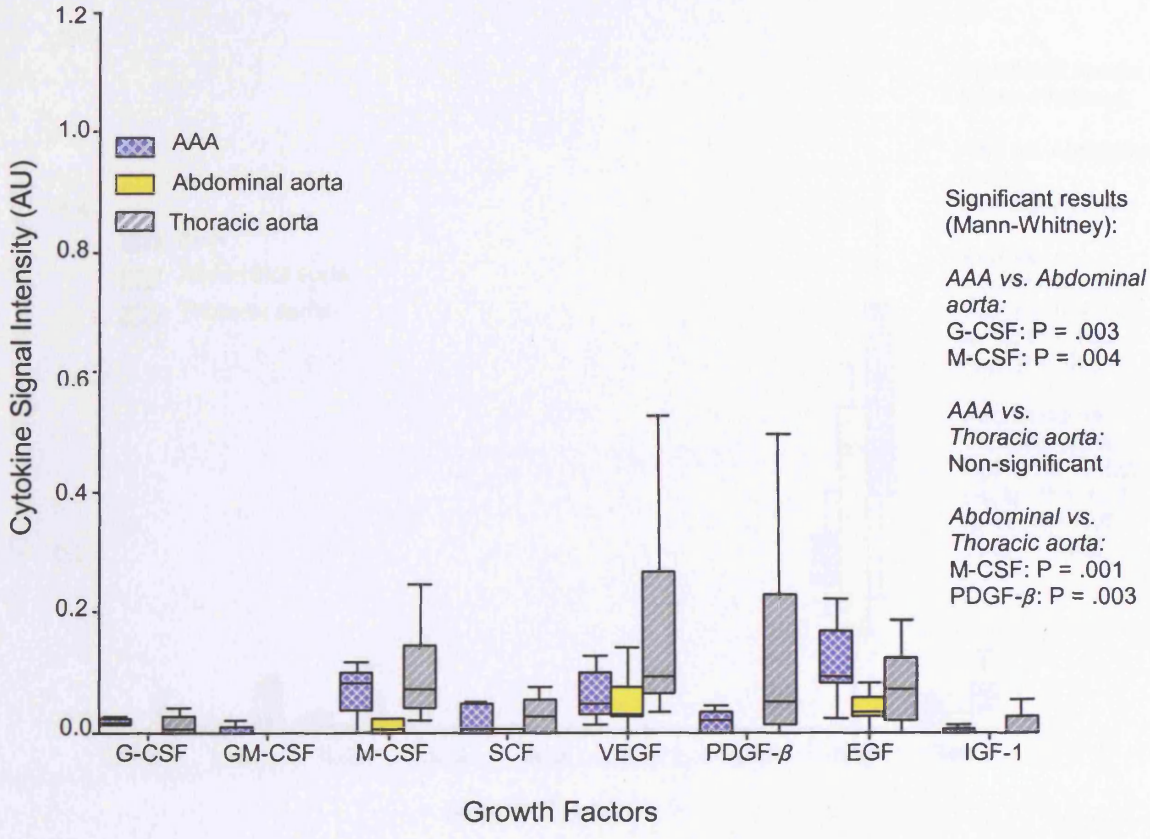


Figure 6-7. Differential expression of growth factors in the AAA. The figure displays the expression of growth factors in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each growth factor. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each cytokine. The significant results ($P < .01$) are displayed on the graph.

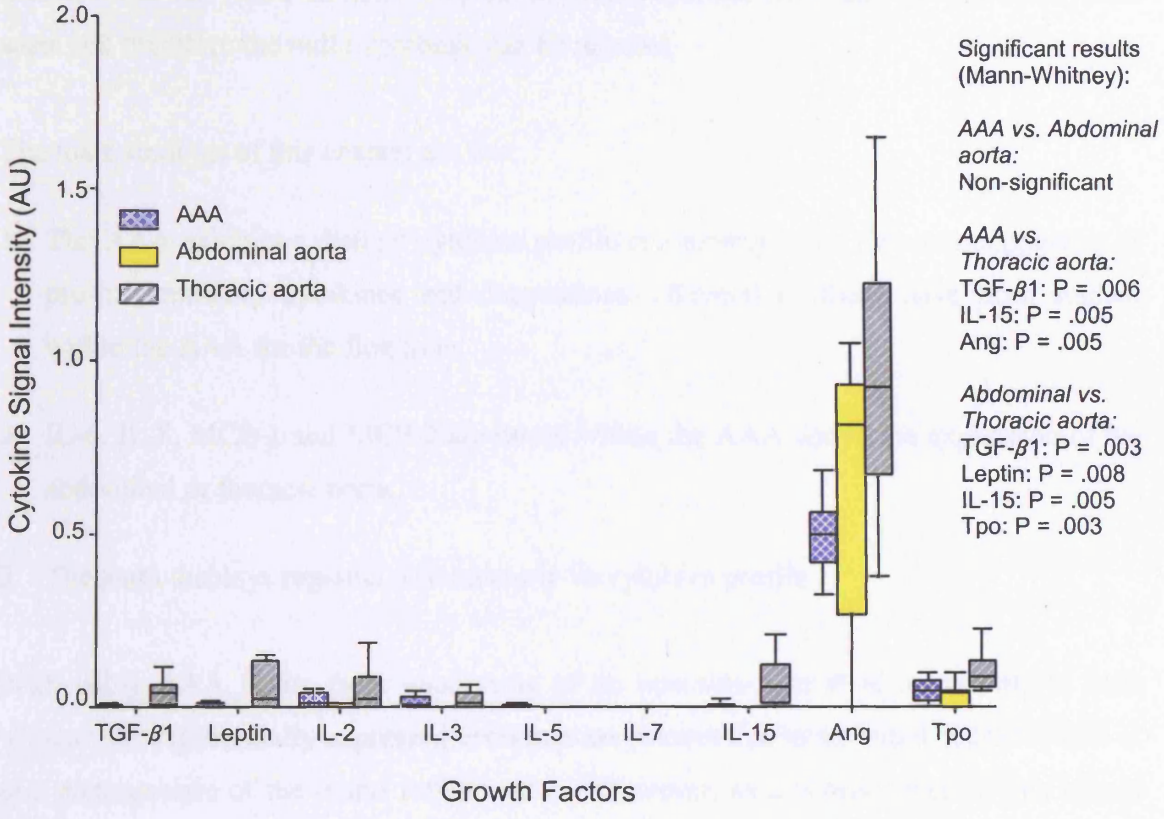


Figure 6-8. Differential expression of growth factors in the AAA. The figure displays the expression of growth factors in AAA (n = 10), abdominal aorta (n = 9) and thoracic aorta (n = 12). The boxplots display the median values (horizontal bar) and interquartile ranges (the vertical length of the box) of the data per aortic group for each cytokine. The whiskers represent the range of the data. Mann-Whitney analysis of each paired combination of groups was performed for each cytokine. The significant results ($P < .01$) are displayed on the graph. *Ang*, angiogenin; *Tpo*, thrombopoietin.

6.4 Discussion

The aim of this chapter was to investigate the cytokine expression profile of the AAA compared to non-aneurysmal aorta and address the null hypothesis that there are no differences in cytokine expression between the three groups. The results from this chapter indicate that the AAA exhibits a cytokine profile distinct from that of non-aneurysmal aorta and therefore the null hypothesis can be rejected.

The main findings of this chapter are that:

1. The AAA exhibits a distinct cytokine profile comprising of an elevated expression of pro-inflammatory cytokines and chemokines. Several of these have been studied within the AAA for the first time.

2. IL-6, IL-8, MCP-1 and MCP-2 are raised within the AAA above the expression of the abdominal or thoracic aorta.

3. The aorta displays regional differences in its cytokine profile.

With using AAA tissue from aneurysms of an operable size it is impossible to state whether the differentially expressed cytokines are present due to an initial causative role or as a consequence of the mural inflammation. However, as a consequence of this altered cytokine profile the presence of these cytokines within the aneurysm wall may contribute to the maintenance and augmentation of inflammation resulting in further tissue damage. This may predispose the aneurysm to further expansion or eventually rupture.

CXC Chemokines

Analysis of the CXC chemokines showed that ENA-78 and GRO were significantly higher in the AAA compared to abdominal aorta. IL-8 was significantly higher in the AAA than in both abdominal and thoracic aorta. ENA-78, GRO, GRO-α, MIG and SDF-1 have not been investigated in the human AAA wall before. Increased expression of IL-8 from AAA explants has been described previously[310] and along with MCP-1 will be discussed in more detail in *Chapter 7. Pro-inflammatory and Chemotactic Cytokine Expression in Abdominal Aortic Aneurysms*. ENA-78, GRO and IL-8 can bind to CXC receptor 1 (CXCR1) and CXCR2. These CXC chemokines act primarily on neutrophils as chemoattractants and

activators, inducing neutrophil degranulation with the release of myeloperoxidase and other enzymes, which may have detrimental effects on the ECM of the AAA. The potential importance of IL-8 and other neutrophil chemoattractants in AAA development was recently highlighted in a study where neutrophil depletion prevented AAA formation in an elastase-induced murine model[165].

CC Chemokines

MCP-1 and MCP-2 were significantly higher in the AAA than in the abdominal or thoracic aorta. RANTES was significantly higher in the AAA than in the abdominal aorta, however it was lower in the AAA than in the thoracic aorta. There may be a regional difference in the expression of RANTES, which was significantly higher in thoracic aorta compared to abdominal aorta. Previous studies have shown MCP-1 to be released by AAA explants in greater quantities than from occlusive or normal aortas[310] and gene arrays have demonstrated a two-fold increase in RANTES expression within the AAA[187]. I-309, MCP-2, MCP-3, MDC, MIP-1δ and TARC have not been studied previously within the human AAA wall. MCPs have roles in activation and chemotaxis of monocytes and activated T-lymphocytes, whilst RANTES functions as a chemoattractant for monocytes, memory T cells, basophils and eosinophils. Increased expression of these chemokines may induce and/or prolong the inflammatory state of the aneurysm wall. An elastase-induced aneurysmal mouse model demonstrated that the chemokines MCP-1 and RANTES were subsequently up-regulated within the wall prior to macrophage infiltration of the aortic wall[312].

Pro-inflammatory Cytokines

This study has shown a significant increase in the expression of IL-1α, IL-1β, TNF-α, TNF-β and oncostatin M between the AAA and abdominal aorta. However, the most highly expressed cytokine within this group was IL-6 which was highly elevated in the AAA above both non-aneurysmal control groups.

This study's findings agree with the previous reports that IL-1β is higher in the AAA than in abdominal aorta from cadaveric organ donors[200, 296]. IL-1β has also been shown to be higher in the circulating serum of patients with AAAs compared to those with coronary heart disease and controls with normal angiograms[300]. The expression of IL-1β within the aneurysm may be damaging to the aortic wall. IL-1β induces the synthesis of

prostaglandin, MMPs and decreases the synthesis of type I procollagen by SMCs[199, 327]. However, IL-1β has also been shown to cause an increase in TIMP-1 synthesis by aneurysmal SMCs[199], suppresses the expression of u-PA and t-PA and up-regulates the expression of PAI-1 by aneurysmal SMCs[183]. The involvement of IL-1 in aneurysm development is unclear as antagonism of the IL-1 receptor failed to prevent post-elastase dilatation in a rat AAA model[322].

TNF-α is widely studied within the AAA and my findings agree with previous reports that this cytokine is higher in the AAA than in aorta controls[200, 297]. TNF-α was also significantly higher in the serum of AAA patients compared to controls[300]. The relevance of TNF-α in aneurysm formation was demonstrated through the use of a TNF-α functional antagonist on an elastase-induced rodent model which, through blocking the action of TNF-α, was protected against aneurysm formation[322]. The role of TNF-α in AAA formation might involve an alternative mechanism than simply increasing MMP expression. A study on the effect of TNF-α on MMP expression in aneurysmal SMCs found that TNF-α did not significantly alter the mRNA levels of MMP-1, MMP-2, TIMP-1 and TIMP-2[199]. TNF-α and IL-α are known to act synergistically to induce the release of IL-8 and MCP-1[328, 329], therefore its expression within the aneurysm may augment and/or prolong the chronic inflammatory state of the aneurysm wall.

TNF-β has not been so extensively studied, however an array study on the mRNA expression of the TNF family reported a significantly higher expression of the TNF-β receptor in the AAA group compared to the organ donor control group[83].

IL-12 and IFN-γ were not found to be significantly different between the aortic groups, although both T_{H1} cytokines were expressed within the aneurysm. Similarly, Davis *et. al.* reported that there was no difference in these cytokines between AAA and AOD at the mRNA level[84]. The expression of IFN-γ in the aneurysm may aid inflammation potentially through its ability to synergistically interact with IL-1α to cause the release of IL-8 and MCP-1[329].

This study demonstrated that oncostatin M was significantly higher in the AAA than in the abdominal aorta. Mural expression of oncostatin M has been identified in the AAA through immunohistochemistry[330]. Oncostatin M is a member of the IL-6 family. It is a

growth and differentiation factor that participates in haematopoiesis and promotes cytokine release. Administration of oncostatin M to mice causes an acute inflammatory reaction including migration of polymorphonuclear leukocytes (PMN), increased adhesion molecule expression and release of PMN activators; IL-6, ENA-78, GRO-α and GRO-β[330]. Interestingly, this work mirrors the array's findings that oncostatin M was significantly higher in the human AAA and was co-expressed with IL-6, ENA-78 and GRO which were all significantly higher in the AAA.

The most highly raised pro-inflammatory cytokine within the AAA was IL-6. Many studies have reported that IL-6 is highly expressed within the AAA[227] with respect to control aorta[298, 299] or AOD[82] and this will be described in more detail in *Chapter 7. Pro-inflammatory and Chemotactic Cytokine Expression in Abdominal Aortic Aneurysms.*

Overall, the increased expression of these pro-inflammatory cytokines may promote a general inflammatory state within the AAA wall resulting in further cascades of cytokine secretion and activities, increased release of proteolytic enzymes and SMC apoptosis.

Anti-inflammatory Cytokines

IL-10 was the only T_{H2} anti-inflammatory cytokine that was significantly raised within the AAA. Previously it has been shown that IL-10 is up-regulated in the AAA[84, 302]. The expression of IL-10 within the aneurysm may be of some significance as IL-10 can decrease the expression of IL-1β, TNF-α and IL-6[331], decreases MMP levels and increases TIMP-1 expression[201]. The expression of IL-10 within the AAA may result from a negative feedback mechanism designed to attenuate the chronic pro-inflammatory response. However, despite the presence of IL-10 the pro-inflammatory cytokines were increased within the AAA. The T_{H2} cytokines IL-4 and IL-13 were not significantly expressed within the AAA. IL-13 has not been previously studied within the aneurysm. Previous studies have not detected the expression of IL-4 in the AAA or by AAA derived-T-lymphocytes[161, 299].

Whilst several studies have debated the predominance of a T_{H1}/T_{H2} response, see *Chapter 3.5.2 Anti-inflammatory Cytokines*, a recent report has suggested the cytokine panel is indicative of a T_{H0} response[301]. This response is produced from natural killer cells and natural killer T-cells which secrete both T_{H1} (IFN-γ, IL-2) and T_{H2} cytokines (IL-4 and IL-

5). From the results of the array it is difficult to conclusively state which response dominates within the aneurysm. Whilst IL-10 was increased within the aneurysm the other major T_{H2} cytokines IL-4, IL-5 and IL-13 were not expressed. IFN-γ and IL-12 were expressed in the aneurysm and TNF-α was raised which would suggest a T_{H1} response, if it were not for the expression of IL-10. The difficulty in ascertaining the dominance of one response over the other may arise from the use by this study, and previous studies, of homogenised aneurysm tissue from full-thickness wall biopsies. Whilst providing an overview of the cytokine microenvironment within the wall small subtle changes are masked by this method and regional changes are lost.

Growth Factors

Overall growth factors were not highly expressed within the aneurysm, with the exception of angiogenin. G-CSF and M-CSF were the only growth factors significantly higher in the AAA than the abdominal aorta. The expression of these cytokines have not been studied previously within the human AAA wall, although M-CSF was up-regulated in apoE$^{-/-}$ mice treated with angiotensin II to induce AAA formation[332]. G-CSF and M-CSF are growth, differentiation and activating factors for neutrophils or macrophages, respectively. As activated neutrophils and macrophages have the potential to release proteolytic enzymes, an increase in G-CSF and M-CSF expression in AAAs may indirectly promote AAA wall degradation. The expression of these growth factors, coupled with the co-expression of IL-8, MCP-1 and MCP-2 suggests that the inflammatory cells, neutrophils and macrophages, may play an important role in AAA development and could potentially be recruited into the AAA wall where they could undergo differentiation and activation.

Several of the growth factors studied on the array, EGF, SCF, leptin, IL-3, IL-7, angiogenin and thrombopoietin, had not been investigated in AAAs before. With the exception of TGF-β1, and to a lesser extent VEGF and IL-5, growth factors have been largely ignored in AAA development. Experimental haemodynamic studies on the effects of blood flow on the expression of GM-CSF, PDGF-β and VEGF within the rodent AAA are described in *Chapter 3.5.4 Growth Factors*. As mentioned, one study reported the increased expression of VEGF in the human AAA compared to AOD[317]. Analysis of VEGF expression between aneurysmal fibroblasts and dermal fibroblasts also revealed that VEGF was significantly higher in the AAA group[333]. However, this study found VEGF was expressed in all aortic groups and was not significantly different between the AAA

and the abdominal or thoracic aorta. The array results showed that IL-2 was not expressed in aortic tissue, a result supported by other studies on IL-2 expression in human AAAs[299, 302]. This study did not detect any expression of IL-5 in aortic tissue. Previously, one study reported the expression of IL-5 within the AAA whilst it was not expressed within normal aorta[302].

Several cytokines, TGF-β1, IL-15 and angiogenin, were significantly higher in thoracic aorta than in the AAA but were not differentially expressed between AAA and abdominal aorta. Previously, TGF-β1 has been shown to be non-differentially expressed between the AAA and AOD[82] and IL-15 has been reported to be lower in AAAs than in AOD[302]. IL-15 has been shown to inhibit the expression of IL-8 and MCP-1 by epithelial cells[334]. In this study IL-15 was not expressed in the aneurysm and therefore the increased levels of IL-8 and MCP-1 may be partly explained by the absence of this inhibitory pathway. The higher expression of TGF-β1 and IL-15 in thoracic aorta than abdominal aorta or AAA suggests that the thoracic aorta may have its own distinct cytokine profile.

The use of an array system for cytokine detection has made it possible to investigate a larger repertoire of cytokine proteins than has previously been studied within the AAA wall. This study chose to investigate cytokine expression at the protein level because whilst cDNA arrays provide useful information on expression at the transcriptional level, proteins are the effector molecule and mRNA expression does not always correlate with protein expression. The use of protein arrays has also allowed for the simultaneous detection of 42 cytokines under identical laboratory conditions. This reduces the experimental variation in multiple cytokine detection and provides a more accurate profile. The combination of simultaneously screening multiple cytokines has several advantages. Firstly, it has allowed this study to identify potential pathways of cytokine synergism and antagonism. Secondly, any downstream changes in cytokine expression resulting from an alteration in the expression of a specific cytokine can be simultaneously detected. Although a casual link between these two events cannot be assumed, the arrays have provided a general overview as to the inflammatory state of the AAA wall. Finally, in this study, the array technique has identified alterations in the overall cytokine expression profile of the AAA, including novel changes that have not been detected previously.

However, there are several limitations to this study. Firstly, the AAA specimens were obtained at surgery and therefore are established aneurysms. This limits the results to an interpretation of the state of the developed aneurysm wall which is undergoing expansion and at risk of rupture, but cannot be used to determine the earlier stages of aneurysm formation. In order to determine if specific cytokines had a causative role in AAA pathogenesis an animal model would be required, although arguably exogenous induction of an aneurysm is not part of the natural formation of an AAA. An additional point is that despite efforts to recruit kidney donors at the upper range for donations this patient group is generally younger than aneurysm patients. To compensate for this difference and to provide a control group with similar clinical features as the AAA group thoracic aorta was used as second control. The thoracic aorta group also acted as a control group to compensate for the effects of surgery on cytokine expression.

This study used homogenised full-thickness aneurysm wall biopsies to provide an overview of cytokine expression within the AAA wall. However, as mentioned, such a method can mask small changes in regional cytokine expression. It would be interesting to obtain a detailed analysis of cytokine expression involving the dissection of the layers of the aneurysm wall to provide topological information on cytokine expression, this may identify small changes in regional cytokine expression, which were masked by analysing the whole tissue.

Whilst arrays provide useful information in allowing the mass screening of protein or mRNA to initially identify changes in an expression profile, protein or mRNA expression is expressed in relative terms. Once the protein of interest has been identified a more detailed analysis involving absolute quantitative analysis and localisation of the individual protein is required.

In conclusion, this is the first study to investigate the simultaneous expression of 42 cytokine proteins within the AAA. It has described the cytokine profile of the AAA and has highlighted the increased expression of several pro-inflammatory cytokines and chemokines within the AAA. This combination of cytokines may maintain the chronic inflammatory nature of the AAA and favour proteolysis of the ECM resulting in further weakening of the aortic wall. The highly elevated expression of IL-6, IL-8, MCP-1 and MCP-2 above both control groups suggests that they are involved in end-stage AAA

pathogenesis. The role these cytokines may have in AAA degeneration remains to be determined. The remainder of this thesis is dedicated to defining the expression of these cytokines within the AAA.

Chapter Seven

Pro-inflammatory and Chemotactic Cytokine Expression in Abdominal Aortic Aneurysms

Chapter 7. Pro-inflammatory and Chemotactic Cytokine Expression in Abdominal Aortic Aneurysms

7. Pro-inflammatory and Chemotactic Cytokine Expression in Abdominal Aortic Aneurysms

7.1 Introduction

The previous chapter identified several pro-inflammatory and chemotactic cytokines which were elevated in the aneurysm above non-aneurysmal aorta. Four cytokines: IL-6, IL-8, MCP-1 and MCP-2 were raised in the AAA above the normal expression of both abdominal and thoracic aorta.

Human IL-6 is a single glycoprotein chain of about 26kDa and 212 amino acids, with two potential N-linked glycosylation sites. IL-6 mediates its biological effect through the IL-6 receptor, which consists of two chains, IL-6Rα and gp130. The IL-6Rα chain is specific to IL-6 and is involved in ligand binding, whilst the gp130 chain is the signal transducing peptide of the receptor complex. IL-6 is a potent pleiotropic inflammatory cytokine that mediates a plethora of physiological functions including: the differentiation of lymphocytes, cell proliferation, cell survival and amelioration of apoptotic signals[335, 336]. Additionally, IL-6 plays a pivotal role in the acute-phase response, with IL-6 stimulating the expression of C-reactive protein and fibrinogen[337].

The biologically active form of IL-8 is an 8.4kDa protein containing 72 amino acids. It is secreted as an 8.9kDa protein of 77 amino acids and it has been shown that this form can be processed by MMP-9 to the 8.4kDa form. This results in a 10- to 27-fold increase in neutrophil activation, as measured by an increase in intracellular Ca^{2+} concentration, secretion of MMP-9 and chemotaxis. This increased potency correlated with enhanced IL-8 binding to neutrophils and increased signalling through CXCR1[308]. IL-8 is a pro-inflammatory CXC chemokine. Biological functions for IL-8 include plasma exudation, neutrophil accumulation, general granulocytophilia *in vivo*[338], chemotaxis for recruitment of leukocytes by diapedesis through endothelial spaces[339], degranulation of neutrophils[340], respiratory burst response[341] and mobilisation of intracellular Ca^{2+} *in vitro*[342]. IL-8 has also been shown to stimulate angiogenesis by promoting proliferation and chemotaxis of endothelial cells[343].

MCP-1 and -2 are initially synthesised as a 99 amino acid polypeptide containing a hydrophobic amino terminal signal sequence of 23 amino acids. After cleavage of the signal peptide the mature protein of 76 amino acids is secreted. MCP-2 has a 62% sequence homology with MCP-1. MCP-1 and -2 belong to the CC chemokine family and signal through the CCR2 receptor. Additionally, MCP-2 signals through the CCR receptors: CCR1, CCR3 and CCR5 suggesting at least some functional difference between the two ligands. The MCPs chemoattract and activate monocytes, but they also stimulate T-lymphocytes, basophils, natural killer cells, dendritic cells and vascular SMCs[344-346]. MCP-2 has a greater effect on sub-types of leukocytes, including eosinophils and basophils, than MCP-1, and hence MCP-2 expression may be responsible for broadening the inflammatory response to infection[347]. The MCPs are not chemotactic for neutrophils.

The aim of this chapter is to verify the array's findings in relation to these cytokines using ELISAs, a well-established quantitative technique for measuring specific proteins, on a larger AAA patient group.

The null hypothesis is that there are no significant differences in the quantitative expression of IL-6, IL-8, MCP-1 and MCP-2 between the AAA, the abdominal aorta and the thoracic aorta.

7.2 Methods

Specimens from the infrarenal aorta were obtained from patients undergoing open AAA repair (n = 25). Two non-aneurysmal control groups were used: abdominal aorta from cadaveric kidney donors (n = 15) and thoracic aorta obtained from patients undergoing aorto-coronary bypass graft operations (n = 10). The characteristics of the three groups are shown in Table 7-1. All tissue was snap frozen in liquid nitrogen upon collection and stored at –80°C for batch analysis.

Table 7-1. Demographics of the study population.

Risk Factor	AAA ($n = 25$)	Abdominal aorta* ($n = 15$)	Thoracic aorta ($n = 10$)
Median age (range) / years	73 (61-84)	54 (23-74)	66 (50-76)
Male sex, n (%)	24 (96)	11 (73)	8 (80)
Smoking history, n (%)	22 (88)	12 (80)	8 (80)
Hypertension, n (%)	18 (72)	-	10 (100)
Myocardial infarction, n (%)	5 (20)	-	6 (60)
Angina, n (%)	7 (28)	-	8 (80)
Cerebrovascular accident, n (%)	3 (12)	-	1 (10)
Diabetes, n (%)	0 (0)	-	0 (0)
Median AAA diameter (range) /mm	64 (55-93)	n/a	n/a

* Only a brief medical history was supplied with the abdominal aorta from kidney donors.

As described in *Chapter 5.4-5.5*, specimens were prepared for the ELISA experiments by homogenisation and manipulation of each sample to a total protein concentration of 1mg/ml. All samples and controls were aliquoted into single-use aliquots to avoid protein denaturation resulting from multiple-freeze thaws. A fixed concentration of tissue lysate was incubated in each ELISA well to allow for a direct comparison of the results between the wells. The tissue lysate was incubated on pre-coated ELISA plates for IL-6, IL-8 and MCP-1, as described in *Chapter 5.7 ELISA* and *Appendix II.* A pre-coated MCP-2 kit was not available and therefore the results section in this chapter (*7.3.4 Monocyte Chemoattractant Protein-2*) describes the stages involved in the development of this assay. The final protocol followed for MCP-2 is described in *Chapter 5.7.3 MCP-2 ELISA.*

For each assay, positive and negative control samples were processed. Homogenising buffer was used as the negative control. The average OD readings for the negative control were used to blank the tissue lysate readings to account for any light absorption relating to the homogenising solution. Positive controls for IL-8 and IL-6 were supplied by Biosource Int., however, they did not produce an MCP-1 control for their MCP-1 ELISA. Therefore an MCP-1 positive AAA specimen was used as a positive control for determining intra-assay variation within the MCP-1 experiment. The AAA control specimen used had been shown by a previous MCP-1 ELISA to contain high levels of MCP-1. For the MCP-2

assay a recombinant MCP-2 protein, purchased from PeproTech EC, was used as a positive control.

Duplicate positive controls were run on each assay to ensure that all assay readings were within an acceptable degree of variation (see inter-assay variation results below). The intra-assay variation for each cytokine assay was also determined to ensure that the variation within an assay was also acceptable.

For each cytokine the data from the three aortic groups were compared using the Kruskal-Wallis test and further analysed by a series of Mann-Whitney *U* tests. A P value of <.05 was used to determine a significant difference for the Kruskal-Wallis test. To correct for multiple comparisons the P value used to determine statistical significance for the Mann-Whitney tests was corrected using the Bonferroni method. This method involved dividing the P value of <.05 by the number of pairwise comparisons. For this chapter the number of possible pairwise comparison was three (AAA vs. abdominal aorta, AAA vs. thoracic aorta and thoracic aorta vs. abdominal aorta). Therefore using the Bonferroni correction method an adjusted P value of <.017 (0.5/3 = 0.17) was used to determine a significant difference for the results of the Mann-Whitney *U* test[326]. To investigate the effect of the difference in ages between the three aortic groups on cytokine expression, the results of the IL-6 analysis, as an example, were adjusted for age. The IL-6 concentrations obtained from the ELISA were logged to make the data more normally distributed. A generalised linear model was used to compare the mean IL-6 concentration between aortic group, adjusting for age.

7.3 Results

7.3.1 Interleukin-6

The intra- and inter-assay variation for the IL-6 ELISAs are displayed in Table 7-2. The intra-assay variation results were determined from measuring the IL-6 content of the positive control in 7 wells on the same ELISA plate. Within the same assay the positive control had a mean value of 151.7 ± 9.7pg/ml and the coefficient of variation between the concentrations of the control in the wells was 6.4%. Duplicate wells of the positive control were incubated on each plate and gave a measure of the variability between assays. The

Table 7-2. The intra- and inter-assay variation of the IL-6 ELISAs.

Intra-assay Variation		Inter-assay Variation	
Well Number	IL-6 Control Concentration (pg/ml)	Plate Number	IL-6 Control Concentration (pg/ml)
1	163.1	1	126.3
2	156.7	2	161.3
3	149.9	3	155.1
4	144.1	4	159.1
5	150.9	5	151.7
6	161.7		
7	135.9		
Mean	151.7	**Mean**	150.7
SD	9.7	**SD**	14.1
Coefficient of Variation (%)	6.4	**Coefficient of Variation (%)**	9.4

SD, standard deviation.

inter-assay variability, based on five assay results, was found to be 9.4%. This level of variability is within acceptable limits.

The results of the measurement of IL-6 within the AAA wall and the aortic wall of the non-aneurysmal abdominal and thoracic aorta are shown in Figure 7-1, with the actual values given in Table 7-3. As the standard curves could not be extrapolated beyond the lowest standard concentration without affecting the accuracy of the results obtained some of the control concentrations could not be quantified by the ELISA (Table 7-3). Therefore these values were recorded as "less than the lowest standard concentration". In the case of IL-6, these were recorded as < 7.8pg/ml. As this data were analysed through median values and non-parametric statistical methods based on rank order this did not affect the outcome of the Kruskal-Wallis and Mann-Whitney analyses. Both these statistical tests work by placing all the values in rank order, then adding up the sum of ranks for each group and dividing the sum of ranks by the number of values in that group to gain the mean rank. The statistical difference between these mean rank values is then determined. Therefore the actual values of the data count only towards its rank order.

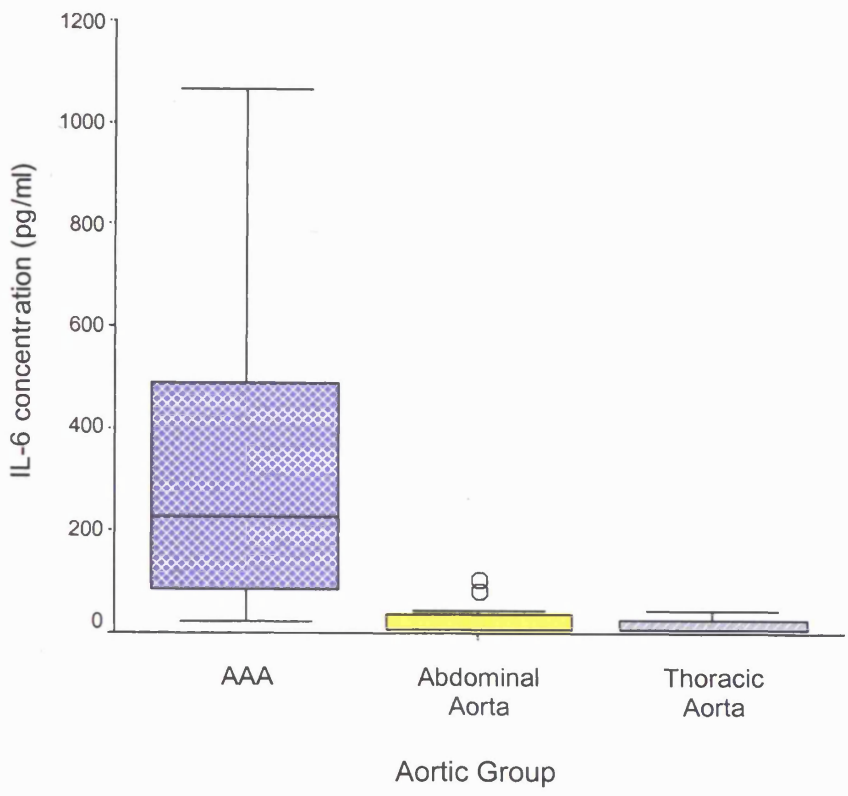


Figure 7-1. IL-6 expression within AAA (*n*=25), abdominal aorta (*n* =15) and thoracic aorta (*n*=10). The boxplots display the median value (horizontal bar) and the interquartile range (the vertical length of the box) of the data per aortic group. The whiskers represent the range of the data. The circles display outlier values.

Table 7-3. Data values for Figure 7-1.

Aortic Group	Number of Cases	Median (pg/ml)	IQR (pg/ml)		Range (pg/ml)	
			1st quartile	3rd quartile	Minimum value	Maximum value
AAA	25	226.7	81.9	514.9	21.9	1067.0
Abdominal Aorta	15	<7.8	< 7.8	40.5	<7.8	102.6*
Thoracic Aorta	10	<7.8	<7.8	27.7	<7.8	43.2

* Outlier on the graph. *IQR*, interquartile range.

Table 7-4. Statistical analyses for Figure 7-1.

Aortic Group	Number of Cases	Kruskal-Wallis	Mann-Whitney Analyses		
			AAA vs. Abdominal Aorta	AAA vs. Thoracic Aorta	Abdominal vs. Thoracic Aorta
		Mean Rank	Mean Rank	Mean Rank	Mean Rank
AAA	25	37.16	27.28	22.88	-
Abdominal Aorta	15	14.97	9.20	-	13.77
Thoracic Aorta	10	12.15	-	5.80	11.85
P Values	-	<.001	<.001	<.001	.458

Kruskal-Wallis analysis revealed that there was a significant difference between at least two of the population medians (P <.001). Mann-Whitney analysis identified that median IL-6 expression was significantly higher in the AAA compared to both non-aneurysmal abdominal aorta (226.7pg/ml vs. <7.8pg/ml, P <.001) and thoracic aorta (226.7pg/ml vs. <7.8pg/ml, P <.001) (Table 7-4).

The results of the age-adjusted analysis of IL-6 expression are shown in Tables 7-5 to 7-7. The back-transformed, age-adjusted means for each aortic group are displayed in Table 7-5. The mean age-adjusted IL-6 concentration in the AAA was 233.5pg/ml, compared to 13.7pg/ml for non-aneurysmal abdominal aorta and 11.9pg/ml in the thoracic aorta. From the statistical analysis of the three aortic groups there is strong evidence to suggest a difference in the mean IL-6 concentration after adjusting for age (P<.0001) (Table 7-6a). Table 7-6b shows that age did not have an effect on IL-6 expression, when the data was adjusted for aortic group (P = .524).

Table 7-7 displays the back-transformed, differences between the least square means (Ratios) for each pairwise comparison of the aortic groups. There was strong evidence of a difference between AAA and non-aneurysmal abdominal aorta (Ratio 17.05; P <.0001),

and strong evidence of a difference between AAA and thoracic aorta (Ratio 19.61; P <.0001), but no evidence of a difference between non-aneurysmal abdominal aorta and thoracic aorta (Ratio 1.15; P = .750). Thus mirroring the previous results for IL-6 expression in aortic tissue, which were not adjusted for age.

Table 7-5. Least squares means of IL-6 concentration (adjusted for age)

Aortic Group	Back-transformed IL-6 concentration (pg/ml)	
	Mean	95% Confidence Interval
AAA	233.45	147.09 to 369.56
Abdominal Aorta	13.68	7.26 to 25.77
Thoracic Aorta	11.89	6.36 to 22.22

Table 7-6a. Type III sum of squares - Effect of aortic group on IL-6 expression (adjusted for age)

Covariate	Degree of Freedom	Type III Sum of Square	Mean Square	F Value	P Value
Aortic Group	2	66.46	33.23	34.53	<.0001

Table 7-6b. Type III sum of squares - Effect of age on IL-6 expression (adjusted for aortic group)

Covariate	Degree of Freedom	Type III Sum of Square	Mean Square	F Value	P Value
Age	1	0.40	0.40	0.41	.524

Table 7-7. Pairwise comparison of the difference between the least squares means for IL-6 (age-adjusted)

Pairwise Comparison	Ratio	95% Confidence Interval	P-Value
AAA vs. Abdominal Aorta	17.05	7.00 to 41.51	<.0001
AAA vs. Thoracic Aorta	19.61	8.94 to 43.00	<.0001
Abdominal Aorta vs. Thoracic Aorta	1.15	0.48 to 2.77	.750

7.3.2 Interleukin-8

The intra- and inter-assay variation for the IL-8 ELISAs are displayed in Table 7-8. The intra-assay variation across 7 wells on the same plate was determined as for IL-6. Within the same assay the positive control had a mean value of 104.3 ± 4.4pg/ml and the coefficient of variation in the concentration of the control between the wells was 4.3%. Duplicate wells of the positive control were incubated on each plate and gave a measure of the variability between assays. The inter-assay variability, based on five assay results, was found to be 7.5%. This level of variability is within acceptable limits.

Table 7-8. The intra- and inter-assay variation of the IL-8 ELISAs.

Intra-assay Variation		Inter-assay Variation	
Well Number	IL-8 Control Concentration (pg/ml)	Plate Number	IL-8 Control Concentration (pg/ml)
1	102.5	1	99.1
2	98.2	2	110.0
3	107.9	3	117.1
4	111.1	4	118.4
5	100.1	5	104.3
6	104.4		
7	106.0		
Mean	104.3	**Mean**	109.8
SD	4.4	**SD**	8.3
Coefficient of Variation (%)	4.3	**Coefficient of Variation (%)**	7.5

SD, standard deviation.

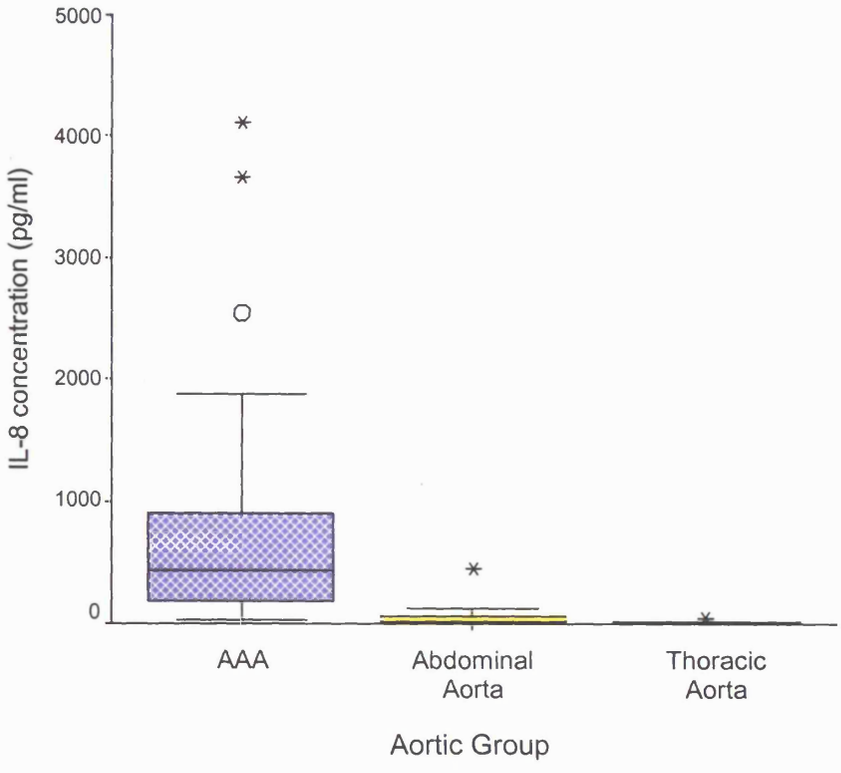


Figure 7-2. IL-8 expression within AAA (n=25), abdominal aorta (n =15) and thoracic aorta (n=10). The boxplots display the median value (horizontal bar) and the interquartile range (the vertical length of the box) of the data per aortic group. The whiskers represent the range of the data. The circles display outlier values, the asterisks highlight extreme outliers.

The results of the measurement of IL-8 within the AAA wall and the aortic wall of the non-aneurysmal abdominal and thoracic aorta are shown in Figure 7-2, with the actual values given in Table 7-9.

As for IL-6, some of the lower values of the control specimens were below the standard curve. Their OD values were greater than the background OD values demonstrating a presence of these cytokines within the controls specimens, but at a level below 15.6pg/ml, the lowest concentration on the standard curve. Several of the values were classed as outliers, as defined by SPSS as having a value over 1.5 times greater than the interquartile range. All values were included in the statistical analysis.

Table 7-9. Data values for Figure 7-2.

Aortic Group	Number of Cases	Median (pg/ml)	IQR (pg/ml)		Range (pg/ml)	
			1st quartile	3rd quartile	Minimum value	Maximum value
AAA	25	438.6	174.6	1063.5	30.2	4110.9*
Abdominal Aorta	15	<15.6	<15.6	74.4	<15.6	450.3*
Thoracic Aorta	10	<15.6	<15.6	<15.6	<15.6	43.7*

* Outlier on the graph. *IQR*, interquartile range.

Table 7-10. Statistical analyses for Figure 7-2.

Aortic Group	Number of Cases	Kruskal-Wallis	Mann-Whitney Analyses		
			AAA vs. Abdominal Aorta	AAA vs. Thoracic Aorta	Abdominal vs. Thoracic Aorta
		Mean Rank	Mean Rank	Mean Rank	Mean Rank
AAA	25	36.96	27.0	22.96	-
Abdominal Aorta	15	16.53	9.67	-	14.87
Thoracic Aorta	10	10.30	-	5.60	10.20
P Values	-	<.001	<.001	<.001	.061

Kruskal-Wallis analysis revealed that there was a significant difference between at least two of the population medians (P <.001). Mann-Whitney analysis identified that median IL-8 expression was significantly higher in the AAA compared to both non-aneurysmal abdominal aorta (438.6pg/ml vs. <15.6pg/ml, P <.001) and thoracic aorta (438.6pg/ml vs. <15.6pg/ml, P <.001) (Table 7-10).

7.3.3 Monocyte Chemoattractant Protein-1

The intra-assay variation for the MCP-1 ELISA is displayed in Table 7-11. The intra-assay variation across 8 wells on the same plate was determined using aliquots of the positive AAA control. Within the same assay the positive control had a mean value of 1279.7 ± 232.8pg/ml and the coefficient of variation in the concentration of the control between the wells was 18.2%. A measure of inter-assay variability couldn't be given as the MCP-1 results were obtained from only two ELISAs.

Table 7-11. The intra-assay variation of the MCP-1 ELISA.

Well Number	MCP-1 Control Concentration (pg/ml)
1	1029.6
2	1383.7
3	1474.2
4	1651.9
5	1400.1
6	994.4
7	1175.3
8	1128.5
Mean	1279.7
SD	232.8
Coefficient of Variation (%)	18.2

SD, standard deviation.

The results of the measurement of MCP-1 within the AAA wall and the aortic wall of the non-aneurysmal abdominal and thoracic aorta are shown in Figure 7-3, with the actual values given in Table 7-12.

MCP-1 was expressed in all of the aortic samples. Despite an 18.2% variability within the plate the overall expression of MCP-1 was much greater in the AAA than in the non-aneurysmal abdominal (9-fold) or thoracic aorta (19-fold), with minimal overlapping of the data. It is therefore unlikely that the data could have resulted simply from this variation.

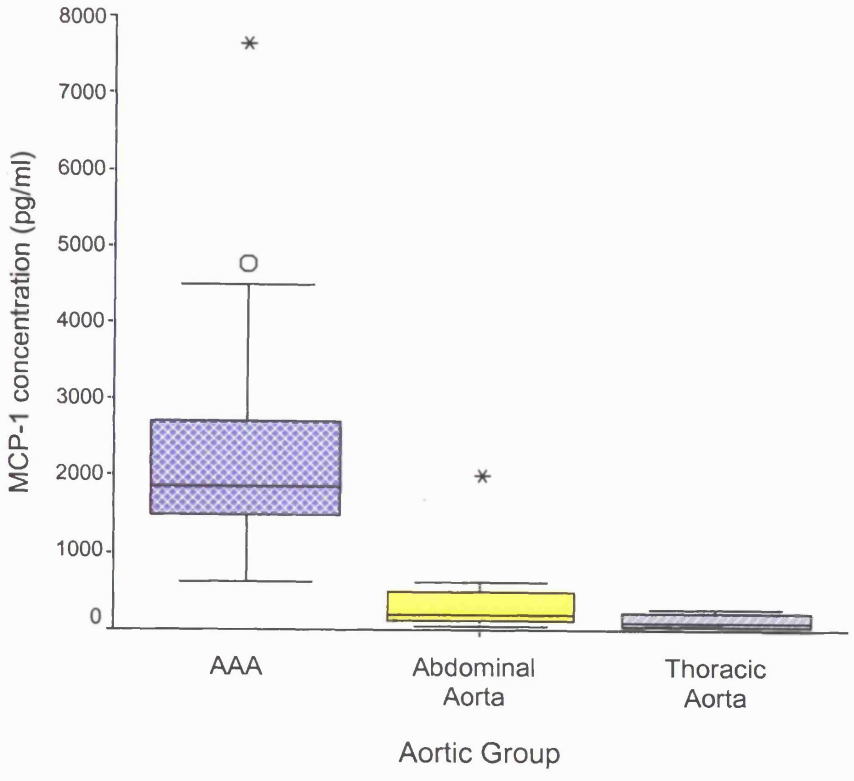


Figure 7-3. MCP-1 expression within AAA (*n*=25), abdominal aorta (*n* =15) and thoracic aorta (*n*=10). The boxplots display the median value (horizontal bar) and the interquartile range (the vertical length of the box) of the data per aortic group. The whiskers represent the range of the data. The circles display outlier values, the asterisks highlight extreme outliers.

Table 7-12. Data values for Figure 7-3.

Aortic Group	Number of Cases	Median (pg/ml)	IQR (pg/ml)		Range (pg/ml)	
			1st quartile	3rd quartile	Minimum value	Maximum value
AAA	25	1869.1	1496.6	2713.4	622.7	7649.5*
Abdominal Aorta	15	198.8	99.6	621.5	61.5	2001.0*
Thoracic Aorta	10	96.9	61.5	238.6	61.5	272.1

* Outlier on the graph. *IQR*, interquartile range.

Kruskal-Wallis analysis revealed that there was a significant difference between at least two of the population medians (P <.001). Mann-Whitney analysis identified that the median MCP-1 expression was significantly higher in the AAA compared to both non-aneurysmal abdominal aorta (1869.1pg/ml vs. 198.8pg/ml, P <.001) and thoracic aorta (1869.1pg/ml vs. 96.9pg/ml, P <.001). Once corrected for multiple comparisons the expression of MCP-1 was not significantly different between abdominal and thoracic aorta (P = .047) (Table 7-13).

Table 7-13. Statistical analyses for Figure 7-3.

Aortic Group	Number of Cases	Kruskal-Wallis	Mann-Whitney Analyses		
			AAA vs. Abdominal Aorta	AAA vs. Thoracic Aorta	Abdominal vs. Thoracic Aorta
		Mean Rank	Mean Rank	Mean Rank	Mean Rank
AAA	24	36.38	26.38	22.50	-
Abdominal Aorta	15	17.17	9.80	-	15.37
Thoracic Aorta	10	9.45	-	5.50	9.45
P Values	-	<.001	<.001	<.001	.047

P <.017 is significantly different.

7.3.4 Monocyte Chemoattractant Protein-2

Initial experimental work involved developing an ELISA for MCP-2 and validating its accuracy. The original protocol used was similar to the protocol described in *Chapter 5.7.3 MCP-2 ELISA* with the following major differences: (1) the plate was coated with 1μg/ml of capture antibody and left overnight at RT to adhere to the plate, (2) the SA-HRP was added at a dilution of 1/200, (3) the range of MCP-2 concentrations used in the standard curve were 62.5-4000pg/ml. The standard curve that was attained using the original protocol is shown in Figure 7-4. The coefficient of determination (R^2), which demonstrates how well the data fits to the line, was 0.9876. The closer that the R^2 value is to the value of 1.0 the better the data fits the curve.

The MCP-2 concentration in a sample of eight AAA specimens was measured in order to check that the OD values would fit on the standard curve. The OD value of the lowest standard on the curve at 62.5pg/ml was 0.1095. However, the OD values obtained from the AAA specimens were lower than this (Table 7-14). Therefore the concentration of MCP-2 in the AAA specimens could not be measured by the assay described in the original DuoSet protocol.

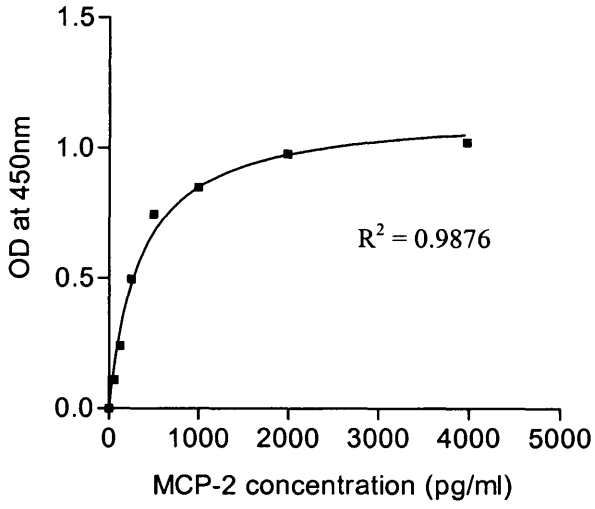


Figure 7-4. The standard curve (62.5pg/ml-4000pg/ml) produced from initial experiments. The ELISA was performed using 1μg/ml capture antibody, 50ng/ml detection antibody and 1/200 dilution of SA-HRP.

Table 7-14. The OD values of the lowest standard and AAAs tested on the original ELISA.

Background corrected OD value of 62.5pg/ml standard	Background corrected OD value of AAA specimens	Verdict
0.1095	0.057	Off the standard curve
	0.097	Off the standard curve
	0.012	Off the standard curve
	-0.005	Off the standard curve
	0.047	Off the standard curve
	0.009	Off the standard curve
	0.008	Off the standard curve
	-0.021	Off the standard curve

Testing the Effect of Varying Reagent Concentrations on Assay Sensitivity

It was necessary to optimise the MCP-2 assay to attain a standard curve that extended below 62.5pg/ml. The test protocol consisted of comparing concentrations of capture antibody (1µg/ml, 5µg/ml); detection antibody (50ng/ml, 100ng/ml); and SA-HRP (1/50, 1/200). The additional concentrations were selected as they were calculated to be the maximum concentrations of the reagents that could be used with the amount of reagents that were supplied with the assay and leave sufficient reagents for two full ELISA plates to measure the concentration of MCP-2 within the aortic specimens. The grid in Table 7-15 displays the various combinations of antibodies and SA-HRP tested. The rest of the protocol was performed as described in *Chapter 5.7.3 MCP-2 ELISA*. The standard concentrations tested were incubated in the wells as displayed in Table 7-15. The lowest standard of 62.5pg/ml was diluted two-fold, twice, to extend the standard curve down to 15.6pg/ml.

Table 7-15. Grid experiment for testing the effect of capture, detection antibody and SA-HRP concentration on the detection signal produced.

	SA-HRP: 1/50		**SA-HRP: 1/200**		**SA-HRP: 1/50**		**SA-HRP: 1/200**	
Detect AB 50ng/ml	4000	62.5	4000	62.5	4000	62.5	4000	62.5
	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3
	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
	0	0	0	0	0	0	0	0
Detect AB 100ng/ml	4000	62.5	4000	62.5	4000	62.5	4000	62.5
	31.3	31.3	31.3	31.3	31.3	31.3	31.3	31.3
	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
	0	0	0	0	0	0	0	0
	Capture AB 1µg/ml		**Capture AB 1µg/ml**		**Capture AB 5µg/ml**		**Capture AB 5µg/ml**	

SA-HRP, streptavidin-horseradish peroxidase; *Detect AB,* detection antibody; *Capture AB*, capture antibody. The concentrations of the MCP-2 standards contained within each well are mapped on the grid (pg/ml).

Table 7-16. Optical density (OD) readings for the grid experiment (Table 7-15).

	SA-HRP: 1/50		**SA-HRP: 1/200**		**SA-HRP: 1/50**		**SA-HRP: 1/200**	
Detect AB 50ng/ml	0.794	0.265	*0.463*	*0.124*	*2.708*	*0.375*	1.989	0.203
	0.091	0.090	*0.066*	*0.069*	*0.151*	*0.137*	0.093	0.085
	0.065	0.080	*0.059*	*0.048*	*0.086*	*0.068*	0.058	0.054
	0.091	0.069	*0.084*	*0.049*	*0.061*	*0.047*	0.047	0.045
Detect AB 100ng/ml	0.848	0.207	0.467	0.127	2.683	0.355	1.865	0.170
	0.108	0.110	0.073	0.082	0.150	0.139	0.097	0.088
	0.058	0.066	0.051	0.075	0.087	0.076	0.072	0.052
	0.041	0.050	0.052	0.079	0.077	0.067	0.039	0.040
	Capture AB 1µg/ml		**Capture AB 1µg/ml**		**Capture AB 5µg/ml**		**Capture AB 5µg/ml**	

Original conditions. Largest improvement in detection signal.

Table 7-17. Signal to noise ratio for the grid experiment (Table 7-15).

	SA-HRP: 1/50		**SA-HRP: 1/200**		**SA-HRP: 1/50**		**SA-HRP: 1/200**	
Detect AB 50ng/ml	9.9	3.3	7.0	1.9	50.1	6.9	43.2	4.4
	1.1		1.0		2.7		1.9	
	0.9		0.8		1.4		1.2	
	1.0		1.0		1.0		1.0	
Detect AB 100ng/ml	18.6	4.6	7.1	1.9	37.3	4.9	47.2	4.3
	2.4		1.2		2.0		2.3	
	1.4		1.0		1.1		1.6	
	1.0		1.0		1.0		1.0	
	Capture AB 1µg/ml		**Capture AB 1µg/ml**		**Capture AB 5µg/ml**		**Capture AB 5µg/ml**	

Signal to noise = standard curve OD/background OD.

Original conditions. Largest improvement in detection signal.

The OD results of the assay are shown in Table 7-16 and the signal to noise ratios are shown in Table 7-17. The "signal" was a measure of the OD values obtained from the standards (4000, 62.5, 31.2 and 15.6pg/ml), which were treated with different concentrations of the antibodies and SA-HRP. The background "noise" OD values were obtained from wells containing only diluent buffer. Increasing the concentration of the capture antibody had the greatest effect on the signal to noise ratio. Increasing the SA-HRP increased the signal strength, however at higher capture antibody concentrations it also increased the background signal. The highest signal to noise ratio was achieved by the combination of 5μg/ml of capture antibody, 50ng/ml of detection antibody and 1/50 dilution of SA-HRP. Using these conditions the assay was able to detect 31.2pg/ml, which was previously undetectable (signal to noise ratio of 2.7 vs. 1.0). At 15.6pg/ml the signal was too close to the background noise (signal to noise ratio of 1.4). Therefore it was possible to extend the standard curve down to 31.2 pg/ml, a two-fold dilution further than under the previous conditions. The highest standard 4000pg/ml was removed from the standard curve as previous work had shown that the aortic samples expressed MCP-2 at the lower range of the standard curve. The new standard curve therefore ranged from 31.2pg/ml – 2000pg/ml (Figure 7-5).

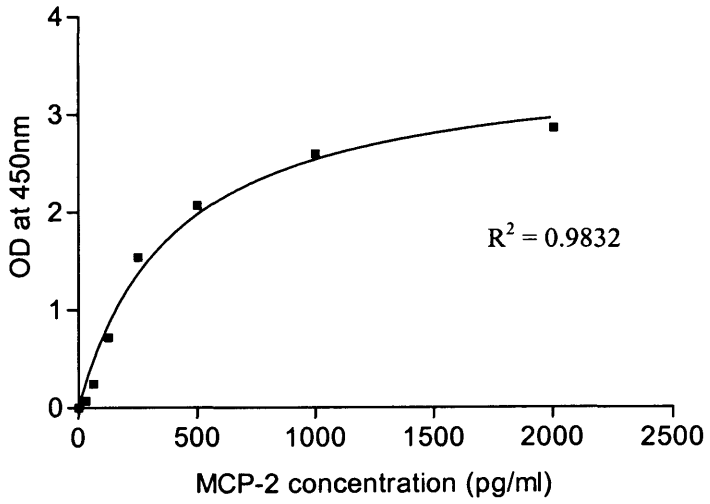


Figure 7-5. The standard curve (31.2pg/ml-2000pg/ml) for MCP-2 under the new assay conditions. The ELISA was performed using 5μg/ml capture antibody, 50ng/ml detection antibody and 1/50 dilution of SA-HRP.

Testing the Accuracy of the Modified Standard Curve under the New Assay Conditions

Having altered the standard curve and the conditions of the assay it was important to determine that the standard curve could be used to accurately calculate unknown concentrations. This was assessed by performing another ELISA with the new standard curve (31.2pg/ml-2000pg/ml) and backplotting the OD values of the standards. Essentially, this involved treating the standards as if they were of an unknown concentration and using the standard curve to calculate their concentration from their OD values. The backplotted concentration was then compared to the known concentration of the standard to measure the accuracy of the standard curve. Additionally the linearity of dilution was assessed using serial two-fold dilutions of the MCP-2 positive control.

Testing the Standard Curve (31.2pg/ml-2000pg/ml)

1) Backplotting:

The results of backplotting the OD values of the standards are shown in Table 7-18. The differences between the actual concentrations of the standards and the concentrations obtained from the curve should be within ±10% of the expected values. At the lower end of the standard curve the differences between the expected and observed concentrations were too high, therefore using this to provide an accurate calculation of unknown samples would produce highly erroneous results.

Table 7-18. Comparison between the actual concentrations of the standards and the backplotted concentrations.

MCP-2 Standards		
Expected Concentration (pg/ml)	Observed Concentration (pg/ml)	% Difference
0.0	0.0	0.0
31.2	8.4	-73.1
62.5	29.8	-52.3
125	101.8	-18.6
250	306.4	+22.6
500	556.9	+11.4
1000	1086.1	+8.6
2000	1642.6	-17.9

2) Linearity of Dilutions:

The results of the linearity of serial dilutions of the positive control sample are shown in Table 7-19. Table 7-19 displays the concentration of the non-diluted MCP-2 positive control (688.1pg/ml) and shows the concentration that would be expected from this control when diluted in a linear manner. The observed concentration displays the results that were actually obtained from the ELISA. If the ELISA could have accurately measured the MCP-2 concentration of the diluted samples then the linearity of the dilution should have been maintained. Therefore multiplying the observed data by its dilution factor should have equalled approximately 688.1pg/ml. As shown in Table 7-19 the results are not linear. The recovery of MCP-2 is expressed in the last column as a percentage of the amount of MCP-2 measured by the ELISA out of the expected amount. If the assay had accurately detected a linear dilution in the control then the values in the last column should have been approximately 100%.

Table 7-19. The assessment of the linearity of dilution on the observed MCP-2 concentration.

Dilution Factor	Expected Concentration*	Observed Concentration	Observed Concentration x Dilution Factor	% of Expected Concentration
1	688.1	688.1	688.1	100
1:2	344.1	239.9	479.8	69.7
1:4	172.0	91.5	366.0	53.2
1:8	86.0	29.8	238.4	34.6

* Expected concentration based on two-fold serial dilutions of the highest concentration of the positive control.

The results show that the amount of MCP-2 measured by the assay was much lower than it should have been. The deviation of the results from the expected values is most likely due to the inaccuracies in the standard curve, as shown through backplotting. Therefore this standard curve (31.2pg/ml–2000pg/ml) would have been unsuitable for measuring MCP-2 in the aortic samples.

For the purpose of measuring the concentration of MCP-2 in aortic tissue only the lower linear section of the standard curve was required. Therefore the results of the previous assay were re-assessed just using the linear section of the curve (31.2pg/ml-250pg/ml).

Testing the Standard Curve (31.2pg/ml-250pg/ml)

Figure 7-6 displays the linear section of the standard curve.

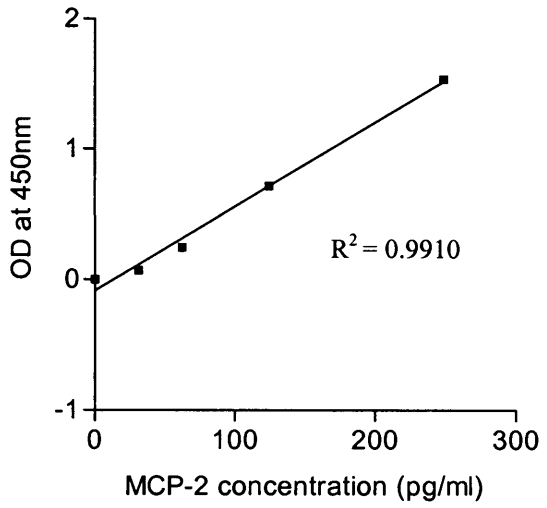


Figure 7-6. The standard curve (31.2pg/ml-250pg/ml) for MCP-2. The ELISA was performed using 5μg/ml capture antibody, 50ng/ml detection antibody and 1/50 dilution of SA-HRP.

1) Backplotting:

To test the accuracy of this standard curve the standard OD values were backplotted. The results are shown in Table 7-20. The differences between the actual concentrations of the standards and the concentrations obtained from the curve are much closer without the distortion of the line by the curve at higher concentrations.

Table 7-20. Comparison between the actual concentrations of the standards and the backplotted concentrations.

MCP-2 Standards		
Expected Concentration (pg/ml)	Observed Concentration (pg/ml)	% Difference
0.0	0.0	0.0
31.2	24.8	-20.5
62.5	51.7	-17.3
125	125.4	+0.32
250	253.3	+1.32

2. Linearity of Dilutions:

The OD data from the serially diluted positive control were re-analysed on the linear standard curve (31.2pg/ml-250pg/ml). Table 7-21 displays the concentration of the highest MCP-2 control that fitted the linear standard curve (220.6pg/ml) and shows the expected concentrations resulting from serial dilutions of this control. The observed concentrations displayed in the table came from the re-analysis of the OD data on the linear standard curve. The observed concentrations multiplied by their dilution factors resulted in final concentrations similar to that of the neat control. The results showed that the observed concentrations of the diluted control were within 93.7-105% of the expected concentration. Therefore the assay was able to measure MCP-2 concentration sufficiently accurately across a dilution range. As the same OD values were used as for the previous standard curve the difference in results cannot relate to experimental variation but from the inaccuracy in the previous standard curve.

Table 7-21. The assessment of the linearity of dilution on the observed MCP-2 concentration.

Dilution Factor	Expected Concentration*	Observed Concentration	Observed Concentration x Dilution Factor	% of Expected Concentration
1	220.6	220.6	220.6	100
1:2	110.3	116.2	232.4	105
1:4	55.2	51.7	206.8	93.7

* Expected concentration based on two-fold serial dilutions of the highest concentration of the positive control.

Using the linear section of the standard curve it was possible to obtain accurate concentration readings. The limited upper concentration range of 31.2pg/ml-250pg/ml was unlikely to be a problem for measuring MCP-2 in the aortic samples as the earlier ELISA had shown that overall the expression of MCP-2 in AAAs was at the lower end of the concentration range. When it was attempted to extend the standard curve down to 15.6pg/ml the signal to noise ratio was similar to that of the blank and therefore the array was unable to quantify MCP-2 at concentrations less than 31.2pg/ml, although this was an improvement on 62.5pg/ml. Additional minor improvements to increase the detection signal strength included increased incubations times, incubation temperatures and shaking the plates during incubations.

Intra-assay Variation

The intra-assay variation for the optimised MCP-2 ELISA is displayed in Table 7-22. The intra-assay variation across 16 wells on the same plate was determined using an aliquot of the positive AAA control. Within the same assay the positive control had a mean value of 71.7 ± 7.5pg/ml and the coefficient of variation in the concentration of the control between the wells was 10.5%. A measure of the inter-assay variability of the assays used to measure MCP-2 within the aortic specimens could not be given as only two plates were used. The positive control was incubated on each assay to provide a qualitative indication that the assay had worked.

Table 7-22. The intra-assay variation of the MCP-2 ELISA.

Well Number	MCP-2 Control Concentration (pg/ml)
1	74.4
2	75.8
3	55.9
4	66.3
5	67.9
6	60.1
7	70.0
8	67.5
9	77.5
10	70.4
11	80.3
12	79.4
13	81.9
14	77.6
15	65.3
16	76.7
Mean	71.7
SD	7.5
Coefficient of Variation (%)	10.5

SD, standard deviation.

ELISA Measurement of MCP-2 in Aortic Specimens

Having improved the sensitivity and validated the linearity of the extension of the curve (31.2-250pg/ml) an ELISA assay was done on the aortic specimens to measure the concentration of MCP-2 within the samples (Table 7-23).

Table 7-23. The MCP-2 concentrations measured in aortic specimens

Concentration of MCP-2 (pg/ml)		
AAA	Abdominal Aorta	Thoracic Aorta
162.5	126.8	-
125.1	42.5	-
72.3	100.5	-
53.0	-	-
76.6	-	-
72.9	-	-
29.2	-	-
31.8	-	-

Most of the samples had OD values lower than the standard curve despite the extension of the curve and their concentration remained undetermined. Out of 25 AAA specimens the concentration could only be determined for 8 AAA specimens. The concentration was measured in 3 abdominal aorta specimens out of 15 samples and none of the thoracic aortic specimens could be measured. The concentrations are shown in Table 7-23. There was not sufficient data to allow for a comparison of the expression of this cytokine in AAAs and other aortic tissue.

7.4 Discussion

The aim of this chapter was to investigate the expression of IL-6, IL-8, MCP-1 and MCP-2 within the AAA and non-aneurysmal aorta in order to support the array findings. The null hypothesis was that there are no significant differences in the quantitative expression of any of these cytokines between the AAA, abdominal or thoracic aorta.

The main finding of this chapter is that:

1. IL-6, IL-8 and MCP-1 are significantly increased within the AAA.

MCP-2 was investigated but for experimental reasons it was not possible to measure its concentration. Therefore the null hypothesis can be rejected for IL-6, IL-8 and MCP-1, but not for MCP-2.

Initial experiments involved validating the IL-6, IL-8 and MCP-1 ELISAs with regards to intra- and inter-assay variability and the variation was found to be within an acceptable limit. The intra-assay variation was much higher in the MCP-1 assay than for IL-6 and IL-8. However, the intra-assay variation of MCP-1 was determined with a positive AAA homogenate sample as opposed to a purified IL-6/IL-8 protein solution. Whilst in part the difference in intra-assay variation between the ELISAs could be a physical difference between the assays, it is also likely to reflect the different matrices used to measure intra-assay variation. As the MCP-1 control was a positive homogenate sample there is a potential issue of antigen to antibody accessibility with steric hindrance from other proteins and cellular components compared to that of a purified protein solution. However, as the samples to be tested by the ELISAs are homogenates this might actually be a more appropriate method for determining the intra-assay variability of the test conditions. As discussed in the results section, the difference between the expression of MCP-1 in the AAA group and the controls is so great it is unlikely to be affected by the intra-assay variation.

It was possible to assess the inter-assay variation for IL-6 and IL-8 because multiple plates were used as the assays had to be repeated a number of times before the AAA samples were sufficiently diluted enough to be measured by the standard curve. Fewer plates were required for the MCP-1 assay as all AAA samples were diluted down 10-fold prior to performing the ELISA and, as with MCP-2, only two plates were required to measure all the aortic samples. Therefore it wasn't possible to measure the inter-assay variation between the plates for MCP-1, however this was immaterial for MCP-2.

Whilst the previous ELISAs had been purchased as fully validated, ready-to-use assays it had not been possible to purchase an MCP-2 kit. Performing the ELISA by the

manufacturer's protocol did not detect any MCP-2 and a series of experiments were conducted to optimise the protocol. There are many parameters which influence the results obtained in an ELISA including: antibody quality and concentration, incubation times, incubation temperatures, detection reagent quality and concentration, and substrate type and quality. The substrate used in the assay was supplied by R&D Systems and had been validated for use with this assay. It was therefore unlikely to be a major factor influencing the sensitivity of the assay. The key factors that could be manipulated were identified as the concentration of capture and detection antibodies used (both supplied with the development assay) and SA-HRP concentration. Having increased the sensitivity of the assay and extended the linearity of the standard curve down to 31.2pg/ml the new conditions were validated. However, it was found that the full standard curve produced inaccurate results when backplotted and that the results of the serial dilution of the control did not reflect the dilution series. Assessment of the linear section of the curve found that this section was able to accurately determine the concentration of the backfitted standards and the serially diluted control. Whilst this was used subsequently to determine MCP-2 concentration in the aortic samples it did consist of only four standard curve points. My concern with this protocol is that with such a limited number of points the standard curve is at a greater risk of being distorted by an erroneous measurement of one of the standards, for example due to a pipetting error. This would directly affect the accuracy of the results obtained. Despite this, the standard curves used to measure MCP-2 within the aortic specimens both had R^2 values close to 1.0, demonstrating that the standard points were closely fitted to the line. However, despite increasing the sensitivity of the assay and validating the extension of the standard curve from 62.5pg/ml down to 31.2pg/ml, it wasn't sufficient to measure MCP-2 expression within the aortic groups.

The expression of IL-6, IL-8 and MCP-1 within the AAA wall have been briefly mentioned in *Chapter 6.4 Discussion*. The arrays and ELISAs have shown that IL-6 is significantly higher in the AAA than in the non-aneurysmal controls. IL-6 has been described as being highly expressed within the AAA wall by several studies[82, 227, 298, 299]. Elevated circulating levels of IL-6 have also been described within AAA patients compared to coronary heart disease patients or normal subject controls[300]. Rohde *et. al.* reported that serum IL-6 was positively associated with aortic diameter in non-aneurysmal subjects and suggested that IL-6 may be involved in the early phase of AAA formation[348]. IL-6 mRNA has been reported to be elevated in the AAA wall compared to AOD[82],

although one study on the expression of IL-6 protein did report it to be lower in the AAA compared to AOD[84]. Jones *et. al.* found the levels of IL-6 were significantly higher in the iliac arteries compared to the brachial arteries and concluded that the AAA was a source of circulating IL-6[227]. The cascade of cytokines found within the AAA is thought to arise from some unknown primary injury and one possible mechanism is hypoxia resulting from intraluminal thrombus. Interestingly, hypoxia, a condition of which the infrarenal aorta is prone to, partly due to the reduced number of vasa vasorum in the abdominal aorta, induces the expression of IL-6 by vascular cells[349].

This thesis has shown that IL-8 and MCP-1 are raised within the AAA compared to non-aneurysmal aorta. Previously, both these chemokines have been shown to be increased in the AAA compared to occlusive and normal aorta[310]. The increase in IL-8 expression also occurs at the gene level. A gene array study of 1176 gene products within the AAA wall found IL-8 to be raised 11-fold when compared to normal cadaveric organ donors[187]. The IL-8 receptor CXCR2 has also been shown to be increased within the human AAA[350]. An increase in IL-8 and its receptor means that through IL-8 signal transduction this chemokine could potentially increase chemotaxis into the aneurysmal wall. IL-8 has been shown to be chemotactic for human endothelial cells[351] and inflammatory cells, especially neutrophils[339]. IL-8 achieves this through its ability to induce cellular expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[352] and through the provision of a chemotactic signal. IL-8 can also induce neutrophil degranulation[308] and the release of MMP-9 contained within these granules[323]. IL-8 therefore has the potential to release lytic enzymes within the AAA wall which could result in aortic wall damage.

A potential role for MCP-1 in AAA formation is supported by evidence from an angiotensin II induced AAA model (in apo $E^{-/-}$ deficient mice) where those mice with the additional knockout of CCR2, the receptor for MCP-1, suppressed the formation of AAAs compared to those with CCR2[353]. MCP-1 was shown to be up-regulated within the AAA wall of an elastase-induced mouse model prior to macrophage infiltration of the aortic wall[312]. IL-8 and MCPs have been shown previously to act synergistically to achieve maximal neutrophil migration at suboptimal IL-8 levels[354]. It is therefore possible that the increased expression of both IL-8 and MCP-1 within the AAA wall may synergistically augment neutrophil chemotaxis into the AAA wall. It may be that subsequent to an initial

injury, chemokine expression results in the infiltration of inflammatory cells which results in chronic inflammation and increased proteolysis. MCP-1 can induce the release of specific enzymes, such as Granzyme A, *N*-acetyl *β-D*-glucosaminidase and MMP-9[347]. This enables cells to digest ECM components in order to migrate into tissues. MCP-1 has also been shown to up-regulate the expression of CD11b and CD11c integrin on monocytes, enabling the monocytes to interact with endothelial cells during inflammation[355, 356]. Therefore the expression within the AAA of IL-8 and MCP-1 could prove detrimental not only due to increased chemotaxis but through the release of matrix degrading enzymes to enhance migration.

In conclusion, this chapter has been able to confirm that IL-6, IL-8 and MCP-1 are raised within the AAA. However, it was unable to measure the expression of MCP-2 in aortic tissue. An alternative method to measure MCP-2 expression is required. Analysing the expression of MCP-2 at the gene level might provide further information and RT-PCR is a more sensitive method of analysis than ELISAs. An extension of this work could involve investigating the expression of all of these cytokines at the gene level to support the ELISA work and also to investigate if there are any discrepancies between protein and genomic expression. The role of the chemokines IL-8 and MCP-1 in AAA wall damage may extend from their involvement in chemotaxis, not only for recruitment of inflammatory cells, but also through the release of MMPs to enhance cellular migration across the ECM. The following chapter characterises the site of expression of IL-8 and MCP-1 within the AAA wall and investigates the association between these chemokines and inflammation.

Chapter Eight

Chemokine Expression and the Inflammatory Infiltrate in the Abdominal Aortic Aneurysm

Chapter 8. Chemokine Expression and the Inflammatory Infiltrate in the Abdominal Aortic Aneurysm

8. Chemokine Expression and the Inflammatory Infiltrate in the Abdominal Aortic Aneurysm

8.1 Introduction

The previous chapter confirmed the higher expression of IL-6 and the chemokines IL-8 and MCP-1 within the AAA compared to controls. This chapter focuses on the expression of IL-8 and MCP-1 within the AAA wall due to an interest in the potential role of chemokines in the pathogenesis of the AAA. IL-6 was not studied further. This chapter describes the cellular nature of the inflammatory infiltrate, the regional expression of these chemokines and their co-localisation with the infiltrating leukocytes.

IL-8 is normally expressed by multiple cell types including fibroblasts[357], endothelial cells[329], lymphocytes[358], macrophages[359, 360] and PMN[360]. MCP-1 is expressed by many different cell types including SMCs[361], fibroblasts[362], endothelial cells[363] and mononuclear cells[362, 363]. In unstimulated cells the production of IL-8 and MCP-1 is low. The main aim of this chapter is to identify the sites of expression of these chemokines within the AAA and identify if these chemokines co-localise with the inflammatory infiltrate. As one of the main characteristics of chemokines is their chemotactic property the association between the extent of inflammation within each sample and the expression of IL-8 and/or MCP-1 is also examined.

In order to determine the co-localisation of IL-8 and MCP-1 the inflammatory infiltrate within the AAA is examined. As discussed in *Chapter 2.4.4 Inflammatory Infiltrate*, the inflammatory infiltrate of the AAA is chiefly formed from T-lymphocytes, B-lymphocytes, plasma cells, neutrophils, macrophages and dendritic cells[156-158]. This chapter also includes a characterisation of the inflammatory infiltrate of the AAA to provide an overview of the inflammatory state of the specimens studied by the ELISAs. Changes in the cellular composition of the inflammatory infiltrate from AAAs with increasing levels of inflammation are investigated.

The null hypotheses tested in this chapter are:

1. **There are no significant differences in the cellular composition of the inflammatory infiltrate between AAAs with different grades of inflammation.**
2. **There is no significant difference in the extent of mural inflammation between AAAs expressing increasing levels of IL-8 or MCP-1.**
3. **In the event that (2) is disproved: There are no significant differences in the cellular composition of the inflammatory infiltrate between AAAs expressing increasing levels of IL-8 or MCP-1.**

8.2 Methods

The histology and characterisation of the extent of inflammation within the AAA was assessed through immunohistochemistry and general staining, as described in *Chapter 5 Materials & Methods*. Abdominal aorta was used to provide a comparative example of the histology of the non-diseased aorta. Thoracic aorta was not examined due to its different anatomical structure to abdominal aorta. The characteristics of the AAA (n = 21) and abdominal aorta (n = 16) groups are shown in Table 8-1. All samples were fixed in 10% formalin and stored in paraffin wax.

Tissue was sectioned and stained as described in *Chapter 5*. To summarise, sections from all the specimens were subjected to H&E and EVG staining to examine their histology. The inflammatory infiltrate was characterised using immunohistochemistry to stain for CD3, CD4, CD8, CD20 and CD68. The localised expression of IL-8 and MCP-1 were visualised using antibodies specific to these chemokines.

Table 8-1. Demographics of the study population.

Risk Factor	AAA (n = 21)	Abdominal aorta* (n = 16)
Median age (range) / years	73 (61-84)	56.5 (23-74)
Male sex, n (%)	19 (90)	11 (69)
Smoking history, n (%)	18 (86)	13 (81)
Median AAA diameter (range)/mm	67 (55-93)	n/a

* Only a brief medical history was supplied with the abdominal aorta from kidney donors.

8.2.1 Microscopic Evaluation

Each staining, with the exception of the specific lymphocyte stainings, was assigned a score from 0 to 3. Lymphocytes (CD3, CD4, CD8 and CD20) were graded based on the percentage of positively stained cells within the infiltrate. The results were graded according to the extent of staining present under a high-powered field of view (magnification x250) and independently assessed by two observers, including a consultant pathologist. All stainings were performed in duplicate and negative slides were treated with the appropriate isotype control and/or PBS to check for non-specific binding of the antibodies and detection system.

1. Inflammation and Extracellular Matrix Degradation:

The overall level of inflammation and extent of ECM degradation within the aneurysm wall, as a whole, was assessed by light microscopic examination of the H&E (Table 8-2) and EVG (Table 8-3) staining, respectively. The following scoring systems were used:

Table 8-2. H&E grading

Score	Definition
0	No inflammatory cells visible,
1	Mild inflammation with diffused inflammatory cells,
2	Moderate inflammation with the appearance of dense follicles (1-3 follicles),
3	Extensive inflammation with $\geq$ 4 follicles or such extensive infiltration that the follicles have merged.

Table 8-3. EVG grading

Score	Definition
0	No discernable loss of elastin, intact elastic lamellae,
1	Mild loss of elastin, fragmented lamellae visible,
2	Moderate loss of elastin, widely fragmented lamellae,
3	Extensive loss of elastin, very little elastin present.

2. Characterisation of the Inflammatory Infiltrate:

The cellular composition of the inflammatory infiltrate within each layer of the aorta was determined through a series of stainings. Specific lymphocytes were identified through

staining for CD3 (T-lymphocytes), CD4 (T_H-lymphocytes), CD8 (T_C-lymphocytes) and CD20 (B-lymphocytes). The percentages were categorised into the scores shown in Table 8-4.

The extent of macrophage infiltration was measured through CD68 staining. The scoring system that was used is shown in Table 8-5.

3. Assessment of the Chemokine Stainings:

The IL-8 and MCP-1 stainings were given an overall grade for each specimen based on the scale shown in Table 8-5. Additionally, it was recorded which layers were positively stained, whether the infiltrate was stained and which cells were positively stained.

Table 8-4. Lymphocyte scoring system

Score	Percentage of cells positively stained (%)
0.0	0
0.5	>0<2
1.0	≥2<10
1.5	10
2.0	>10<25
2.5	25
3.0	>25<50
3.5	50
4.0	>50<75
4.5	75
5.0	>75<100

Table 8-5. Macrophage/IL-8/MCP-1 scoring system

Score	Definition
0	No visible staining
1	Mild staining
2	Moderate staining
3	Extensive staining

8.2.2 Statistical Analysis

All data is presented as medians with interquartile ranges. Statistical analysis of the changes in cellular composition with increasing inflammation or IL-8 expression was performed using a Kruskal-Wallis test, followed by a series of Mann-Whitney *U* tests. The association between the level of inflammation and chemokine expression was measured using a Chi-square test. A P value of <.05 was used to determine a significance difference for the Kruskal-Wallis and Chi-square test. To correct for multiple comparisons of the data by Mann-Whitney tests a P value of <.017 was used to determine statistical significance, as determined through the Bonferroni method (*Chapter 7.2 Methods*).

8.3 Results

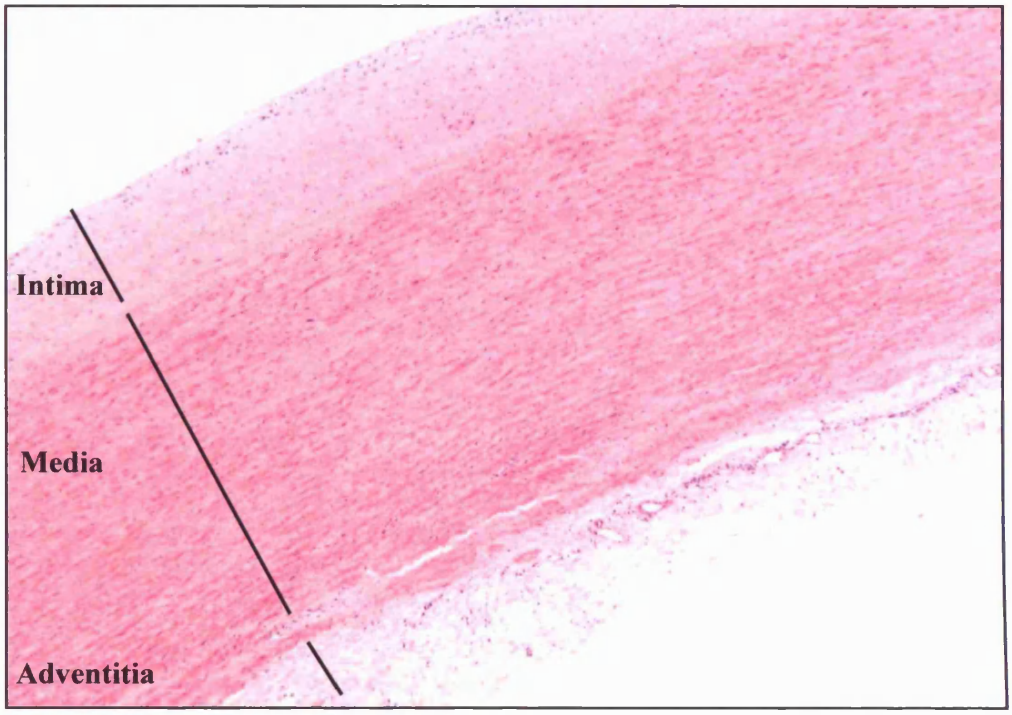
8.3.1 Inflammation within the Abdominal Aortic Aneurysm Wall

H&E Staining

The overall inflammatory status of the AAA wall was determined using H&E staining. Figure 8-1 shows H&E staining of the AAA and control abdominal aortic wall. Nuclei are stained a purple/blue by the haematoxylin dye, whilst collagen and other connective tissues are stained pink by the eosin dye. Figure 8-1*A* shows a cross-section of the abdominal aorta. The control demonstrates the highly organised structure of the aorta with a clear distinction between the layers. The media is broad, much thicker than the adventitia, and shows the regular layering of the elastic lamellae between layers of SMCs. The connective tissue within the adventitia can be seen as an irregular arrangement of fibres. Figure 8-2*A* shows in more detail the arrangement of the ECM of the medial layer.

A comparison of the wall structure between the abdominal aorta and that of the AAA (Figure 8-1*B*) shows that the AAA has lost its layered structure, including a thinning of the media and a thickening of the adventitia. The most notable feature in Figure 8-1*B* is the intense infiltration of this layer by inflammatory cells. Figure 8-2*B* highlights the loss of ECM within the medial layer of the AAA. Compared to the abdominal aorta there is a notable loss of SMCs within the media and the ECM appears fragmented.

A) Abdominal Aorta



B) AAA

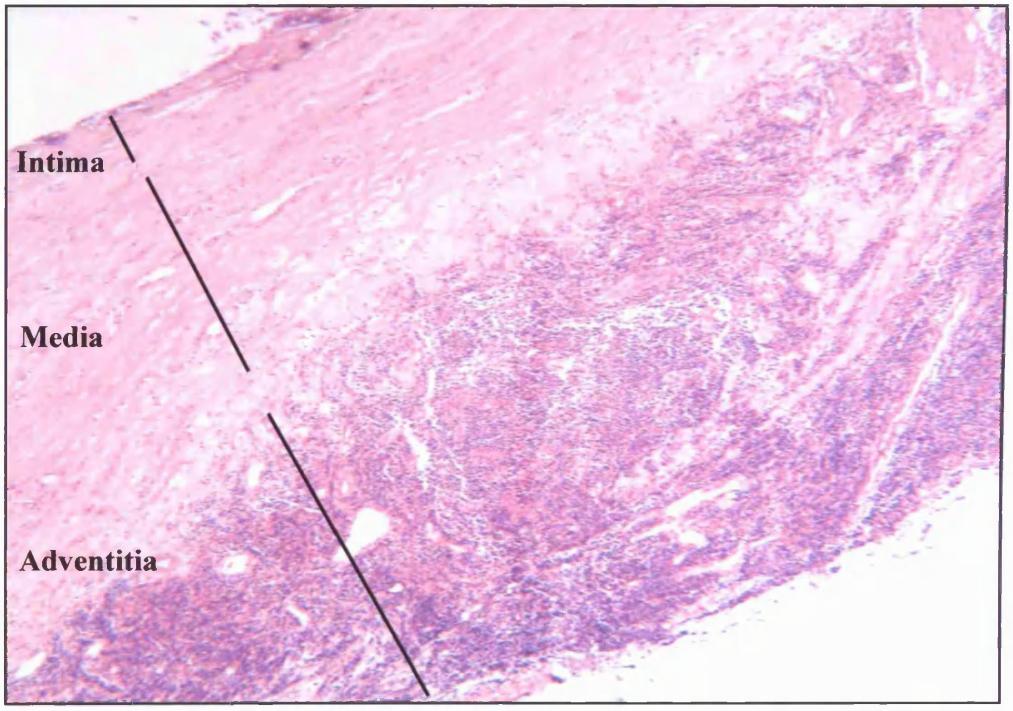
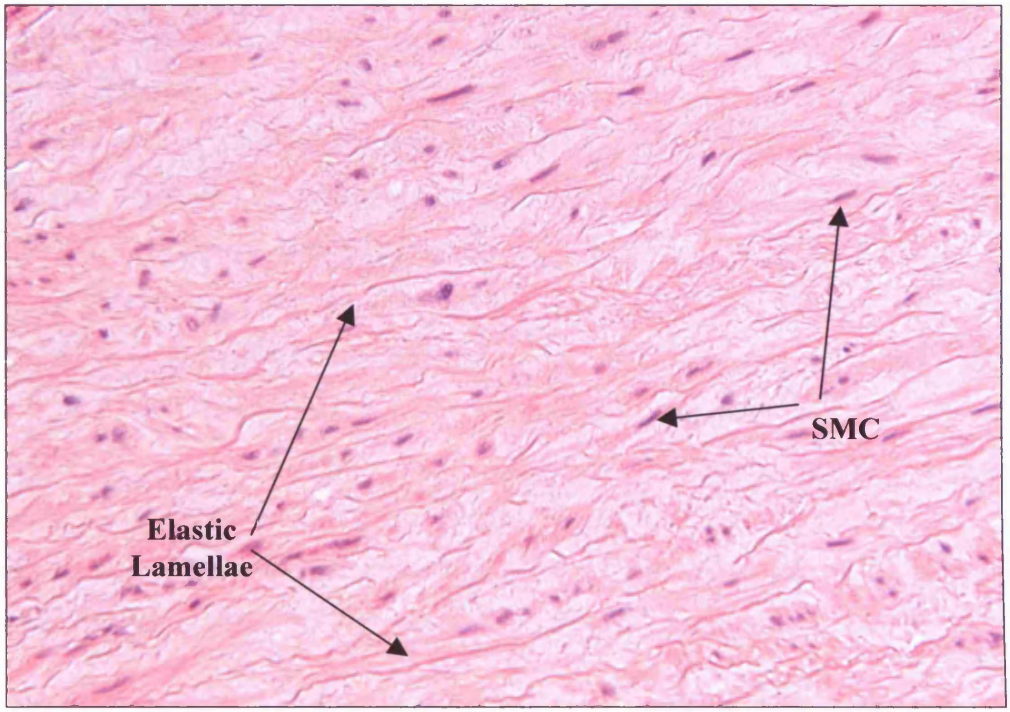


Figure 8-1. The inflammatory nature of the AAA wall, as demonstrated by H&E staining. (A) The H&E staining of the abdominal aorta demonstrates the three-layered organisation of the aorta. (B) The H&E staining of the AAA shows the extensive inflammation within the aneurysm wall, including a mass infiltration of the adventitia. Magnification: x40.

A) Abdominal Aorta - Media



B) AAA – Media/Adventitia Border

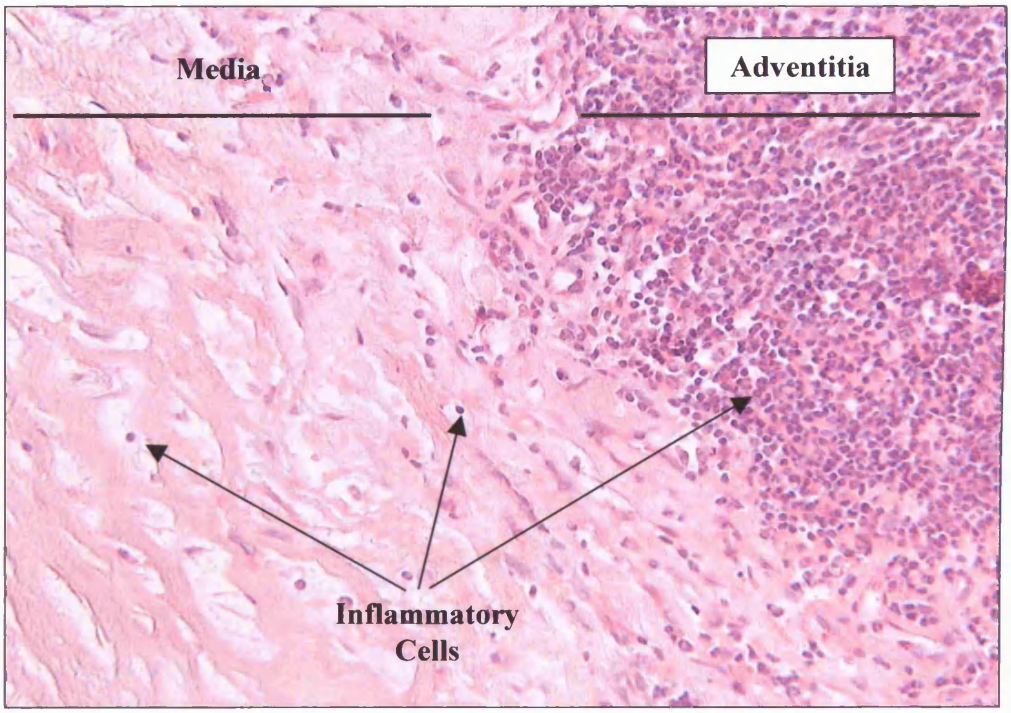


Figure 8-2. The changes within the media of the AAA wall, as demonstrated by H&E staining. (A) The H&E staining of the abdominal aorta demonstrates the organisation of the elastic lamellae between layers of SMCs within the media. (B) The H&E staining of the AAA shows the disruption of the media accompanied by a loss of SMCs and the infiltration of the adventitia, and occasionally the media, by inflammatory cells. Magnification: x250.

Inflammatory Grading of the Aortic Groups

The H&E staining of the AAA group demonstrated that the infiltrate was predominantly found in the adventitia and occasionally in the media. The extent of inflammation varied between the AAA specimens from cases of mild inflammation to extensive inflammation (interquartile range = 1-3) (Figure 8-3 & Table 8-6). In mild cases of inflammation the inflammatory cells were found dispersed throughout the adventitial layer. However, with increasing inflammation the infiltrate formed dense follicles. On average the AAAs showed moderate inflammation (median score = 2). Grading the control tissue on the same scale as the AAAs showed that on average abdominal aortic samples did not shows signs of inflammation (median inflammatory score = 0). The control group displayed an inflammatory score ranging from no inflammation to mild inflammation in 50% of the abdominal aortic specimens (interquartile range = 0-1). Some control samples did exhibit a moderate infiltration of the intima associated with a thickening of the intimal layer.

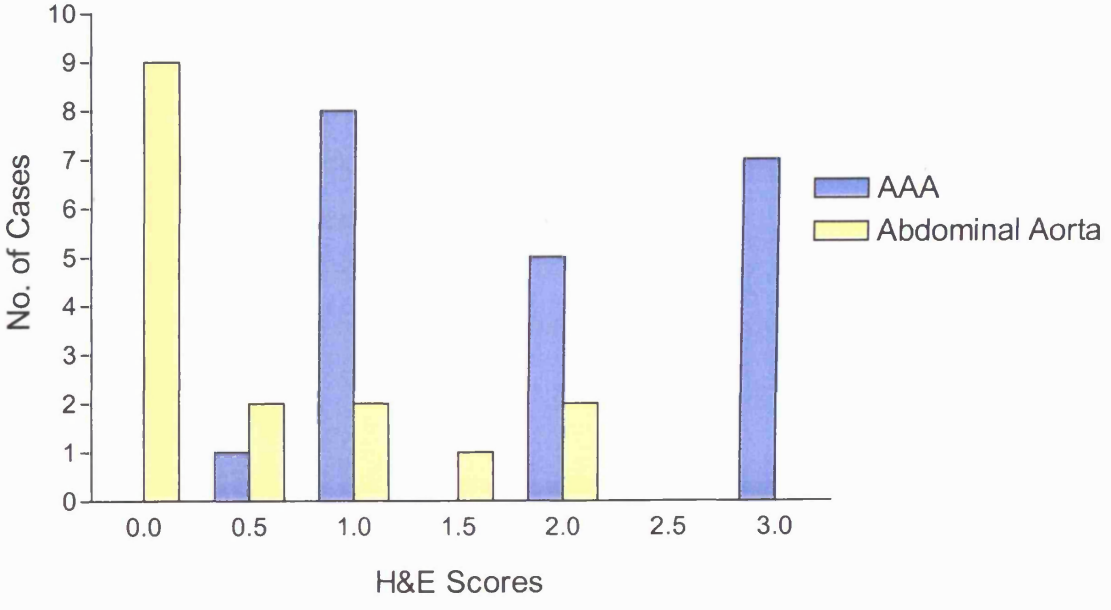


Figure 8-3. The distribution of the H&E scores for the AAA and the abdominal aorta groups. The majority of the abdominal aortic samples did not display any inflammation. The AAA samples displayed some inflammation, with the intensity varying from mild to extensive inflammation.

Table 8-6. Quantification of the extent of inflammation and loss of elastin

Stain	AAA		Abdominal Aorta	
	Median	Interquartile Range	Median	Interquartile Range
H&E	2	1-3	0	0-1
EVG	2	2-3	0	0-0

EVG Staining

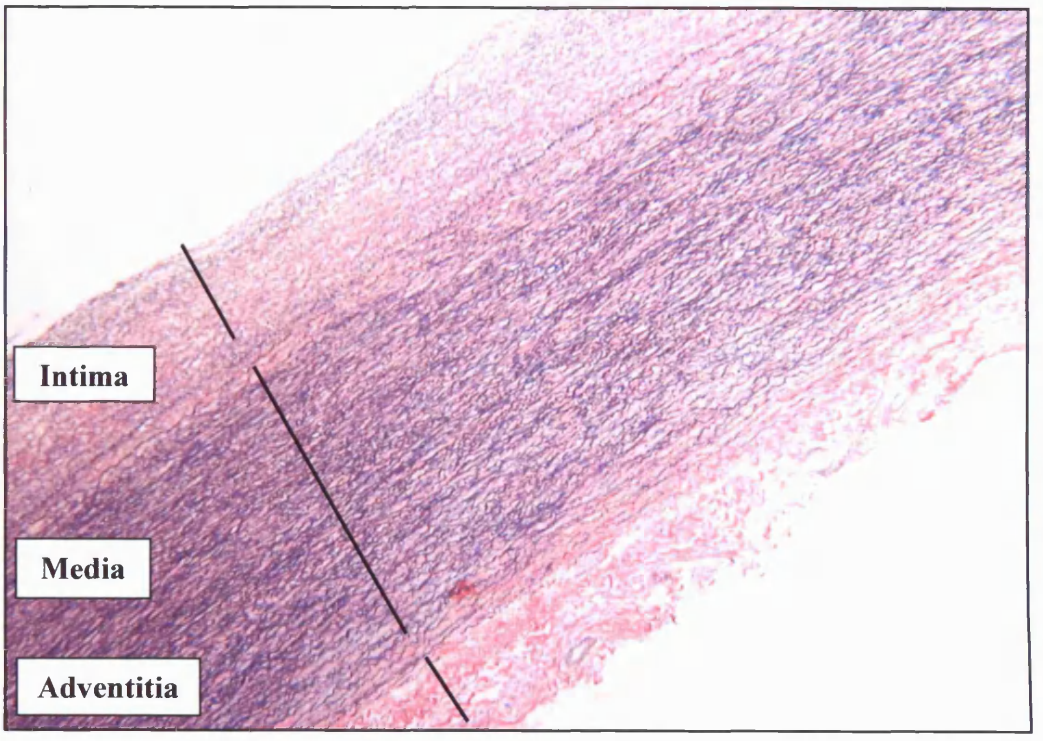
Figure 8-4 shows EVG staining of the AAA and control abdominal aorta wall. The EVG stains elastin black, collagen red and cytoplasm yellow. The EVG staining of the control abdominal aorta clearly shows the three layers of the abdominal aorta and demonstrates the arrangement of the ECM fibres (Figure 8-4*A*). The most notable feature is the black striations of elastic lamellae within the media and the elastic fibres within the adventitia. Figure 8-5*A* demonstrates the arrangement of the elastic lamellae within the media in more detail. The pink/red collagen fibres are arranged in a regular manner between the elastic lamellae to provide a well-organised connective tissue with the properties of high tensile strength and elasticity.

The EVG staining of the connective tissue within the AAA revealed disorganisation of the media and adventitia (Figure 8-4*B*). The red collagen fibres are present but in an irregular arrangement and the highly ordered elastic lamellae are absent from the media. The yellow mass that can be seen within this figure is the inflammatory infiltrate. Figure 8-5*B* provides a magnified image of the changes described within the media of the AAA.

EVG Grading of the Aortic Groups

Collectively, EVG staining of the AAA group demonstrated widespread fragmentation of elastic fibres and depletion of elastin (Figure 8-6 & Table 8-6). Overall the AAA group demonstrated a moderate loss of elastin (median EVG score = 2). An interquartile score range of 2-3 demonstrated that 50% of samples had suffered a moderate to extensive loss of elastin. Comparatively, the control group did not suffer a discernable loss of elastin (median score = 0), with an interquartile range of 0-0 (Figure 8-6 & Table 8-6).

A) Abdominal Aorta



B) AAA

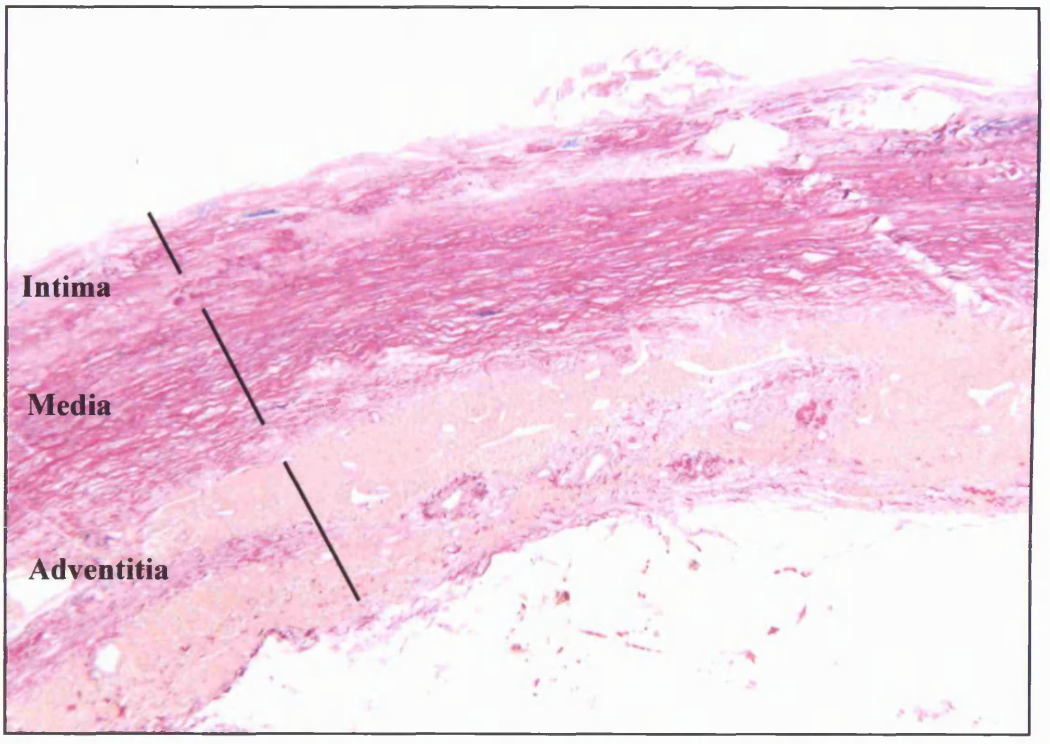
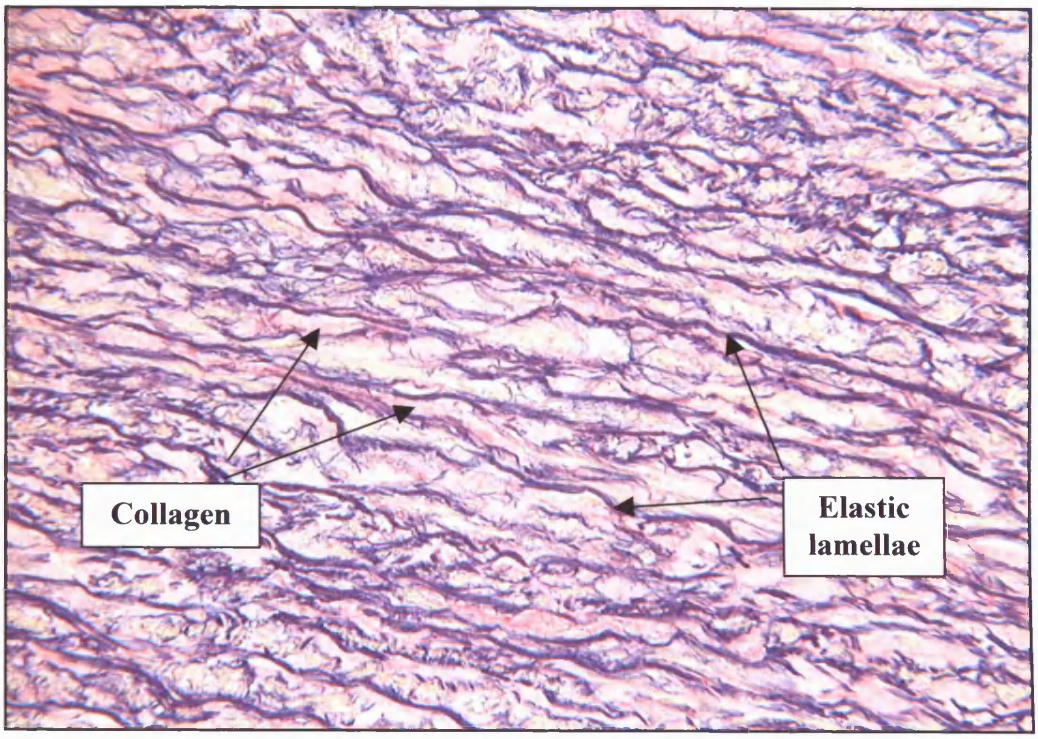


Figure 8-4. The loss of elastin within the AAA and disruption of the ECM organisation, as demonstrated by EVG staining. (A) The EVG staining of the abdominal aorta highlights the regular elastic lamellae arrangement. (B) The EVG staining of the AAA shows the loss of elastin from the media and the adventitia. Magnification: x40.

A) Abdominal Aorta - Media



B) AAA - Media

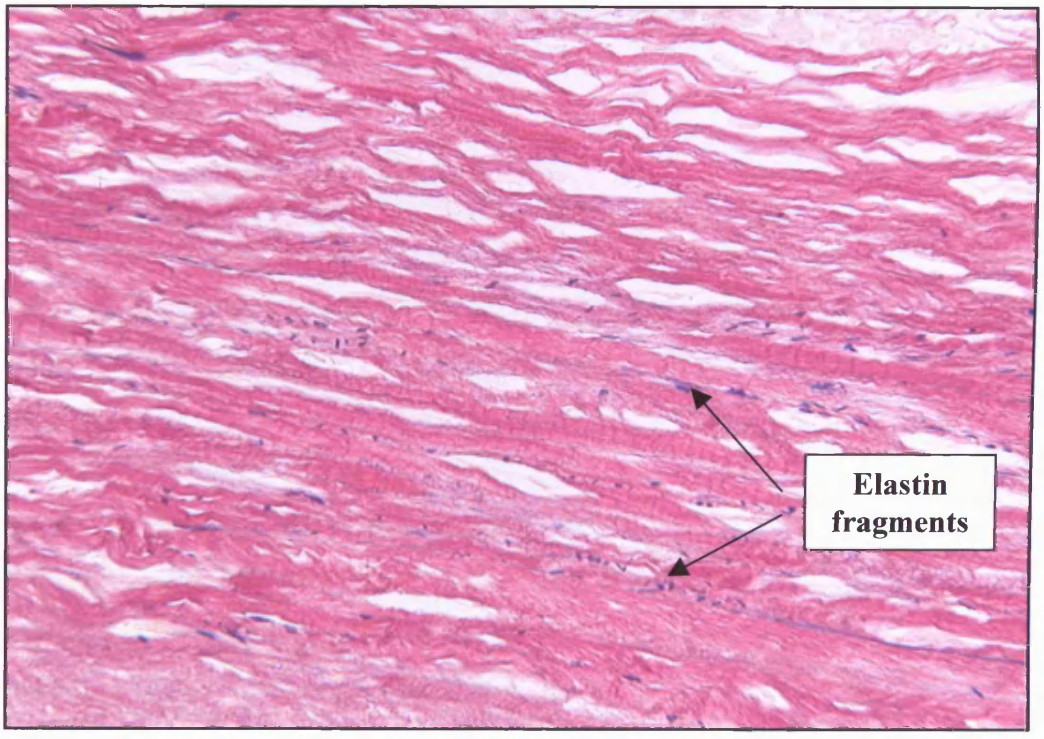


Figure 8-5. The loss of elastin from the media within the AAA, as demonstrated by EVG staining. (A) The EVG staining of the abdominal aorta highlights the regular elastic lamellae arrangement. (B) The EVG staining of the AAA shows the loss of elastin from the media. Magnification: x250.

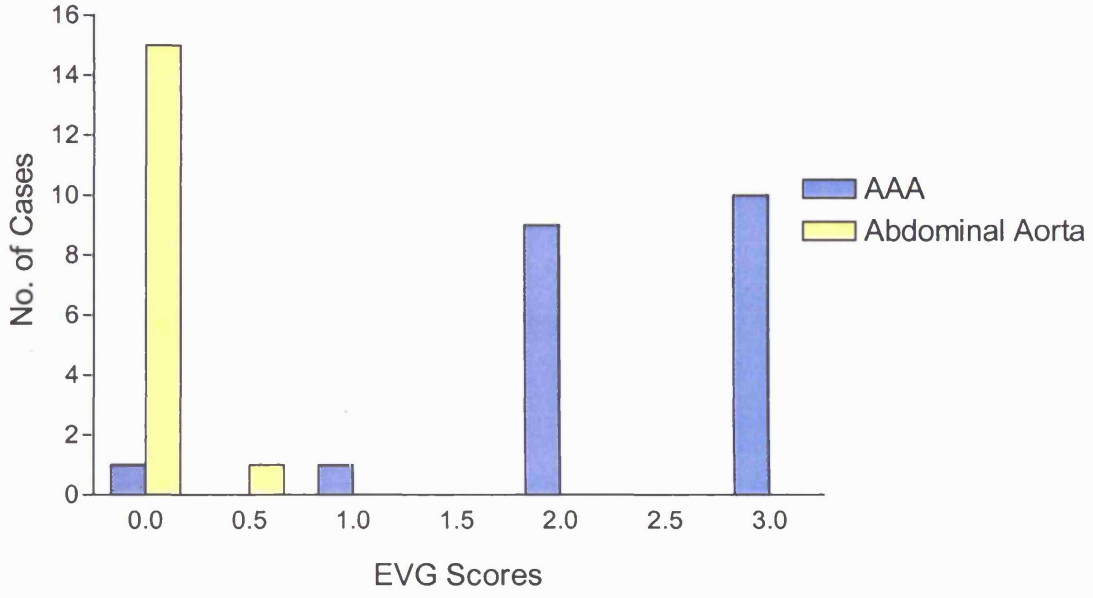


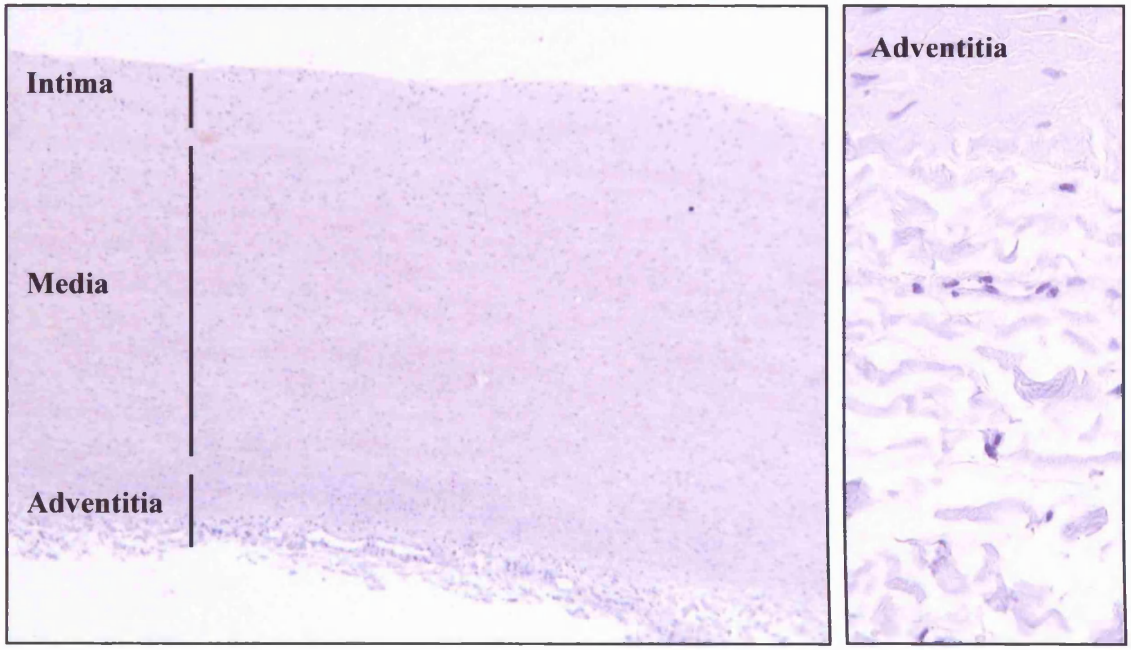
Figure 8-6. The distribution of the EVG scores for the AAA and the abdominal aorta groups. The abdominal aortic samples displayed negligible loss of elastin. The majority of the AAA samples displayed a moderate to extensive loss of elastin.

8.3.2 Characterisation of the Inflammatory Infiltrate

The basic composition of the inflammatory infiltrate within the AAA was studied using a series of CD markers for specific cell types. Staining of the abdominal aorta revealed that overall there was relatively little inflammation within the abdominal aorta (Figures 8-7*A* - 8-10*A* & Table 8-7).

Staining of the AAA revealed that lymphocytes (CD3, CD4, CD8 and CD20) were found almost exclusively in the adventitia, with occasional low level infiltration into the media. Lymphocytes were rarely found within the intima. Conversely, $CD68^{+}$ macrophages were predominantly found within the intima of the AAA, with some macrophages present within the adventitia (Figures 8-7*B* - 8-10*B* & Table 8-8).

A) Abdominal Aorta – CD20



B) AAA – CD20

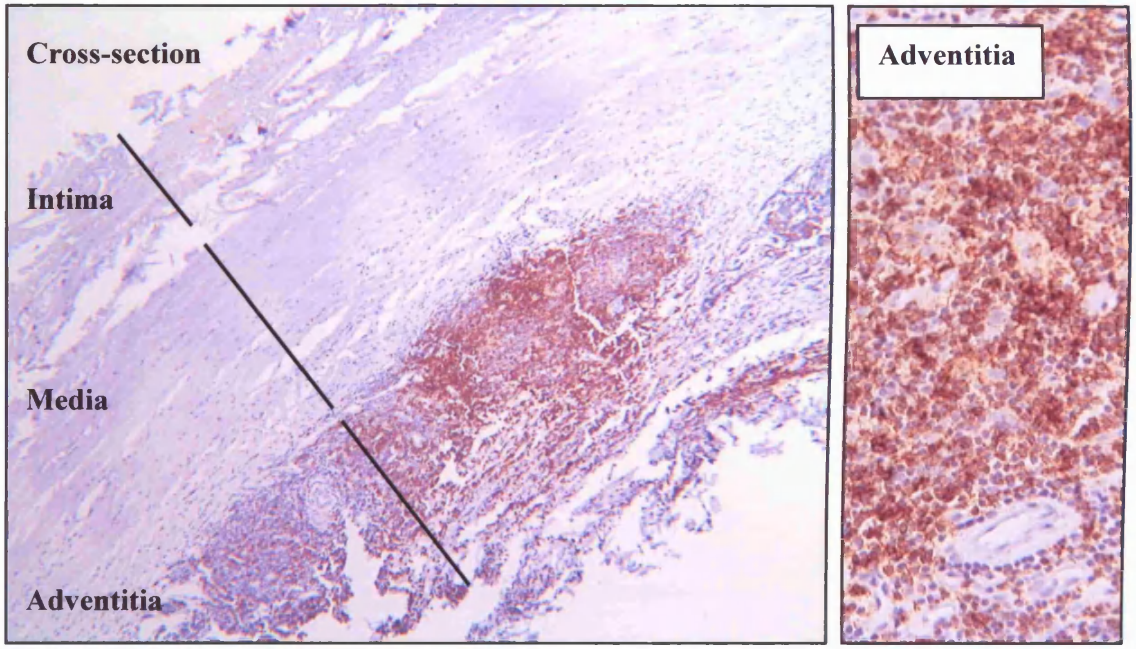
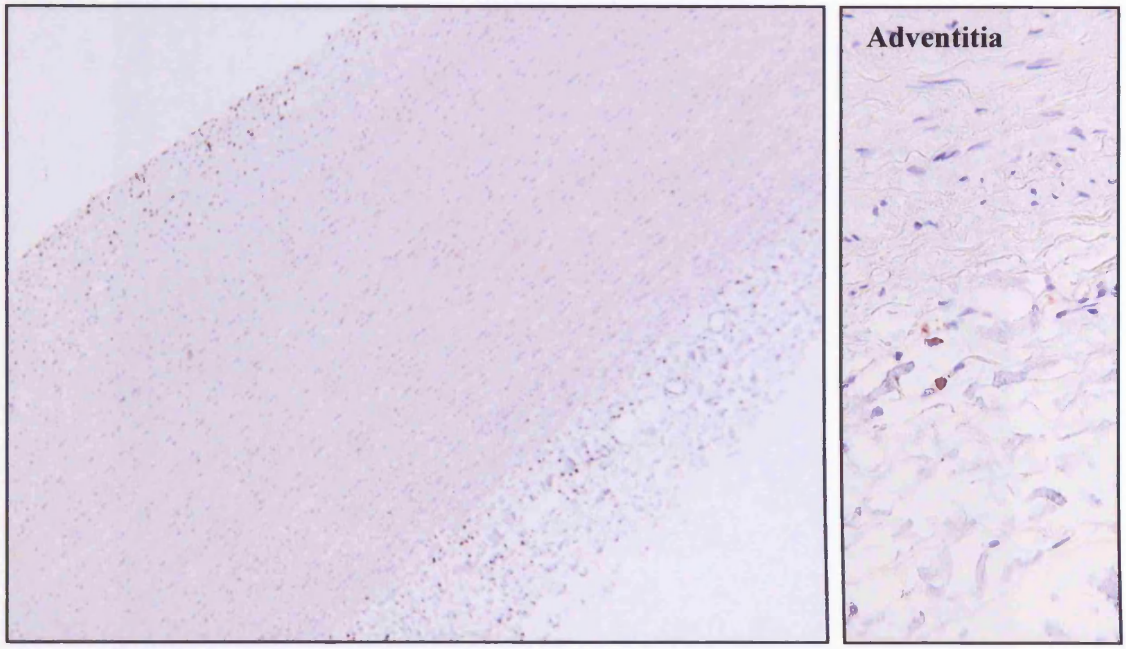


Figure 8-7. CD20 specific staining of the aortic wall of (A) the control abdominal aorta and (B) the AAA. Sections were stained by immunohistochemistry using anti-human CD20 antibodies to detect B-lymphocytes. The left-hand images depict the cross-sectional view of the aortic wall and are orientated with the intima at the top of the cross-section and the adventitia at the bottom (magnification: x40). The right-hand images show CD20 staining of the adventitia (magnification: x250).

A) Abdominal Aorta – CD3



B) AAA – CD3

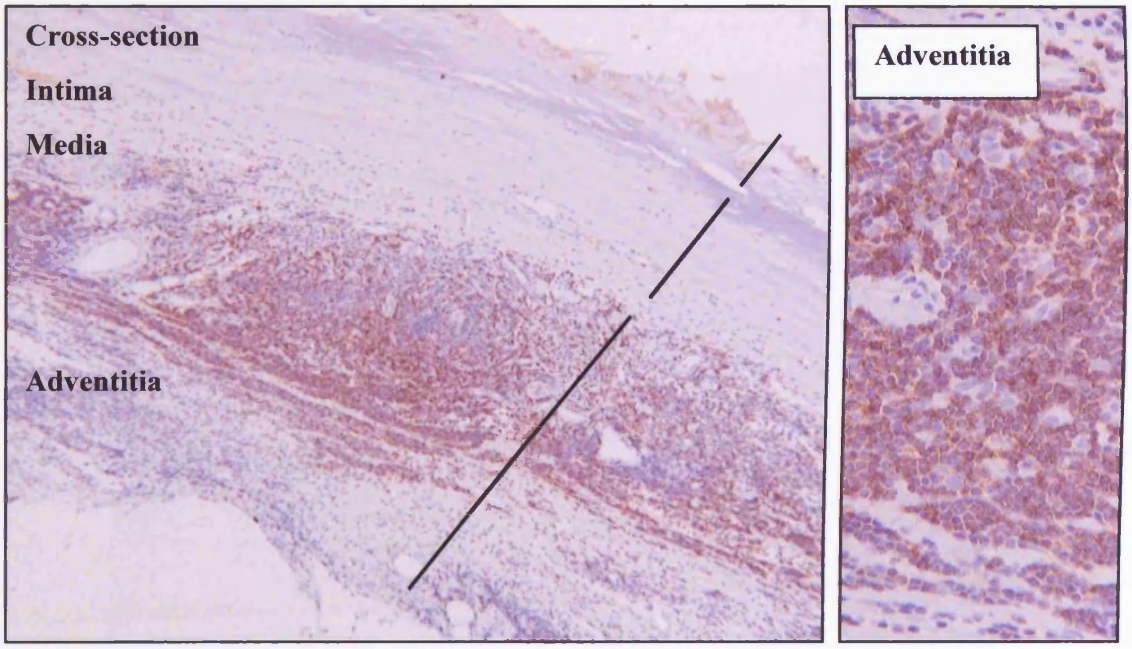
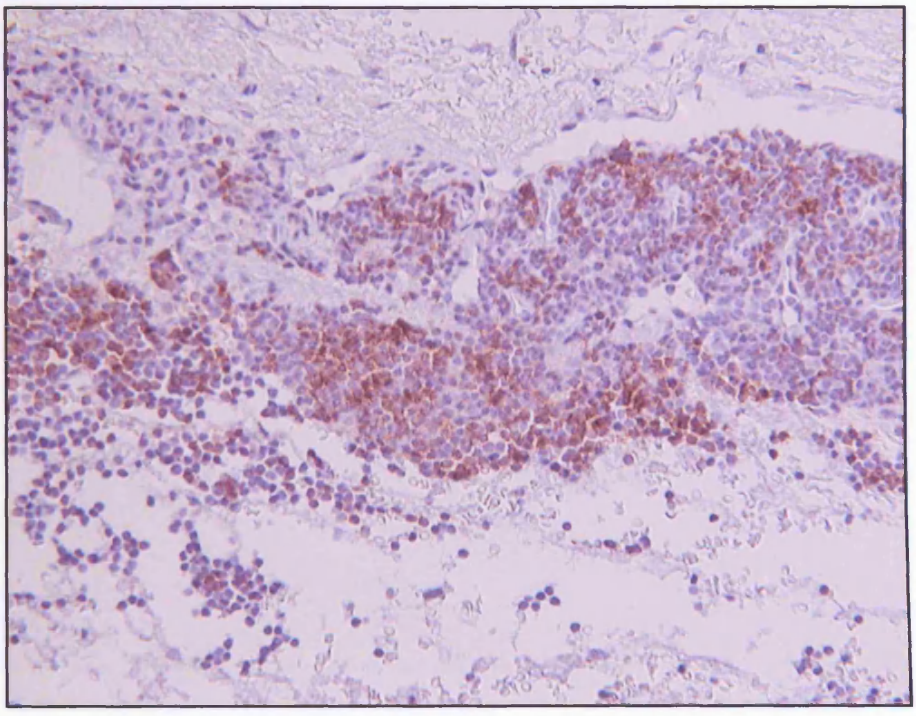


Figure 8-8. CD3 specific staining of the aortic wall of (A) the control abdominal aorta and (B) the AAA. Sections were stained by immunohistochemistry using anti-human CD3 antibodies to detect T-lymphocytes. The left-hand images depict the cross-sectional view of the aortic wall and are orientated with the intima at the top of the cross-section and the adventitia at the bottom (magnification: x40). The right-hand images show CD3 staining of the adventitia (magnification: x250).

A) CD4 – AAA: Adventitia



B) CD8 – AAA: Adventitia

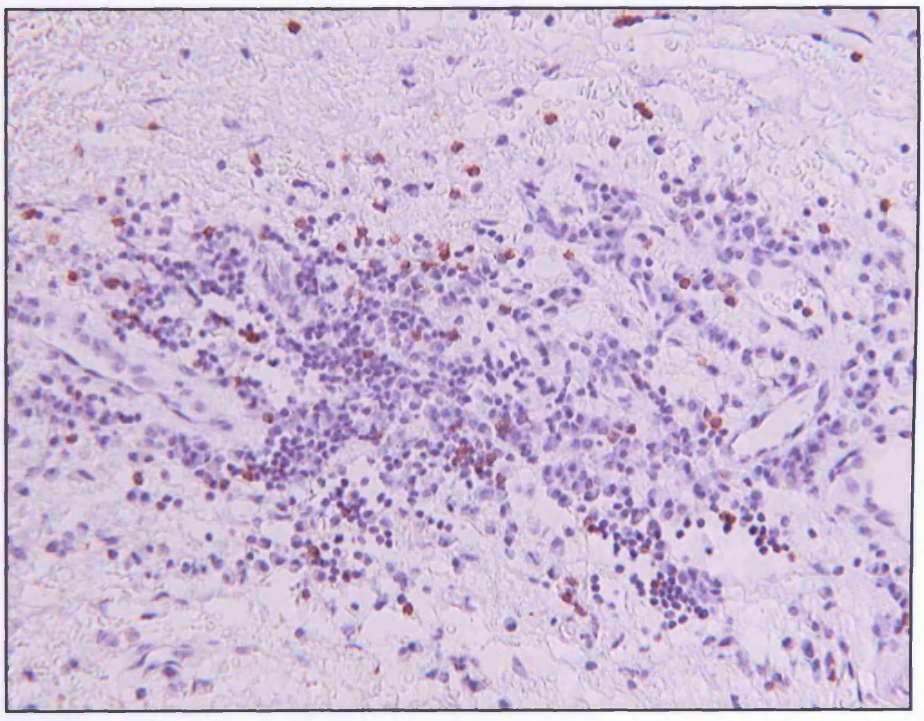
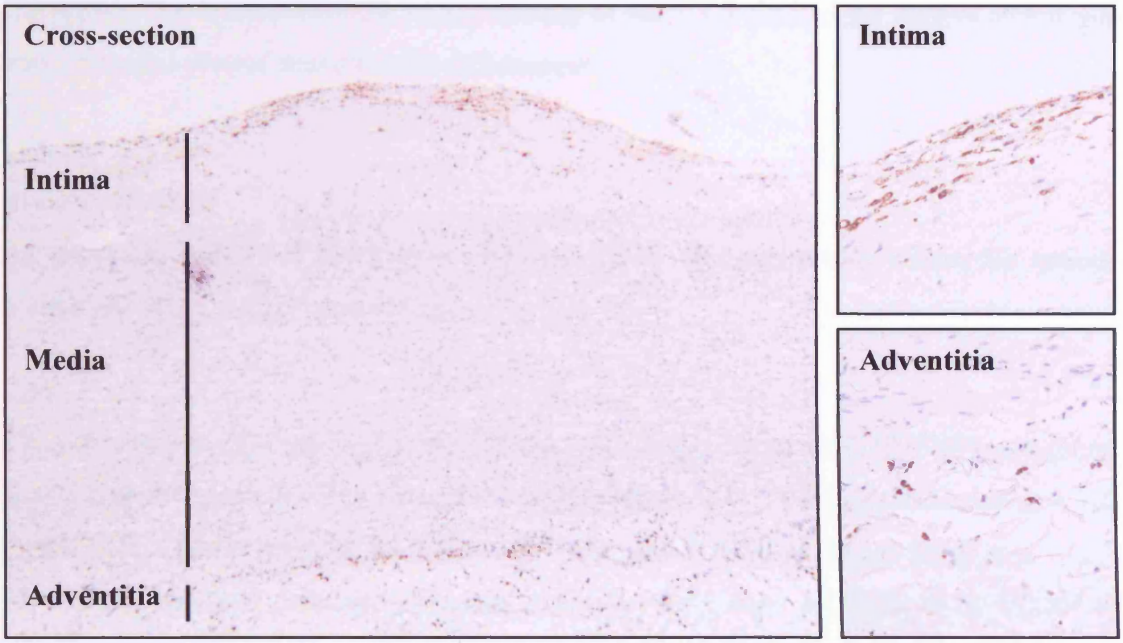


Figure 8-9. Staining of the adventitia within the AAA by immunohistochemistry. AAA sections were treated with (A) anti-human CD4 antibodies to detect T_H-lymphocytes and (B) anti-human CD8 antibodies to detect T_C-lymphocytes. Magnification: x250.

A) Abdominal Aorta – CD68



B) AAA – CD68

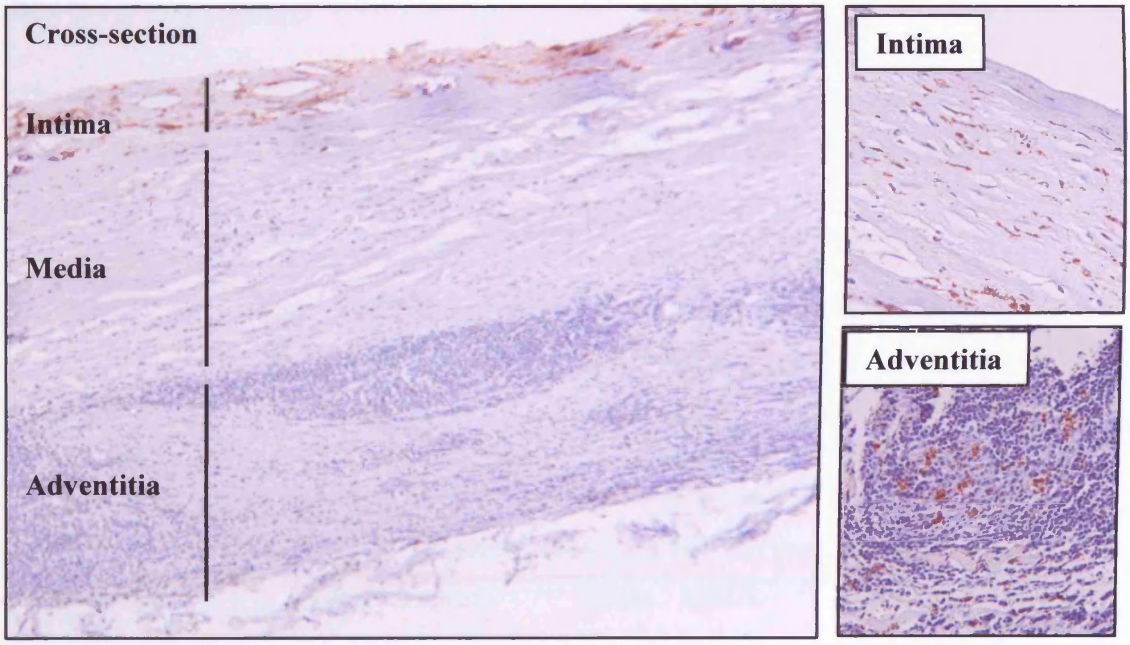


Figure 8-10. CD68 specific staining of the aortic wall of (A) the control abdominal aorta and (B) the AAA. Sections were stained by immunohistochemistry using anti-human CD68 antibodies to detect macrophages. The left-hand images depict the cross-sectional view of the aortic wall and are orientated with the intima at the top of the cross-section and the adventitia at the bottom (magnification: x40). The right-hand images show CD68 positive staining of the intima and adventitia (magnification: x250).

Overall Staining Pattern of the Inflammatory Infiltrate

Comparing the immunohistochemistry staining of the AAA wall to the control abdominal aorta revealed several characteristic differences:

CD20

Abdominal Aorta

As shown in Table 8-7 there were very few $CD20^+$ B-lymphocytes within the control abdominal aortic wall (Figure 8-7*A*).

AAA

The predominant cell type within the infiltrate of the AAA was the $CD20^+$ B-lymphocyte, accounting for approximately 50% of the infiltrating cells (adventitial median score = 3.5) (Table 8-8). The proportion of these cells within the infiltrates ranged from 25% to 50-75% of the infiltrate (interquartile range = 2.5-4). As shown in Figure 8-7*B* $CD20^+$ B-lymphocytes were usually present in dense follicles within the adventitia, or dispersed throughout the layer. Occasionally, in cases of severe inflammation, $CD20^+$ B-lymphocytes were found in the media.

Table 8-7. Grading of the immunohistochemistry stainings for the abdominal aorta group (n = 16)

CD marker	Intima – Median (IQR)	Media - Median (IQR)	Adventitia - Median (IQR)
CD3	0 (0-1.75)	0 (0-2)	0.5 (0-4)
CD4	0 (0-0)	0 (0-0)	0 (0-3)
CD8	0 (0-0)	0 (0-2)	0 (0-1)
CD20	0 (0-0)	0 (0-0)	0 (0-0)
CD68	1 (0.75-2)	0 (0-0)	0 (0-0)

Table 8-8. Grading of the immunohistochemistry stainings for the AAA group (n = 21)

CD marker	Intima - Median (IQR)	Media - Median (IQR)	Adventitia - Median (IQR)
CD3	0 (0-0)	0.5 (0-1)	2 (0.75-3)
CD4	0 (0-0)	0 (0-0.75)	1 (0.5-2.25)
CD8	0 (0-0)	0 (0-0.5)	0.5 (0.25-1)
CD20	0(0-0)	0 (0-0.5)	3.5 (2.5-4)
CD68	2 (1-2)	0 (0-0.75)	1 (0-1)

IQR, interquartile range

CD3

Abdominal Aorta

Whilst lymphocytes were only occasionally present within the control abdominal aortic wall, those that were present were usually of the $CD3^+$ phenotype (Figure 8-8*A*). The median score for $CD3^+$ T-lymphocytes within the adventitia of the abdominal aorta was 0.5, representing 0-2% of the infiltrate. The scores obtained from the abdominal aortic group were either 0/0.5 or at the top end of the scoring system, there were no middle range scores. Thus either $CD3^+$ T-lymphocytes were absent or constituted the majority of the infiltrating cells within the adventitia (interquartile range = 0-4). The other cells that were noted in the adventitia were $CD68^+$ macrophages and, in 3/16 cases, included $CD20^+$ B-lymphocytes. $CD3^+$ T-lymphocytes were occasionally present within the intima and media (Table 8-7) The scores for the media were either 0 or >4 and for the intima were either 0 or >3.5. The interquartile range is 0-2 for the media and 0-1.75 for the intima because the 3rd quartile of the data occurred between the scores of 0 and 4 for the media and 0 and 3.5 for the intima.

AAA

$CD3^+$ T-lymphocytes were also prevalent within the infiltrate and proportionally represented between 10-25% of the infiltrating cells (adventitial median score = 2). There was a degree of variation between the AAA specimens as to the prominence of $CD3^+$ T-lymphocytes within the infiltrate. The interquartile range shows that in 50% of the AAA samples $CD3^+$ T-lymphocytes contributed to between 0-10% and 25-50% of the cellular infiltrate (interquartile range = 0.75-3) (Table 8-8). Whilst overall B-lymphocytes were the predominant cell type of the infiltrate, on an individual basis in some AAA samples T-lymphocytes were the dominant cell type. One sample had a 75-100% proportion of $CD3^+$ T-lymphocytes and in 3/21 AAA samples $CD3^+$ T-lymphocytes constituted 50-75% of the infiltrate. As shown in Figure 8-8*B* the $CD3^+$ T-lymphocytes were found in follicles, or dispersed throughout the layer in milder inflammatory conditions. The T-lymphocytes were also dispersed through the media in cases of severe inflammation.

CD4 & CD8

Abdominal Aorta

In over 50% of the cases neither $CD4^+$ nor $CD8^+$ cells were present within the abdominal aorta (median score = 0). On the occasions where T-lymphocytes were present within the

adventitia generally the CD4 phenotype dominated over the CD8 phenotype (adventitial CD4 interquartile range = 0-3 vs. adventitial CD8 interquartile range = 0-1) (Table 8-7).

AAA

Analysis of the T-lymphocyte subgroups showed that the T-lymphocyte population consisted of mostly follicular $CD4^+$ T_H-lymphocytes, which represented between 2-10% of the infiltration population of the adventitia (median score = 1). The interquartile range shows that 50% of the AAA samples had a $CD4^+$ T_H-lymphocyte population that represented between 0-2% to 10-25% of the inflammatory infiltrate (interquartile range = 0.5-2.25). The second subgroup of T-lymphocytes consisted of $CD8^+$ T_C-lymphocytes. The $CD4^+$ T_H-lymphocyte population was larger than the $CD8^+$ T_C-lymphocyte population (Figure 8-9*A* & *B*). The $CD8^+$ T_C-lymphocyte population comprised between 0-2% of the infiltrate population (median score = 0.5). The interquartile range shows that in 50% of the AAAs the infiltrate consisted of between 0-2% to 2-10% of $CD8^+$ T_C-lymphocytes (Table 8-8). Both subsets were present in the layers in the same pattern as described for $CD3^+$ T-lymphocytes.

<u>CD68</u>

Abdominal Aorta

$CD68^+$ macrophages were the principal inflammatory cell type in the abdominal aorta. Low levels of these macrophages were found frequently within the intima (interquartile range is from a low to moderate presence) (Figure 8-10*A*). However, $CD68^+$ macrophages were rarely found within the adventitia of the abdominal aorta (Table 8-7).

AAA

$CD68^+$ macrophages were predominantly found within the intima where there was a moderate presence of macrophages dispersed throughout the layer (median score = 2). This ranged from a mild to moderate presence in 50% of the samples (interquartile range = 1-2) (Figure 8-10*B* & Table 8-8). Macrophages were rarely seen in the media, however there was a low presence of $CD68^+$ macrophages within the adventitia (median score = 1, interquartile range = 0-1).

8.3.3 Composition of the Inflammatory Infiltrate at Different Levels of Inflammation

The characterisation of the inflammatory infiltrate in the previous section (*8.3.2 Characterisation of the Inflammatory Infiltrate*) described the overall pattern of staining seen for the whole AAA group (n = 21). Within the AAA group there existed a large variation in the extent of inflammation observed between AAA specimens. The composition of the infiltrate within the adventitia of AAAs at different levels of inflammation was examined to determine if there were changes in its cellular nature with increasing inflammation (Tables 8-9 & 8-10).

The $CD20^+$ B-lymphocyte was the predominant cell type in cases of mild to moderate inflammation and the extent of its presence within the infiltrate was fairly consistent regardless of the level inflammation (Kruskal-Wallis, P = .482).

$CD68^+$ macrophages showed a small increased presence within the infiltrate with increasing severity of inflammation. Statistically there was no significant difference in the CD68 staining between the three grades of inflammation (Kruskal-Wallis, P = .062). However, with such small groups examined it may be that the observed increase in macrophages between mild/moderate and severe inflammation would be significant if larger groups were studied.

The presence of $CD3^+$ T-lymphocytes significantly increased with increasing inflammation (Kruskal-Wallis, P = .025). The median T-lymphocyte presence within the infiltrate increased from 2-10% in cases of mild inflammation to 50-75% in severe cases. In cases of severe inflammation, based on the median scores, the predominant cell types were equally B- and T-lymphocytes. Both T-lymphocyte subsets increased with inflammation, however the overall increase in $CD3^+$ T-lymphocytes was chiefly due to the increase in $CD4^+$ T_H-lymphocytes. As for CD68 staining this increase failed to reach significance (Kruskal-Wallis, CD4: P = .053 and CD8: P = .074).

Table 8-9. Comparison of infiltrate composition in the adventitia at different levels of inflammation

Cells	Grade 1 – Mild Median (IQR) n = 9	Grade 2 – Moderate Median (IQR) n = 5	Grade 3 – Severe Median (IQR) n = 7
CD 3^+ T-lymphocytes	1 (0.5-2.25)	2.5 (1.75-2.75)	4 (2-4)
$CD4^+$ T_H-lymphocytes	0.5 (0.25-1.5)	1 (1-1.5)	2.5 (1.5-4)
$CD8^+$ T_C-lymphocytes	0.5 (0-0.75)	1 (0-1.5)	1 (0.5-2)
$CD20^+$ B-lymphocytes	3 (2.5-4)	4 (3-4)	4 (2.5-4)
$CD68^+$ Macrophages	0 (0-1)	0 (0-1.5)	1 (1-3)

Table 8-10. Statistical analysis of Table 8-9

	Kruskal-Wallis (P Values)	Mann-Whitney Analyses (P Values)		
		Grade 1 vs. 2	Grade 2 vs. 3	Grade 1 vs. 3
CD 3^+ T-lymphocytes	**.025**	.068	.162	.018
$CD4^+$ T_H-lymphocytes	.053	.128	.068	.048
$CD8^+$ T_C-lymphocytes	.074	.443	.319	.018
$CD20^+$ B-lymphocytes	.482	.204	.648	.574
$CD68^+$ macrophages	.062	.822	.128	.022

Kruskal-Wallis: P <.05 is significant. Mann-Whitney: Adjusted P <.017 is significant.

Elastin Degradation at Different Levels of Inflammation

The relationship between inflammation and the amount of elastin degradation was assessed. EVG scores were grouped according to level of inflammation, as deemed from the H&E scores. Table 8-11 shows the median EVG scores in each category of inflammation and the results of the Chi-square analysis. In order to include the two AAA samples which exhibited an EVG score of <2 the data from the EVG staining was dichotomised into two groups consisting of those with mild/moderate inflammation (Grade 1-2) and those with severe inflammation (Grade 3). A Chi-square test assessed the two groups at each level of inflammation. There was no significant difference between the amount of elastin degraded at different grades of inflammation (Chi-square, P = .523).

Table 8-11. Comparison between the extent of elastin degradation and the level of inflammation

Stain	Grade 1 – Mild Median (IQR) n = 9	Grade 2 – Moderate Median (IQR) n = 5	Grade 3 – Severe Median (IQR) n = 7
EVG	2 (1.5-3)	3 (2-3)	3 (2-3)

	Chi-square Value	DF	P value
EVG	1.298	2	.523

Chi-square: P <.05 is significant.

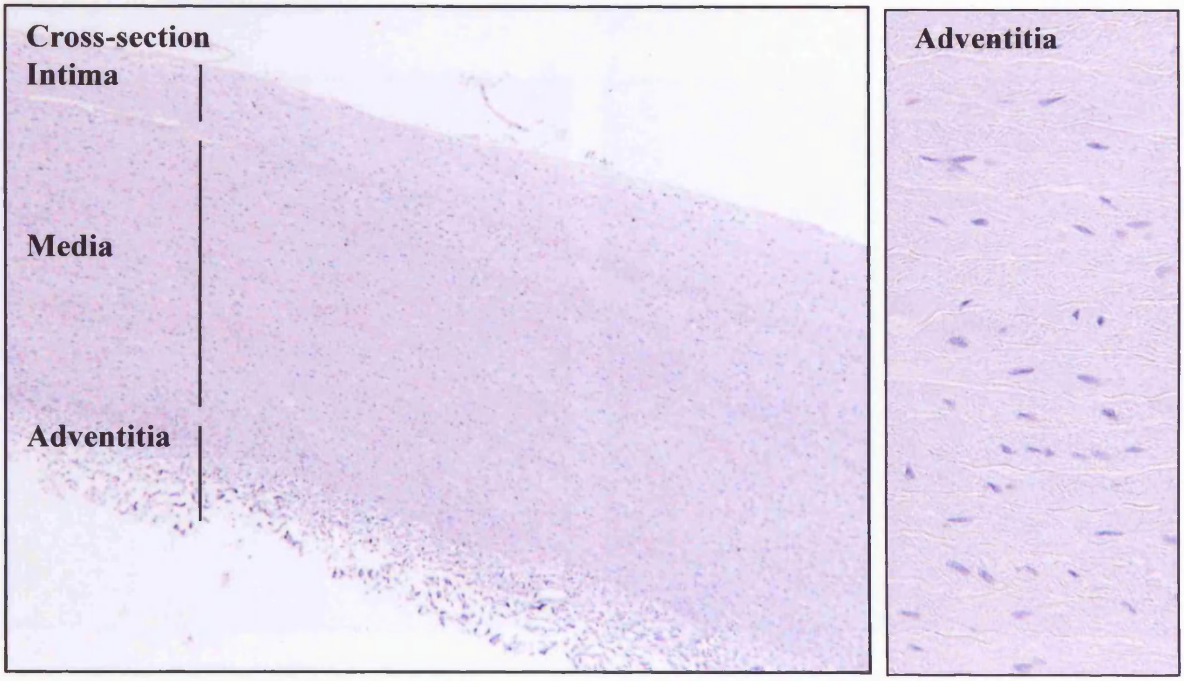
8.3.4 Localised Expression of IL-8 and MCP-1 within the AAA Wall

In order to further characterise the expression of IL-8 and MCP-1 within the AAA wall immunohistochemistry was used to identify the localised expression of these cytokines.

IL-8

Figure 8-11 shows a comparison of the expression of IL-8 in the AAA wall compared to the abdominal aortic wall. IL-8 was associated with the inflammatory infiltrate and was predominantly expressed within the adventitial layer (100% of all AAA specimens showed positive adventitial staining). Figure 8-12*A* shows that IL-8 expression is localised to the follicles within the adventitia. Figure 8-12*B* & *C* demonstrate that the follicles are predominantly formed from $CD20^+$ B- and $CD3^+$ T-lymphocytes. This suggests that IL-8 may be expressed by lymphocytes, although it wasn't possible to distinguish between the two lymphocyte populations.

A) Abdominal Aorta – IL-8



B) AAA – IL-8

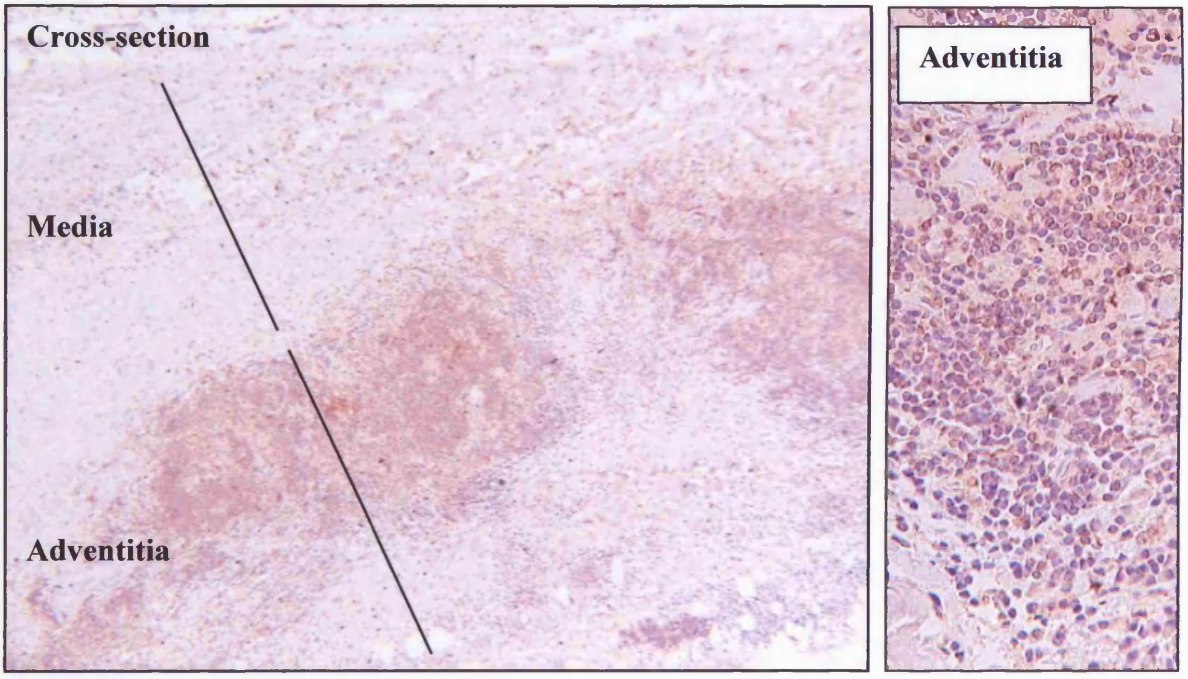
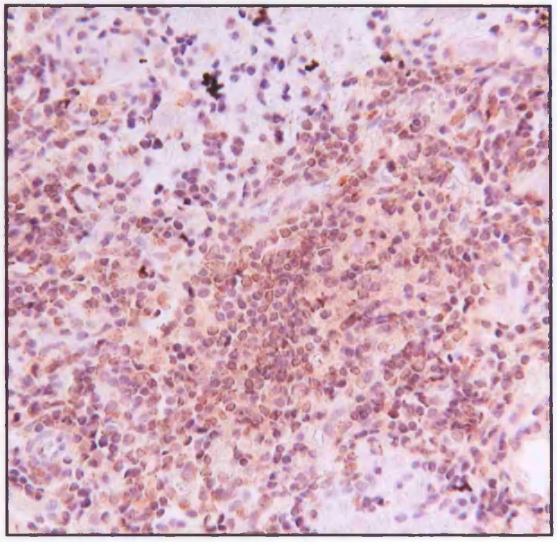
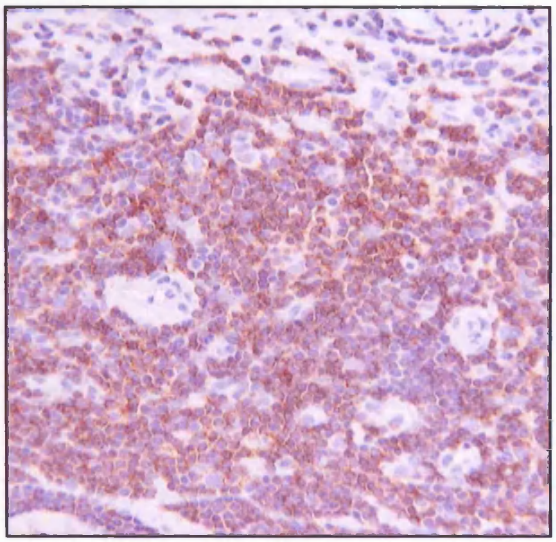


Figure 8-11. IL-8 specific staining of the aortic wall of (A) the control abdominal aorta and (B) the AAA. Sections were stained by immunohistochemistry using anti-human IL-8 antibodies. The left-hand images depict the cross-sectional view of the aortic wall (magnification: x40). The right-hand images show IL-8 staining of the adventitia (magnification: x250).

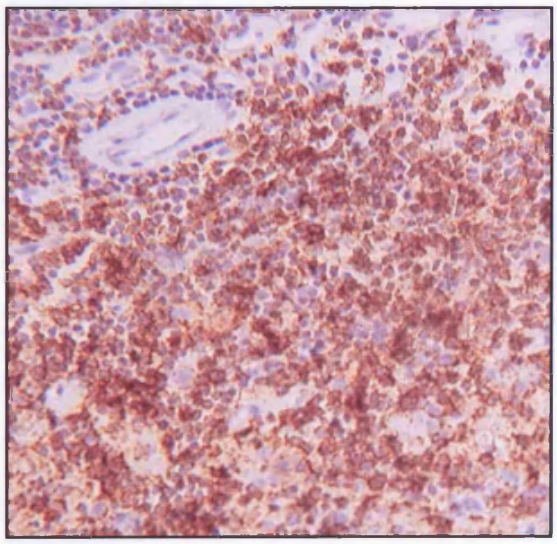
A) IL-8



B) CD3



C) CD20



D) IL-8 Negative Antibody (IgG_1 isotype)

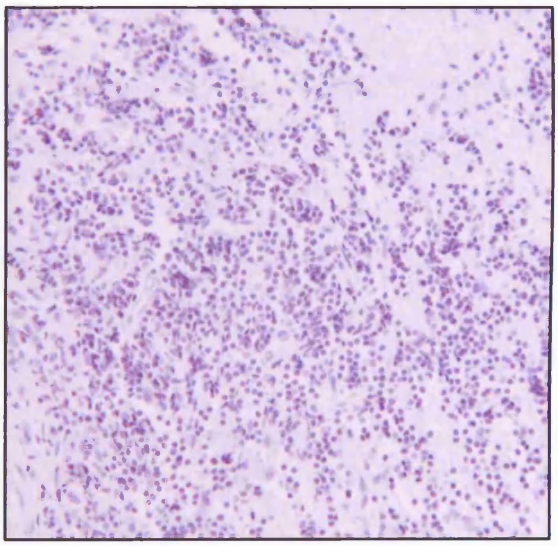


Figure 8-12. Localisation of IL-8 in the AAA. Sections were stained for (A) IL-8; (B) $CD3^+$ T-lymphocytes; (C) $CD20^+$ B-lymphocytes; (D) IL-8 negative control stained with a non-specific IgG_1 isotype antibody. IL-8 staining was follicular. Analysis of the follicles showed that they were formed from $CD3^+$ T-lymphocytes and $CD20^+$ B-lymphocytes. A-C shows that IL-8 co-localised to lymphocytes, although it wasn't possible to distinguish between the lymphocyte populations (magnification: x250). The negative control demonstrates a lack of non-specific binding of the IL-8 antibodies (magnification: x250).

IL-8 Grading of the Aortic Groups

Figure 8-13 shows the expression of IL-8 within both the AAA and abdominal aorta groups and shows the number of cases within each grade of IL-8 expression. IL-8 was not expressed in the majority of control abdominal aorta samples (interquartile range = 0-0.25) (Figure 8-13 & Table 8-12). The expression of IL-8 in the AAA group exhibited a wider distribution of scores and was more highly expressed (median score = 1.5, mild/moderate expression). At least 50% of AAA specimens showed a very weak to moderate expression of IL-8 (interquartile range = 0.75-2). Figure 8-13 shows that only 20 AAAs were included in the IL-8 analysis. One AAA specimen was too fragile to survive the high pH antigen retrieval method and despite repeated efforts was either lost from the slide or was too badly disintegrated to grade.

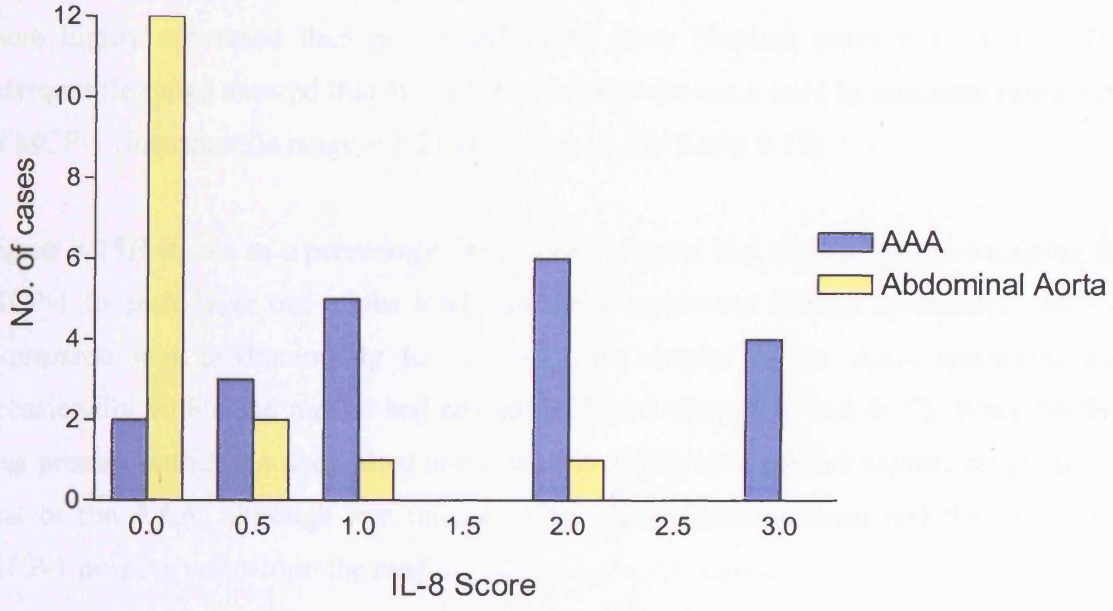


Figure 8-13. The distribution of the scores of the level of IL-8 expression within the abdominal aorta and AAA. IL-8 was either not expressed within the abdominal aortic samples or was expressed at a low level. The expression of IL-8 within the AAA ranged from not being expressed to being highly expressed.

Table 8-12. Group results for the level of expression of IL-8 and MCP-1.

Chemokine	AAA (n = 20)		Abdominal Aorta (n = 16)	
	Median	IQR	Median	IQR
IL-8	1.5	0.75-2	0	0-0.25
MCP-1	1	1-2	0	0-0.5

MCP-1

Figure 8-14 shows a comparison of the expression of MCP-1 in the AAA wall compared to the abdominal aortic wall.

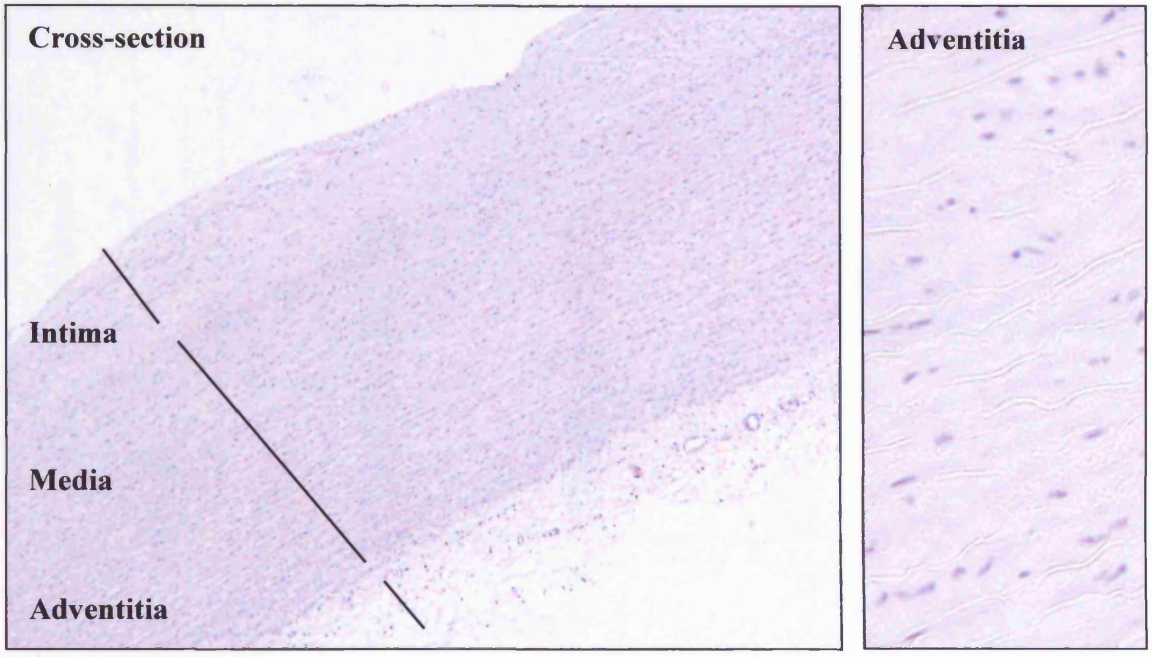
MCP-1 Grading of the Aortic Groups

Figure 8-15*A* summarises the level of expression of MCP-1 within both the AAA and abdominal aorta and shows the number of cases within each MCP-1 grade. MCP-1 was only detected in 31.3% (5/16) of abdominal aortic specimens (median score = 0) (Figure 8-15*A* & Table 8-12). Overall MCP-1 expression ranged from not being expressed to a very low level of expression within the abdominal aorta (interquartile range = 0-0.5). Within the aneurysm group MCP-1 was detected in 90% (19/21) of the AAA specimens. The expression of MCP-1 in the AAA group exhibited a wider distribution of scores and was more highly expressed than in the abdominal aorta (median score = 1 vs. 0). The interquartile range showed that 50% of AAAs had between a mild to moderate expression of MCP-1 (interquartile range = 1-2) (Figure 8-15*A* & Table 8-12).

Figure 8-15*B* shows as a percentage the number of cases that showed positive staining for MCP-1 in each layer out of the total number of positively stained specimens. MCP-1 expression was predominantly found within the intima of the AAA specimens and occasionally within the medial and adventitial layers (Figure 8-16*A* & *C*). When MCP-1 was present within the abdominal aortic wall it displayed a similar expression pattern to that of the AAA, although less intense, with intimal predominance and the occasional MCP-1 positive cell within the media.

Figure 8-16 demonstrates the similar staining pattern of both MCP-1 and CD68 antibodies within the intima and adventitia of the AAA. MCP-1 positive cells were identified as being $CD68^{+}$ macrophages by a consultant pathologist.

A) Abdominal Aorta – MCP-1



B) AAA – MCP-1

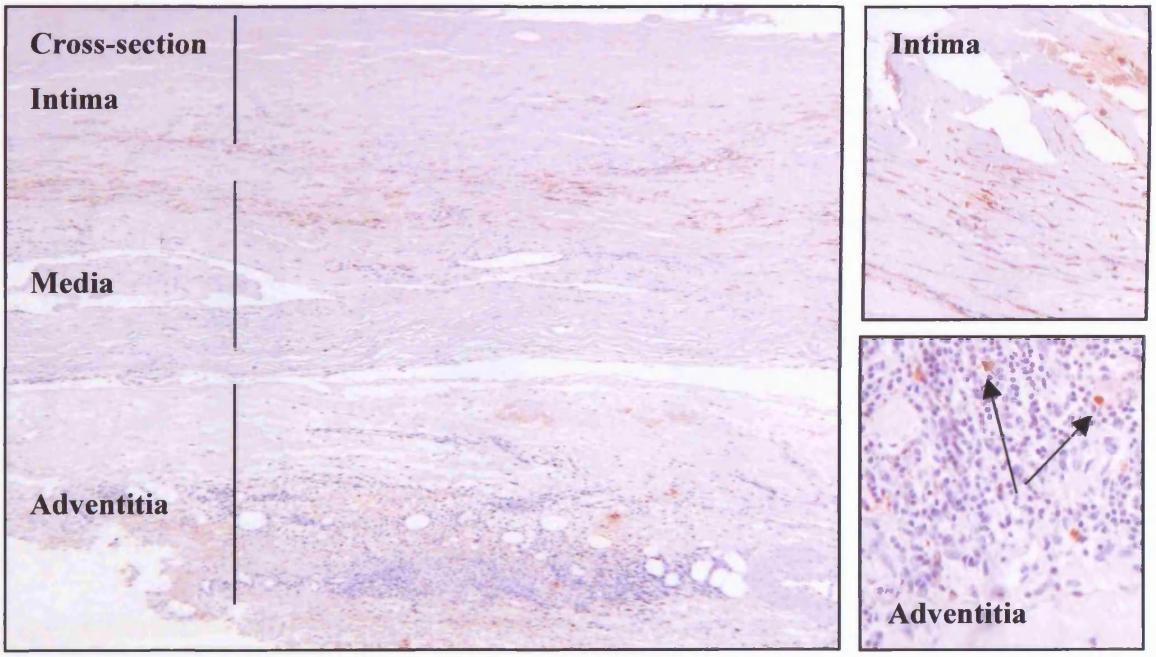
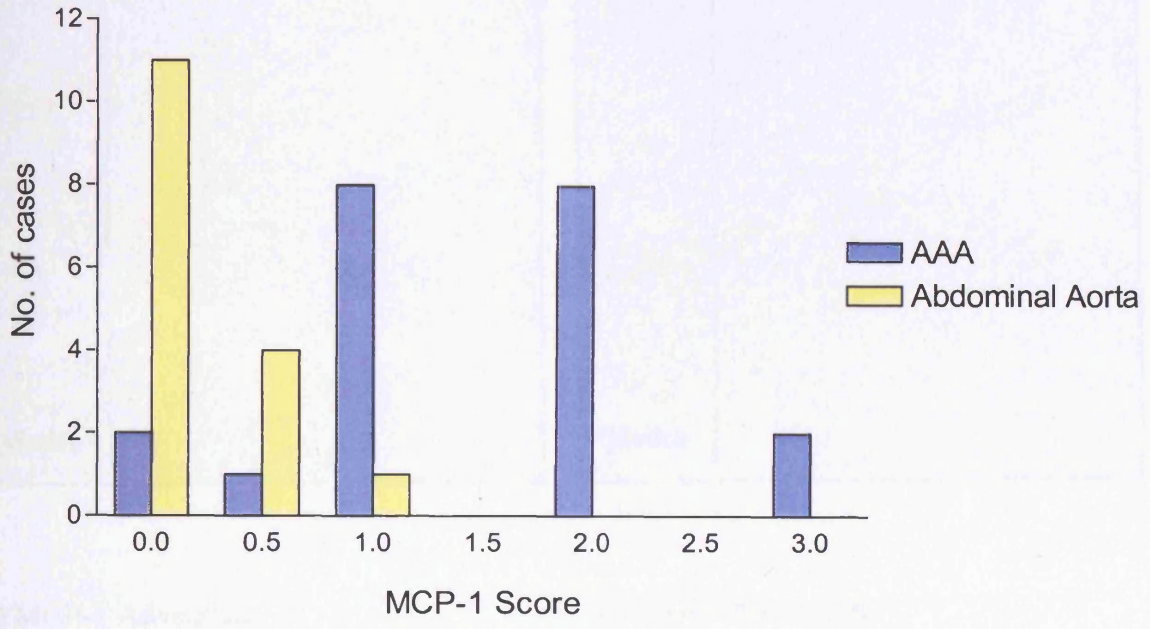


Figure 8-14. MCP-1 specific staining of the aortic wall of (A) the control abdominal aorta and (B) the AAA. Sections were stained by immunohistochemistry using anti-human MCP-1 antibodies. The left-hand images depict the cross-sectional view of the aortic wall, orientated with the intima at the top and the adventitia at the bottom (magnification: x40). The right-hand images show (A) MCP-1 staining of the adventitia in control abdominal aorta, (B) MCP-1 staining of the intima and adventitia in the AAA. The arrows point to solitary MCP-1 positive cells (magnification: x250).

A)



B)

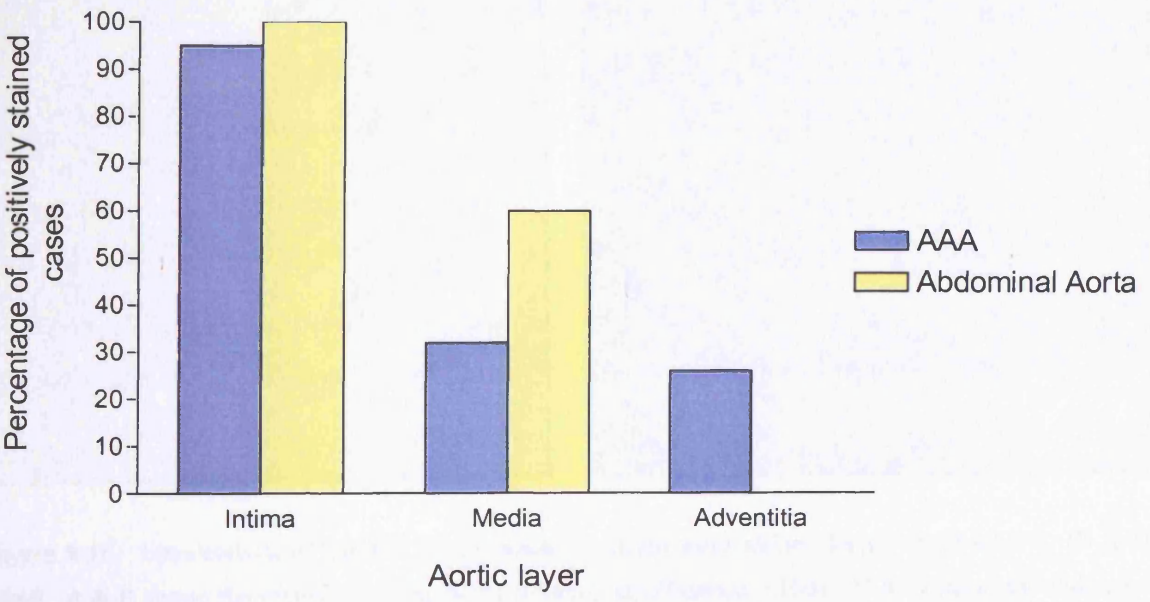
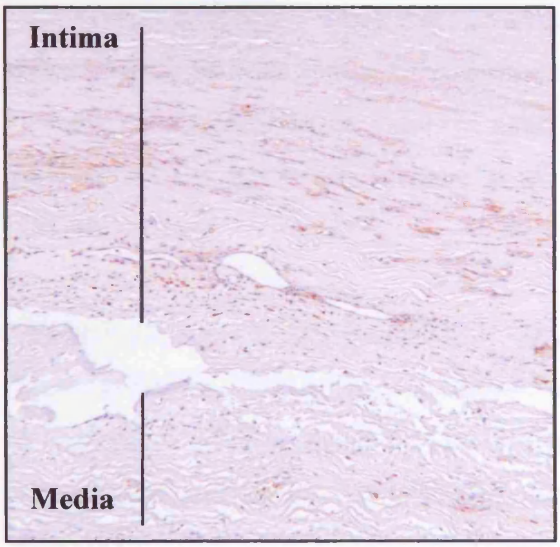
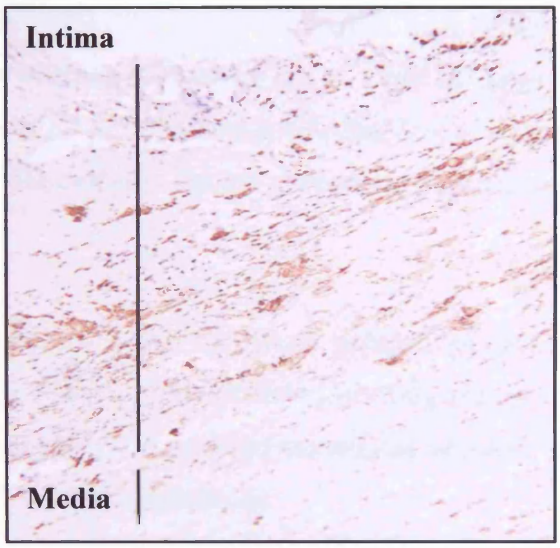


Figure 8-15. MCP-1 expression within the AAA and control abdominal aorta. (A) The bar chart shows the distribution of the scores of the level of MCP-1 expression within the abdominal aorta and AAA. MCP-1 was either not expressed within the abdominal aortic samples or expressed at a low level. The expression of MCP-1 within the AAA ranged from not being expressed to being highly expressed. (B) The bar chart shows the percentage of staining for MCP-1 within each layer out of the total number of positively stained specimens. MCP-1 staining was predominantly intimal based for both the AAA and abdominal aorta, with occasional staining of the media/adventitia.

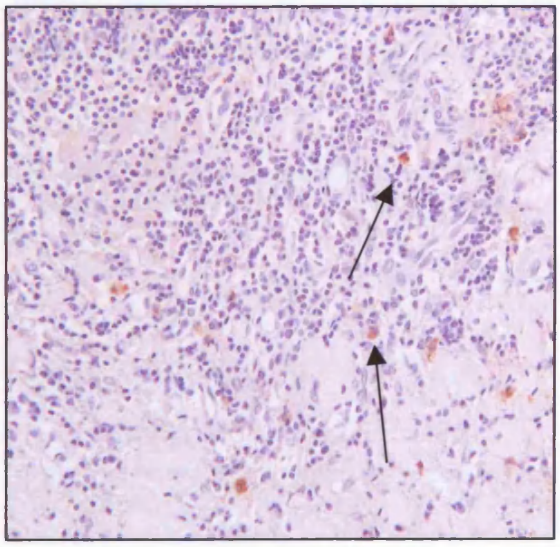
A) MCP-1 – Intima



B) CD68 – Intima



C) MCP-1 Adventitia



D) CD68 - Adventitia

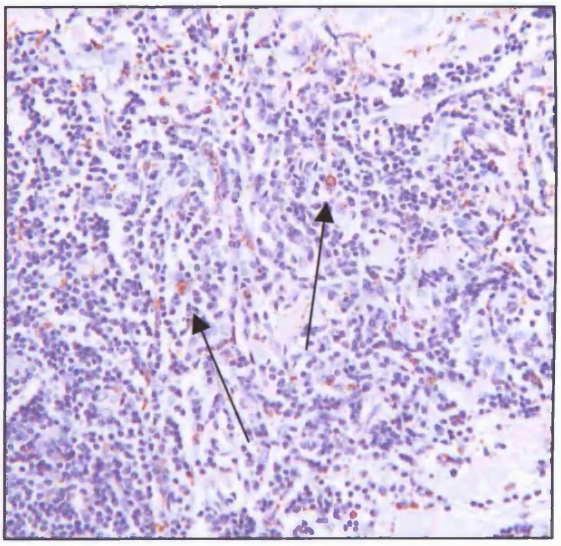


Figure 8-16. Localisation of MCP-1 in the AAA. Sections were stained for (A & C) MCP-1; (B & D) CD68. A & B shows the specific staining of the intima (magnification: x100). C & D shows the staining of solitary positive cells within the adventitia (magnification: x250). The pattern of the MCP-1 staining was mirrored by the staining pattern of the $CD68^{+}$ macrophages. The MCP-1 positive cells were identified as being macrophages by a consultant pathologist.

8.3.5 The Association of AAA Inflammation with the Expression of IL-8 and MCP-1

As these chemokines are potent chemoattractants the AAA specimens were grouped according to IL-8/MCP-1 grade to determine whether IL-8 and/or MCP-1 had an effect on the extent of inflammation observed within the AAA. The two AAAs that had a negative IL-8 or MCP-1 score were included in the mild category for the purpose of performing a Chi-square test.

Table 8-13 displays the median H&E inflammatory scores for AAAs grouped by level of IL-8 expression. The amount of inflammation within AAAs increased in cases of high IL-8 expression. The results of the Chi-square test found a significant association between IL-8 expression and the level of inflammation (P = .043) (Table 8-15).

A comparison between the inflammatory grades of the AAA specimens at different levels of MCP-1 expression did not demonstrate any significant change in inflammation with MCP-1 expression (P = .393) (Table 8-14 & Table 8-15). The final group of MCP-1 expression (grade 3) was really too small for any meaningful analysis.

Table 8-13. The level of inflammation within the AAA wall with increasing levels of IL-8 expression

	IL-8 Grade 1 – Mild *n* = 10	IL-8 Grade 2 – Moderate *n* = 6	IL-8 Grade 3 – Severe *n* = 4
Median H&E Score (IQR)	1.5 (1-2)	1 (1-2)	3 (3-3)

Table 8-14. The level of inflammation within the AAA wall with increasing levels of MCP-1 expression

	MCP-1 Grade 1 – Mild *n* = 11	MCP-1 Grade 2 – Moderate *n* = 8	MCP-1 Grade 3 – Severe *n* = 2
Median H&E Score (IQR)	2 (1-3)	1.5 (1-2.5)	1 (1-1)

Table 8-15. Statistical analysis of Tables 8-13 & 8-14.

	Chi-square Value	DF	P value
IL-8	9.862	4	**.043**
MCP-1	4.097	4	.393

Chi-square: P <.05 is significant.

8.3.6 The Association between IL-8 and the Inflammatory Infiltrate Composition

The inflammatory infiltrate was examined to determine the effect of increasing IL-8 on its composition. Table 8-16 displays the median scores and interquartile ranges of specific cells of the inflammatory infiltrate at each level of IL-8 expression. The results showed a significant increase in $CD3^+$ T-lymphocytes (P = .013) of the $CD4^+$ phenotype (P = .012) with an increase in IL-8 expression (Table 8-16 & 8-17). The percentage of $CD8^+$ T_C-lymphocytes within the infiltrate did not alter with changes in IL-8 expression (P = .447), neither did $CD20^+$ B-lymphocytes (P =.263) nor $CD68^+$ macrophages (P = .085).

Table 8-16. Comparison of infiltrate composition in the adventitia at different levels of IL-8 expression

Cells	Grade 1 – Mild Median (IQR) *n* = 10	Grade 2 – Moderate Median (IQR) *n* = 6	Grade 3 – Severe Median (IQR) *n* = 4
$CD3^+$ T-lymphocytes	1.5 (0.5-2)	2 (1-3)	4 (3.5-4.5)
$CD4^+$ T_H-lymphocytes	0.75 (0-1)	1 (0.5-2.5)	3.5 (2.5-4.5)
$CD8^+$ T_C-lymphocytes	0.5 (0-1)	0.5 (0-1)	1 (0.75-1.5)
$CD20^+$ B-lymphocytes	3 (2.5-4)	4 (3.5-4)	2.75 (2.25-3.5)
$CD68^+$ macrophages	0.25 (0-1)	0 (0-1)	1.5 (1-2.5)

Table 8-17. Statistical analysis of Table 8-16.

	Kruskal-Wallis (P Values)	Mann-Whitney Analyses (P Values)		
		Grade 1 vs. 2	Grade 2 vs. 3	Grade 1 vs. 3
$CD\ 3^+$ T-lymphocytes	**.013**	.272	.040	**.005**
$CD4^+\ T_H$-lymphocytes	**.012**	.221	.041	**.005**
$CD8^+\ T_C$-lymphocytes	.447	.911	.273	.245
$CD20^+$ B-lymphocytes	.263	.265	.138	.374
$CD68^+$ macrophages	.085	.763	.094	.032

Kruskal-Wallis: P <.05 is significant. Mann-Whitney: Adjusted P <.017 is significant.

8.4 Discussion

The main aims of this chapter were to characterise the cellular nature of the inflammatory infiltrate and to investigate the localised expression of IL-8 and MCP-1 within the AAA wall. Additionally, the relationship between these chemokines and the infiltrating leukocytes was investigated.

The main findings of the chapter are that:

1. The AAA is associated with a prominent inflammatory infiltrate within the adventitia mostly comprising of lymphocytes. Overall, the predominant cell type was the B-lymphocyte. Macrophages were occasionally present within the adventitia but were predominantly found in the intima.

2. IL-8 co-localised with the infiltrating lymphocytes within the adventitia. MCP-1 co-localised to the $CD68^+$ macrophages within the intima and adventitia.

3. The increasing mural inflammation within the AAA was associated with a change in the composition of the infiltrate resulting from an increase in T-lymphocytes.

4. Increasing IL-8 expression, but not MCP-1, was associated with an increase in inflammation. The increase in IL-8 expression was associated with an increase in T-lymphocytes, including T_H-lymphocytes.

The null hypotheses tested were: (1) there are no significant differences in the cellular composition of the inflammatory infiltrate with increasing inflammation, (2) there is no significant difference in the level of mural inflammation between AAAs with increasing levels of IL-8 or MCP-1 expression, (3) in the event that (2) is disproved, there are no significant differences in the cellular composition of the inflammatory infiltrate between AAAs expressing increasing levels of IL-8 or MCP-1. Therefore the null hypotheses can be rejected for part (1) and part (2 & 3) regarding IL-8. The null hypothesis is accepted for part (2 & 3) regarding MCP-1.

Through a comparison with abdominal aorta it was possible to demonstrate the fundamental changes within the ECM of the AAA wall and the distinct loss of the aorta's highly organised, layered structure. The changes observed within the AAA specimens are supported by previous reports on inflammation within the aneurysm wall[137, 141, 364-366]. The size and distribution of the infiltrate varied between AAAs. Despite the variation in inflammation there was little variation in the quantity of elastin lost between AAA samples, with 90% of samples having suffered from either a moderate or complete loss of elastin by the time of operating. The finding that most AAAs of an operable size had suffered major degradation of elastin is supported by White *et. al.* who reported that the elastin content of aneurysms was substantially depleted irrespective of the size of the aneurysm and concluded that elastolysis is a primary event in aneurysm formation[141]. Analysis of the amount of elastin lost and the level of inflammation within the aneurysm wall did not find an association between the two events. This suggests the amount of elastin degradation is independent of the level of inflammation in the aneurysm wall by this stage of aneurysm development. Previously, Brophy *et. al.* also found that the infiltrate within the AAAs did not codistribute with the loss of elastin[367].

Further analysis of the cellular composition of the infiltrate revealed that overall it was predominantly formed from B-lymphocytes, with the remainder of adventitial infiltration resulting from T-lymphocytes and occasionally macrophages. Several previous studies have reported the predominance of the B-lymphocyte within the adventitia of the AAA[175, 193, 366, 368, 369]. However, whilst Koch *et. al.* reported an increase in T- and B- lymphocytes within the AAA compared to normal abdominal aorta, they found that the T-lymphocytes represented the main infiltrating inflammatory cell population[156]. Several other studies have also reported that the T-lymphocyte forms the main AAA lymphoid population, with B-lymphocytes forming a relevant component of the infiltrate[160, 163]. This study's immunohistochemical analysis revealed that whilst overall B-lymphocytes were the predominant lymphoid population, on an individual basis the composition of the infiltrate varied and in a few cases T-lymphocytes were the predominant inflammatory cell type. Bobryshev *et. al.* reported that whilst the B-lymphocyte represented the predominant cell population within some infiltrates, the T-lymphocyte was the predominant cell type in the majority of inflammatory infiltrates[157, 159]. Analysis of the cellular composition at different degrees of inflammation revealed that whilst the proportion of the B-lymphocyte population within the infiltrate remained fairly constant between the inflammatory grades, the T-lymphocyte population increased. This may, in part, contribute to the variations in the reported infiltrate composition. It also suggests that further ECM changes may result from the progressive recruitment/clonal expansion of T-lymphocytes and their actions.

This study's findings on the prevalence of the $CD4^+$ T-lymphocyte subpopulation over the $CD8^+$ population is supported by several studies[156, 157, 161, 366]. With the increased presence of the T_H-lymphocytes with inflammation there is the potential for an increased release of inflammatory mediators, such as cytokines, within the aneurysm wall. As described in *Chapter 6. The Cytokine Expression Profile of the Abdominal Aortic Aneurysm*, the AAA wall has a higher expression of several pro-inflammatory cytokines and chemokines compared to control abdominal aorta.

The predominance of B-lymphocytes in AAAs with mild inflammation may suggest that these lymphocytes are present within the aneurysm at an earlier stage of aneurysm development, with the development of a T-lymphocyte population occurring later. This may suggest that B-lymphocytes are involved in the early stages of AAA development. One study investigated whether the B-lymphocyte rich infiltrate was a result of an

autoimmune response, however, they found an unrestricted usage of the immunoglobulin variable heavy chain genes which suggested that the B-lymphocyte population was not a result of an autoimmune response[193]. The exact role the lymphocytes play in the pathogenesis of the AAA remains to be elucidated.

The remainder of the infiltrate contained macrophages. Previously, macrophages have been found scattered within the adventitia and media[156, 365]. This study found that macrophages were mostly based within the intima, those macrophages that were present within the adventitia were scattered within lymphoid follicles in a similar pattern as described by Koch *et. al.*[156]. Bobryshev *et. al.* described the intima as being rich in $CD68^+$ macrophages that were diffusely distributed within the adventitia[157, 159]. Ocana *et. al.* reported that macrophages were mostly located in the intima and the inner layer of the media[160]. $CD68^+$ macrophages were also frequently found within the intima of abdominal aorta, although greater numbers were present within the AAA, which suggests that the intimal macrophages may result from the atherosclerotic process and may not be involved in AAA pathogenesis.

In the previous chapters, it has been shown that the chemokines IL-8 and MCP-1 are more highly expressed within the AAA compared to control abdominal aorta. IL-8 was localised to the lymphocyte population within the adventitia of the AAA, whereas the majority of the control abdominal aortas were negative for IL-8. The only other study on the expression of IL-8 within the aneurysm wall however reported that IL-8 positive cells were macrophages and to a lesser extent endothelial cells[310]. It is possible that positive macrophage expression of IL-8 within the infiltrate was masked by the overwhelming positive staining of the lymphocytes, but IL-8 staining was rarely found within the intima, which was the main source of macrophages within the AAAs studied. The MCP-1 positive cells were identified as being predominantly macrophages, as reported previously by Koch *et. al.*[310]. Within the AAA the MCP-1 positive staining localised to the intima and adventitia, mirroring the staining pattern of $CD68^+$ macrophages. MCP-1 was found chiefly within the intima and media of the abdominal aorta. This difference in the localised expression of MCP-1 suggests that the expression of this chemokine in the intima of the abdominal aorta and AAA may, in part, be responsible for the infiltration of macrophages into the intima and may have contributed to atherosclerotic plaque

development within the vessels. However, its expression within the adventitia of the AAA suggests that MCP-1 may also be involved in the pathogenesis of the AAA.

The exact role these chemokines may contribute, if any, to the pathogenesis of the AAA is unknown. Previously, Szekanecz *et. al.* demonstrated that IL-8 induced endothelial cell chemotaxis and suggested this may be important in AAA neovascularisation[351]. MCP-1 was shown to be necessary for aneurysm formation in mice as the targeted deletion of the CCR2 gene, the receptor for MCP-1, suppressed aneurysm formation in apoE knockout mice treated with angiotensin II[353]. A more detailed discussion of the potential role of these chemokines in AAA pathogenesis is given in *Chapter 7.4 Discussion.* The relationship between IL-8 and MCP-1 expression and mural inflammation was investigated. The level of mural inflammation was found to increase with increasing IL-8 expression, suggesting that IL-8 may have a chemoattractant effect on the development of the infiltrate. However, no such relationship was determined for MCP-1 and the infiltrate, suggesting that MCP-1 may have an alternative role in AAA pathogenesis other than chemotaxis, if it is involved. Examination of the association between IL-8 and the composition of the infiltrate revealed a significant increase in T-lymphocytes, including T_H-lymphocytes with increasing IL-8 expression. This suggests that the expression of IL-8 within the aneurysm wall could promote further inflammation and increased degeneration of the aneurysm wall.

A limitation of this chapter was the small study size used to assess the association between inflammation, infiltrate composition and IL-8 expression. Whilst the results show a potential involvement of IL-8 in the development of the infiltrate the small study size means that the statistical results are at a greater risk of being either false positive or negative if the samples used were atypical of the population being studied. Furthermore, whilst immunohistochemistry can identify the location of the IL-8 and MCP-1 protein it can be difficult to interpret whether the cytokine is detected at its cellular source or ligand bound on its target cell. Additionally, as cytokines can be stored in vessels in the ECM it can be difficult to determine specific antibody binding and background staining of connective tissue. However, this latter point was compensated for by using an isotype control to determine non-specific binding of the IL-8 antibody. Future work would have involved using *in situ* hybridisation to determine the cellular source of these cytokines.

In conclusion, this chapter localised the expression of IL-8 and MCP-1 within the aneurysm wall to lymphocytes and macrophages, respectively and identified a potential relationship between IL-8 expression and the infiltration of specific cell types into the aneurysm wall. With the presence of these chemokines within the adventitia and their raised expression within the AAA wall it suggests that they may have a role in aneurysm pathogenesis. The following chapter investigates a potential role for IL-8 in the pathogenesis of AAAs.

Chapter Nine

The Effect of Interleukin-8 on Matrix Metalloproteinase Expression in the Abdominal Aortic Aneurysm

Chapter 9. The Effect of Interleukin-8 on Matrix Metalloproteinase Expression in the Abdominal Aortic Aneurysm

9. The Effect of Interleukin-8 on Matrix Metalloproteinase Expression in the Abdominal Aortic Aneurysm

9.1 Introduction

In the previous chapters chemokines and pro-inflammatory cytokines were shown to be higher in the AAA wall than in control aorta. Four cytokines: IL-8, IL-6, MCP-1 and MCP-2 were prominently elevated in the aneurysm. Whilst the assay system used to detect MCP-2 was insufficiently sensitive to detect this cytokine, ELISA results verified the arrays findings on the expression of IL-8, MCP-1 and IL-6. The expression and role of chemokines within the AAA wall was investigated further using immunohistochemistry to detect the localised expression of IL-8 and MCP-1. IL-8 co-localised with the lymphocytes within the inflammatory infiltrate. MCP-1 co-localised with macrophages and these were predominantly found in the intima and occasionally within the infiltrate. Increasing IL-8, but not MCP-1, was associated with an increase in mural inflammation and changes in the composition of the inflammatory infiltrate. This chapter describes the use of an explant system to investigate a potential role for IL-8 within the aneurysm wall.

Relatively few studies have investigated the possible role(s) for IL-8 within the aneurysm and those studies that have have focused on its chemotactic properties. Szekanecz *et.al.* demonstrated that the chemotactic nature of the conditioned media from aneurysm explants to human aortic endothelial cells could be abolished by treatment of the medium with an anti-IL-8 antibody[351]. No other studies on the possible role for IL-8 within the AAA could be found.

The previous chapters have shown that the aneurysm wall is a highly chemotactic environment, with a strong expression of IL-8 which may have some influence on the level of mural inflammation and composition of the inflammatory infiltrate. It has been reported that the aneurysm wall shows signs of increased angiogenesis compared to control aortas[315, 370] and that angiogenesis is ongoing in the mature aneurysm[316]. IL-8 is also a potent angiogenic factor[343]. Both the development of the infiltrate and angiogenesis require cellular migration usually induced by a chemotactic molecule. However, cellular migration requires not only the inducement of a chemotactic signal but also the physical

migration through a network of extracellular fibres. Vascular SMCs from aneurysm patients have been shown to have better invasive qualities than SMCs from non-aneurysmal patients and this difference was associated with increased MMP-2 synthesis[371]. The over-production of MMPs by migrating cells could potentially result in aneurysm wall damage. Recent reports have shown that specific chemokines can induce the secretion of MMPs[323-325]. There are no published studies about the relationship between IL-8 and MMP expression within the AAA. The aim of this chapter is to investigate the effect of IL-8 on the expression of MMP-2 and MMP-9 within AAA explants.

The null hypothesis is that there are no significant differences in the expression/activation of MMP-2 and MMP-9 between explants treated with IL-8 compared to non-treated controls.

9.2 Methods

In order to test the effects of IL-8 on MMP expression an *in vitro* explant modelling system was used based on the system described by Franklin *et. al.*[372]. Fresh AAA specimens (n = 5) were obtained from theatre and cultured under the conditions described in *Chapter 5.9 Explant Culture*. Briefly, from each AAA specimen four identically sized 8mm explant biopsies were placed into individual wells and immersed in culture medium overnight. This medium was removed and discarded. Fresh medium was added and the explants were incubated overnight. On day 1 the untreated medium was removed and an aliquot of this medium from each explant was snap-frozen for MMP analysis (day 1 – pre-treatment). The remainder was discarded and replaced by 5ml of medium supplemented with IL-8 (the four explants from a single AAA were treated with one of the following concentrations of IL-8: 0ng/ml, 1ng/ml, 10ng/ml, 100ng/ml). The AAA explants were cultured under these conditions for a further six days, with the supplemented medium being changed every 48 hours. At day 7 an aliquot of the medium was taken from each explant and snap-frozen for MMP analysis (day 7 – post-treatment). The MMP concentration in the aliquots taken from each explant at day 1 and day 7 was measured by ELISAs, as described in *Chapter 5.7 ELISAs*. Each aliquot was measured for total and active MMP-2 and MMP-9 concentration. All samples were measured in duplicate on the ELISAs and the duplicate concentrations were averaged. The results are presented as the median values

(interquartile ranges) from five independent experiments. The medium collected at day 7 had been exposed to the explant for 48 hours whilst the medium collected at day 1 had only been exposed for 24 hours. Therefore the day 7 concentrations were divided by two to give the MMP concentration per 24 hours in order for the results to be comparable with day 1. Data were analysed using a Kruskal-Wallis test to determine whether a significant difference existed between the treatment groups and a Mann-Whitney *U* test was used to determine whether there was a significant difference between the day 1 and day 7 concentrations. A P value of $< .05$ was used to indicate a significant difference.

9.3 Results

9.3.1 Effect of IL-8 on MMP-2 Release

Total MMP-2 Expression

The effect of IL-8 on the total expression of MMP-2 within the AAA explant was measured. The freshly cultured AAA explants produced MMP-2 without an exogenous stimulus (Figure 9-1*A*: Pre-treatment). Table 9-1 shows the median concentrations of total MMP-2 secreted by the explants in the four treatment groups, prior to and after IL-8 treatment. Kruskal-Wallis analysis of the concentration of MMP-2 expressed by the different treatment groups prior to treatment (Table 9-1: Pre-treatment) showed that there wasn't a significant difference between any of these groups (Table 9-2: Pre-treatment, P = .262). At this stage there should not have been a difference between the different treatment groups as the explants within the different groups were from the same AAAs.

A comparison between the concentration of MMP-2 secreted by the untreated control explants at day 1 and at day 7 showed a small median decrease by day 7 (Figure 9-1*A* & Table 9-1). However, Mann-Whitney analysis of the data did not determine a significant difference between the concentration of MMP-2 secreted by the same explants at the start and at the end of the culturing period (32.5ng/ml (25.4-40.3ng/ml) vs. 25.1 (23.7-31.3ng/ml), P = .175). Therefore culturing did not significantly affect the expression of MMP-2 and the similar concentrations of MMP-2 at day 1 and day 7 suggests that the explants have retained a similar level of viability in culture.

Figure 9-1*B* shows the effect of increasing concentrations of IL-8 on MMP-2 production. For each AAA, the concentrations of MMP-2 secreted by the three explants that were treated with IL-8 (1, 10, 100ng/ml) were expressed as a percentage of the concentration of MMP-2 secreted by the fourth untreated explant. The results from 5 AAAs were collated and are displayed in Figure 9-1*B* and Table 9-1. Figure 9-1*B* shows that IL-8 did not affect the level of MMP-2 expressed. Figure 9-1*A* shows that the concentration range of MMP-2 measured from explants prior to and after treatment was similar, with all explants constitutively expressing MMP-2. Kruskal-Wallis analysis of the concentration of MMP-2 expressed by the different post-treatment groups failed to determine a significant difference between any of the groups (P = .304) (Table 9-2). IL-8 treatment did not affect MMP-2 expression.

Table 9-1. Effect of IL-8 on total MMP-2 secretion by AAA explants.

IL-8 concentration added (ng/ml)	Number of AAA explants	Concentration of MMP-2 secreted (ng/ml/24 hrs)		Percentage of MMP-2 secreted at day 7 compared to control (%)*
		Pre-treatment	Post-treatment	
0	5	32.5 (25.4-40.3)	25.1 (23.7-31.3)	100.0 (100.0-100.0)
1	5	25.5 (22.0-37.9)	26.3 (23.1-27.2)	94.0 (80.7-112.0)
10	5	31.5 (30.1-43.8)	23.0 (20.1-25.7)	86.7 (74.8-96.7)
100	5	37.1 (30.4-49.6)	24.1 (22.3-26.5)	95.6 (81.9-98.9)

** For each AAA, the concentration of MMP-2 secreted by the three IL-8 treated explants at day 7 is expressed as a percentage of the concentration of MMP-2 secreted by the untreated explant at day 7. The results for all five AAAs are displayed in each column as medians and interquartile ranges.*

Table 9-2. Results of the Kruskal-Wallis statistical analysis of the concentration data in Table 9-1.

	Mean rank for each treatment group				Significance
	0ng/ml	1ng/ml	10ng/ml	100ng/ml	
Pre-treatment	9.60	6.80	11.60	14.00	.262
Post-treatment	12.40	13.20	6.80	9.60	.304

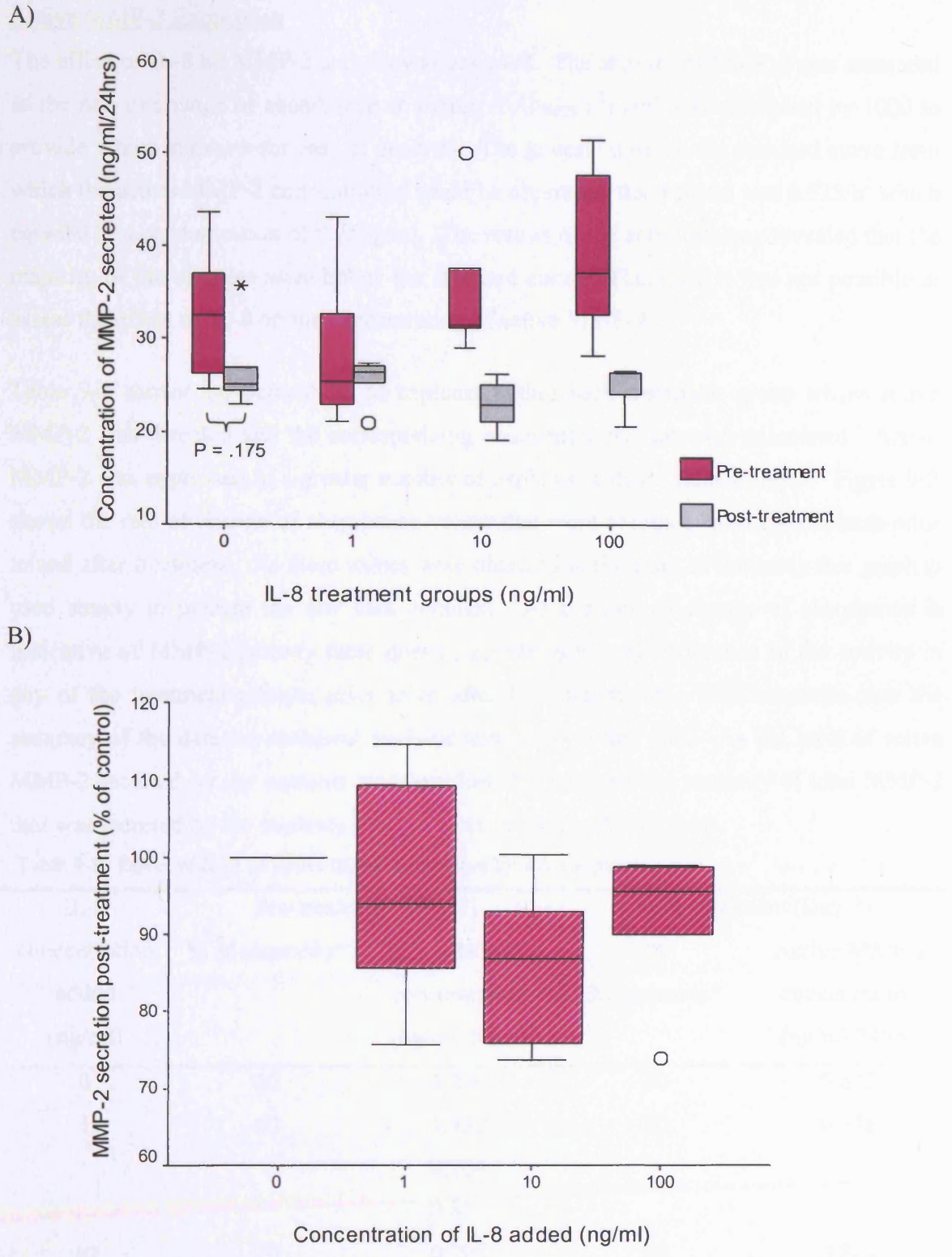


Figure 9-1. The effect of IL-8 on the secretion of total MMP-2 from AAA explants. (A) The concentration of total MMP-2 secreted from explants was measured prior to and after treatment with a single concentration of IL-8 (0, 1, 10, 100ng/ml). (B) For each AAA, the amount of MMP-2 secreted by the treated explants (1, 10, 100ng/ml) at day 7 is expressed as a percentage of the amount secreted by the untreated explant (0ng/ml). The results were obtained from 5 AAAs and are presented as medians (bar), interquartile ranges (box) and ranges (whiskers). O and * represents outliers, as defined in Chapter 7.3.2.

Active MMP-2 Expression

The effect of IL-8 on MMP-2 activity was assessed. The activity of MMP-2 was measured as the rate of change of absorbance at 405nm ($\delta Abs_{405}/h^2$) and was multiplied by 1000 to provide whole numbers for ease of analysis. The lowest value on the standard curve from which the active MMP-2 concentration could be accurately determined was $3.625/h^2$ which equated to a concentration of 0.75ng/ml. The results of the activity assay revealed that the majority of the samples were below the standard curve. Therefore it was not possible to assess the effect of IL-8 on the concentration of active MMP-2.

Table 9-3 shows the percentage of explants within each treatment group where active MMP-2 was detected and the corresponding concentrations that were calculated. Active MMP-2 was expressed in a greater number of explants at day 1 than at day 7. Figure 9-2 shows the rate of change of absorbance values that were obtained from the explants prior to and after treatment. As these values were obtained at the limit of the assay this graph is used simply to present the raw data obtained. As the rate of change of absorbance is indicative of MMP-2 activity there doesn't appear to be any difference in the activity in any of the treatment groups, prior to or after IL-8 treatment. With concerns over the accuracy of the data no statistical analysis was done on this data. As the level of active MMP-2 secreted by the explants was very low this suggests the majority of total MMP-2 that was secreted by the explants was therefore in the pro-MMP form.

Table 9-3. Effect of IL-8 on active MMP-2 secretion by AAA explants.

IL-8 concentration added (ng/ml)	Pre-treatment (Day 1)		Post-treatment (Day 7)	
	% Measurable*	Active MMP-2 concentration (ng/ml/24hrs)	% Measurable*	Active MMP-2 concentration (ng/ml/24hrs)
0	20	1.330	20	0.833
1	60	1.432	20	0.978
		0.764		
		0.833		
10	20	0.798	20	0.824
100	40	0.781	0	
		0.935		

* Percentage of explants secreting measurable levels of active MMP-2.

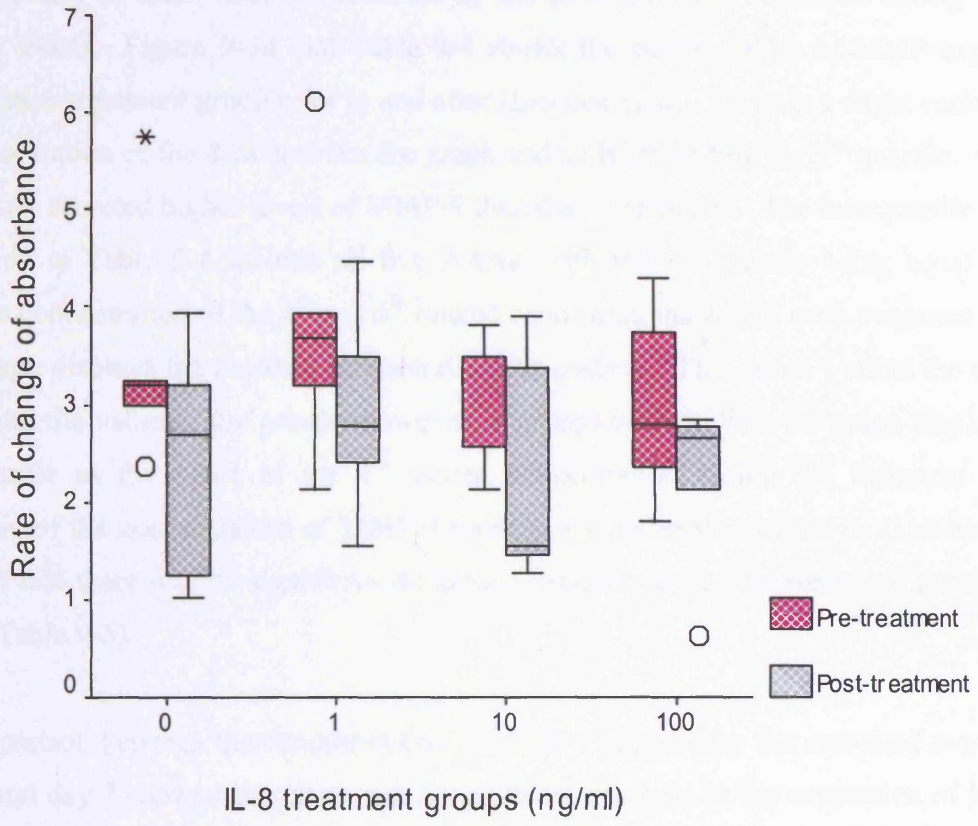


Figure 9-2. The effect of IL-8 on the secretion of active MMP-2 from AAA explants. The rate of change in absorbance at 405nm was proportional to MMP-2 activity in the culture medium from the explants and was measured prior to and after treatment with a single concentration of IL-8 (0, 1, 10, 100ng/ml). The results were obtained from 5 AAAs and are presented as medians (bar), interquartile ranges (box) and ranges (whiskers). O and * represents outliers, as defined in Chapter 7.3.2.

9.3.2 Effect of IL-8 on MMP-9 Release

Total MMP-9 Expression

Whilst total MMP-2 was constitutively expressed by the explants in culture, the concentration of total MMP-9 expressed by the same explants decreased during culture (Figure 9-3*A*). Figure 9-3*A* and Table 9-4 shows the concentration of MMP expressed within each treatment group prior to and after IL-8 treatment. There is a slight variation in the presentation of the data between the graph and table, regarding the 3rd quartile. One of the AAAs secreted higher levels of MMP-9 than the other AAAs. The interquartile ranges displayed in Table 9-4 include all five AAAs, with the 3rd quartile being equal to the average concentration of the 4th and 5th ranked concentrations within each treatment group. The graph displays the extreme concentrations as outliers. This doesn't affect the median or 1st quartile value of the groups, however in groups with outliers the graph displays the 3rd quartile as the value of the 4th ranked concentration within the treatment group. Analysis of the concentration of MMP-9 secreted by the explants prior to IL-8 treatment showed that there was no significant difference between any of the treatment groups (P = .870) (Table 9-5).

A comparison between the concentrations of MMP-9 secreted by the untreated explants at day 1 and day 7 showed that there was a significant decrease in the expression of MMP-9 with time spent in culture (6.9ng/ml (6.5-47.0ng/ml) vs. 1.5ng/ml (0.7-2.6ng/ml), P = .009). As the same explants were still constitutively expressing MMP-2 by day 7 at a level comparable to day 1 it suggests that this decrease in MMP-9 is not the result of a decrease in explant viability. It suggests that MMP-9 expression within the AAA requires a stimulus/stimuli which was not present in this culture system.

Figure 9-3*B* shows the concentration of MMP-9 that was secreted by each treatment group, post-IL-8 treatment, and expressed as a percentage of the control concentration. The outlier values are all from the same AAA. From the graph it can be seen that there was no difference in the expression of MMP-9 irrespective of IL-8 treatment. Kruskal-Wallis analysis confirmed that there was no significant difference between any of the treatment groups (P = .951) (Table 9-5). Therefore IL-8 did not have an effect on MMP-9 expression.

Table 9-4. Effect of IL-8 on total MMP-9 secretion by AAA explants.

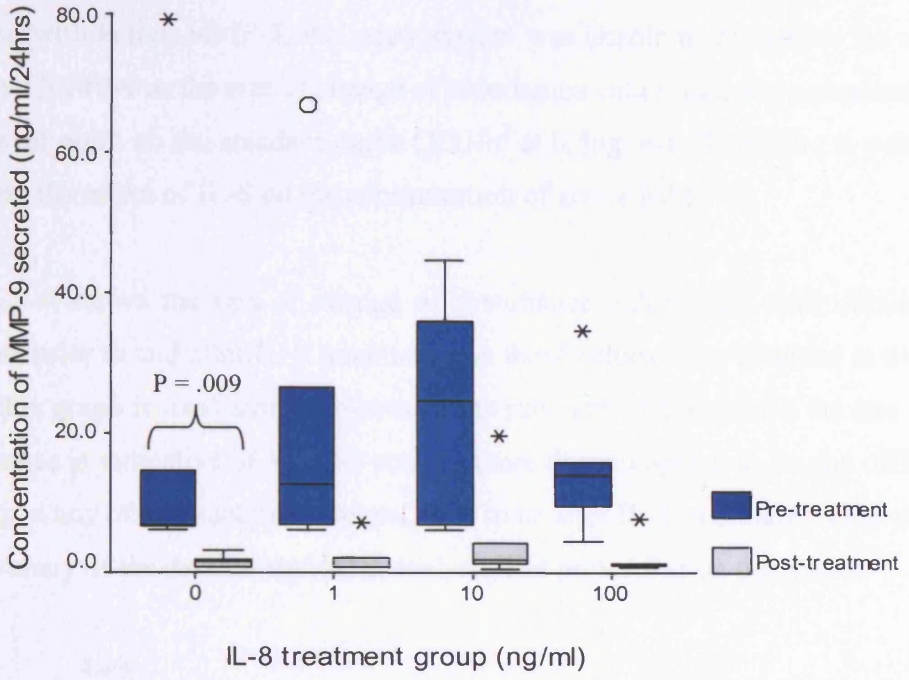
IL-8 concentration added (ng/ml)	Number of AAA explants	Concentration of MMP-9 secreted (ng/ml/24 hrs)		Percentage of MMP-9 secreted at day 7 compared to control (%)*
		Pre-treatment	Post-treatment	
0	5	6.9 (6.5-47.0)	1.5 (0.7-2.6)	100.0 (100.0-100.0)
1	5	12.8 (6.6-46.7)	0.8 (0.8-4.6)	111.0 (56.2-249.3)
10	5	24.6 (6.5-40.5)	1.8 (0.9-12.0)	177.6 (69.0-655.0)
100	5	14.0 (7.0–25.2)	1.2 (1.0–4.5)	142.4 (56.9-288.4)

** For each AAA, the concentration of MMP-9 secreted by the three IL-8 treated explants at day 7 is expressed as a percentage of the concentration of MMP-9 secreted by the untreated explant at day 7. The results of all five AAAs are displayed in each column as medians and interquartile ranges.*

Table 9-5. Results of the Kruskal-Wallis statistical analysis of the concentration data in Table 9-4.

	Mean rank for each treatment group				Significance
	0ng/ml	1ng/ml	10ng/ml	100ng/ml	
Pre-treatment	9.40	9.60	12.20	10.80	.870
Post-treatment	9.50	11.10	11.40	10.00	.951

A)



B)

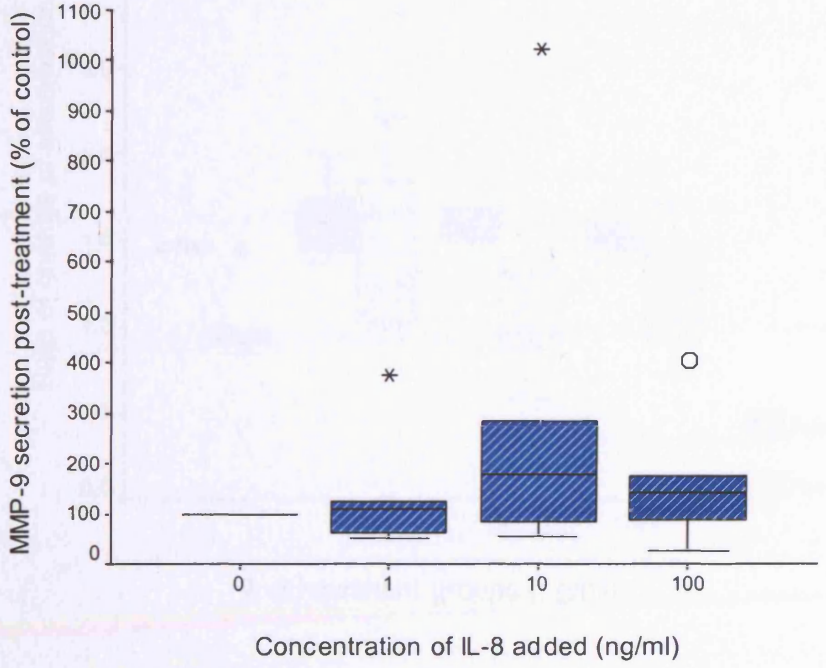


Figure 9-3. The effect of IL-8 on the secretion of total MMP-9 from AAA explants. (A) The concentration of total MMP-9 secreted from explants was measured prior to and after treatment with a single concentration of IL-8 (0, 1, 10, 100ng/ml). (B) For each AAA, the amount of MMP-9 secreted by the treated explants (1, 10, 100ng/ml) at day 7 is expressed as a percentage of the amount secreted by the untreated explant (0ng/ml). The results were obtained from 5 AAAs and are presented as medians (bar), interquartile ranges (box) and ranges (whiskers). O and * represents outliers, as defined in Chapter 7.3.2.

Active MMP-9 Expression

As seen with active MMP-2, the assay system was unable to determine the concentration of active MMP-9 as the rate of change of absorbance values that were obtained were below the lowest point on the standard curve ($3.31/h^2$ at 0.5ng/ml). Therefore it was not possible to assess the effect of IL-8 on the concentration of active MMP-9.

Figure 9-4 shows the rate of change of absorbance values that were obtained from the explants prior to and after IL-8 treatment. As these values were obtained at the limit of the assay this graph is used simply to present the raw data obtained. As the rate of change of absorbance is indicative of MMP-9 activity there doesn't appear to be any difference in the activity in any of the treatment groups, prior to or after IL-8 treatment. With concerns over the accuracy of the data no statistical analysis was performed on this data.

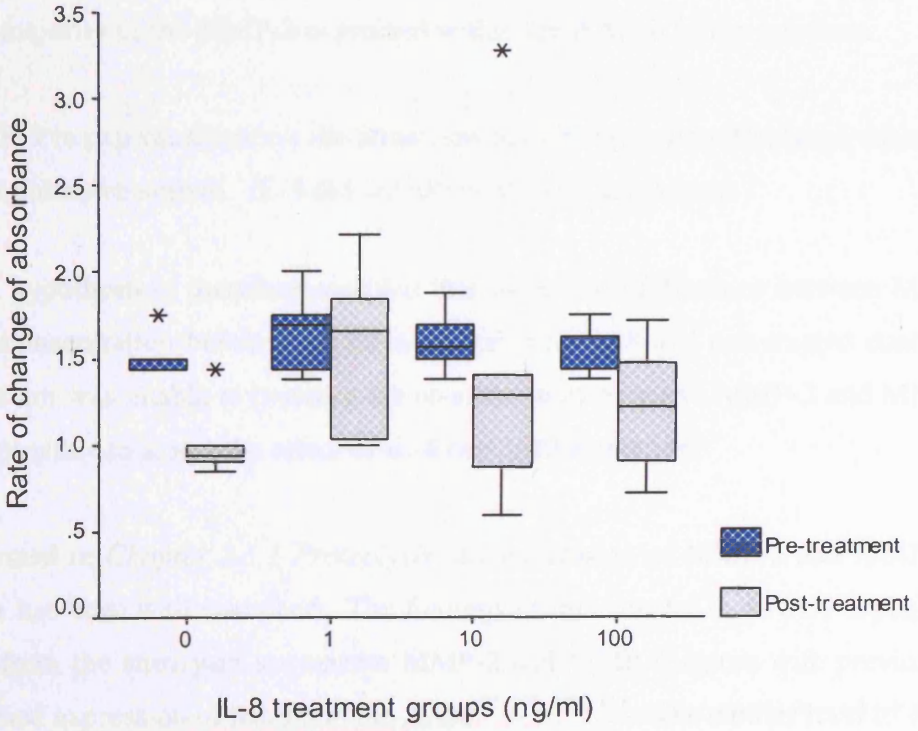


Figure 9-4. The effect of IL-8 on the secretion of active MMP-9 from AAA explants. The rate of change in absorbance at 405nm was proportional to MMP-9 activity in the culture medium from the explants and was measured prior to and after treatment with a single concentration of IL-8 (0, 1, 10, 100ng/ml). The results were obtained from 5 AAAs and are presented as medians (bar), interquartile ranges (box) and ranges (whiskers). * represents extreme outliers, as defined in Chapter 7.3.2.

9.4 Discussion

The aim of this chapter was to investigate the effect of IL-8 on the expression and activation of MMP-2 and MMP-9 in the AAA explant. The null hypothesis was that there are no differences in MMP-2 and -9 concentration and activation between explants treated with IL-8 compared to non-treated controls.

The findings of this chapter are that:

1. The explant model system used remained viable for a 7-day culture period.

2. MMP-2 was produced constitutively by AAA explants but MMP-2 expression was not affected by IL-8.

3. The majority of the MMP-2 expressed within the AAA is in the pro-form.

4. MMP-9 is expressed within the aneurysm but its expression decreased when removed from unknown stimuli. IL-8 did not affect MMP-9 expression.

The null hypothesis is therefore accepted that there is no difference between MMP-2 and MMP-9 concentration between explants treated with IL-8 and non-treated controls. The assay system was unable to measure the concentration of active MMP-2 and MMP-9 so it was not possible to assess the effect of IL-8 on MMP activation.

As discussed in *Chapter 2.5.1 Proteolysis*, the expression of MMP-2 and MMP-9 within the AAA has been well described. The findings of this chapter that AAA explants freshly isolated from the aneurysm sac secrete MMP-2 and MMP-9 agrees with previous reports of increased expression of MMPs in the AAA[171, 173, 184, 373]. The similar level of expression of MMP-2 that was detected in the medium at the beginning and end of the culture period suggests that cellular viability was mostly unaltered in the explants and that MMP-2 is produced constitutively. It has been previously reported that MMP-2 is constitutively expressed by vascular SMCs[85, 154]. Without the need for an exogenous stimulus to induce expression the explants did not respond to treatment with IL-8. IL-8 is a known angiogenic factor, however its precise role in angiogenesis is unclear. One study showed

that MMP-2 production by human umbilical vein endothelial cells (HUVEC) in culture was inhibited by neutralising antibodies to IL-8 and its receptors. In addition endothelial cell migration was also reduced by anti-IL-8 treatment[374]. IL-8 has been shown to up-regulate the expression of MMP-2 and MMP-9 by endothelial cells under the same concentrations as used in this chapter (10, 100ng/ml)[375].

It has also been reported that IL-8 causes the up-regulated expression of MMP-9 mRNA by endothelial cells[375] and increases the release of MMP-9 from neutrophils[323]. Neutrophils store MMP-9 in tertiary granules which are released upon stimulation by IL-8 through the CXCR2 receptor, but not CXCR1[323]. Neutrophils alone produce MMP-9 constitutively, whereas MMP-9 expression is usually induced in response to a proinflammatory stimuli. Relatively few neutrophils are observed within the AAA wall and studies on the expression of MMP-9 have reported the source of MMP-9 to be adventitial macrophages[86]. The expression of MMP-9 within the aneurysm wall has been well studied and, unlike MMP-2, it has not been found to be expressed constitutively within the AAA. This study has found that whilst all the explants secreted MMP-9 at day 1 in culture, after a 7 day period the expression had significantly decreased. This would suggest that the pro-inflammatory nature of the aneurysm wall, as described in *Chapter 6. The Cytokine Expression Profile of the Abdominal Aortic Aneurysm*, in some manner induces MMP-9 expression. However, whilst the aneurysmal wall is a cocktail of pro-inflammatory, chemotactic cytokines and growth factors, as an *in vitro* model the simple addition of IL-8 was not sufficient to induce MMP-9 release. As physiologically cytokines frequently work synergistically future work would have investigated the effect of the combination of IL-8 and MCP-1 on MMP expression. As described in *Chapter 7.4 Discussion* MCP-1 and IL-8 can work synergistically to increase IL-8 potency[354].

With MMP-2 being constitutively expressed within the explants, it may have been that the explants were maximally producing MMP-2 and therefore this would have prevented the study from determining the effect of IL-8 on MMP-2 expression. However, MMP-9 was not being maximally produced by the explants and IL-8 failed to produce a response. As MMP-2 is expressed at a lower level within non-aneurysmal aorta the explant work could be repeated on this tissue to determine whether IL-8 could increase MMP-2 expression.

The advantage of using an explant model is that it provides a general overall view of the effects of IL-8 on MMP expression within the aneurysm wall and allows the opportunity to assess any accompanying changes in the morphology of the explant. As IL-8 did not have a detectable effect on MMP expression morphological changes were not assessed. One advantage of using an explant over cell culture is that it allows the assessment of the effect of IL-8 on MMP expression in a more clinically relevant setting.

One of the disadvantageous of using an explant is the heterogeneity of each explant. Whilst it was ensured that all explants were of a similar size and taken from adjacent sections of the aneurysm sac there would have been a high degree of variation between the explants at the cellular level. This may account for the variation in the results obtained. With the degree of variation from the small numbers of AAAs that were studied statistically it would have been difficult to determine a significant result even if a small genuine difference existed. Furthermore, with the number of different cell populations present within the explant the response of one cell population to IL-8 may have been masked by the constitutive expression of MMP-2 by other cell types, for example SMCs. It is possible that a population of cells does respond to IL-8 but constitutes a small percentage of the cells within the explant, for example neutrophils, and therefore any induced expression would be difficult to detect. Whilst this explant study did not detect a change in MMP expression with IL-8 treatment, future work could involve identifying cells within the aneurysm wall which express IL-8 receptors (CXCR1 and CXCR2) and repeating the experiment on cell cultures of these specific cells. This should reduce experimental variation and increase the level of response to a detectable level. However, it is possible that IL-8 simply does not affect MMP expression. An extension to this work could involve investigating the effect of IL-8 on other proteins involved in inflammation and inflammatory cell recruitment, such as adhesion molecules. With IL-8 localising to the inflammatory infiltrate future work could investigate the effect of IL-8 on lymphocyte activation.

Further work is required to enable the detection of active MMP-2 and MMP-9. One problem with the explant method used was the large volume of medium required to immerse the explant (5ml) and the diluting factor this would have had on the measurement of active MMP-2 and MMP-9. Either the explant methodology needs modifying to reduce the volume of medium used, whilst retaining viability, or that the whole volume is

collected and spun through a column to remove excess liquid and concentrate the proteins. As an alternative method for the detection of active MMP-2 and -9 gelatin zymography could have been used and quantified through densitometric analysis.

In conclusion, IL-8 has been shown in previous studies to induce the expression of MMP-2 and –9, however the treatment of AAA explants with IL-8 did not induce the expression of these MMPs. This may result from the use of AAA explants as an *in vitro* model. The main limitations associated with this method are that the AAA explants may be maximally producing MMPs and that explants are highly heterogeneous. A better explant model system would have been the use of non-aneurysmal aorta, which as seen from the previous chapters expresses MMPs at a lower concentration. However, explants are a difficult method for obtaining reproducible results with limited deviations in the data due to the heterogeneity between the explants. At a cellular levels, despite an investigator's best efforts to ensure identical culturing conditions, there is a high degree of variability between explants. As an alternative to the explant method studies on cell cultures of a single cell type, such as cultured vascular SMCs, can produce less varied results, the experimental conditions can be more tightly controlled, and any response to IL-8 treatment would have been amplified by the increased numbers of responsive cells. An extension of this work could have involved investigating the effect of IL-8 and MCP-1 working individually and in synergy on MMP expression by non-aneurysmal aorta. As an alternative to the explant method the effect of IL-8 on MMP expression by cell types expressing CXCR1 and CXCR2 could have been investigated, particularly on vascular SMCs. Future work would have been extended further to investigate the effect of IL-8 on other inflammatory proteins, adhesion molecules and cellular activation.

Chapter Ten

Conclusions and Future Work

Chapter 10. Conclusions and Future Work

10. Conclusions and Future Work

10.1 Conclusions

The AAA is a common idiopathic disease of Caucasian males over the age of 65 years. Screening studies have shown that AAAs are present in 1.3 to 12.7% of the male population within England[44] and its incidence is rising. The natural history of the disease is to progressively expand until the aneurysm wall ruptures. Rupture of the AAA leads to intra-abdominal haemorrhage, circulatory collapse and death. Currently, the only method of treatment to prevent further aneurysm growth and rupture is through elective surgery. However, surgery is not suitable for all patients, is not indicated for small aneurysms and is associated with a risk of post-operative complications and mortality[7]. Whilst it would be desirable to treat aneurysms pharmacologically at present this treatment is not available. Previous attempts at drug therapy have proved to be unsuccessful[120, 121, 124 127]. In order to develop a suitable drug therapy a better understand of the pathogenesis of the AAA is required.

Aneurysm formation requires the extensive remodelling of the ECM within the aortic wall. A key feature in the development of the aneurysm is the degradation of medial elastin. The aneurysm wall is characterised by an increase in the elastin/collagen ratio, a decrease in medial SMC density and chronic inflammation of the adventitial layer. An inflammatory infiltrate of mononuclear phagocytes and lymphocytes is present within the adventitia. The mechanisms thought to be involved in aneurysm formation can be divided into: biomechanical wall stress, a genetic predisposition, proteolysis and inflammation. It is thought that an initial stimulus for remodelling results in an influx of inflammatory cells and the activation of the proteolytic system within the AAA wall. MMPs, particularly MMP-2 and MMP-9, have been implicated in aneurysm formation and may be responsible for changes observed in the composition of the ECM[173, 177]. MMP expression is regulated by specific cytokines[199-201]. The cells of the infiltrate are known to produce a variety of inflammatory mediators, including cytokines.

Cytokines are proteins involved in cell-to-cell communication and have an important role in regulating the local immune system. In AAAs this control is lost and chronic

inflammation results in extensive wall damage. Several studies have investigated the expression of specific cytokines within the human AAA wall[84, 296, 297, 348]. However, these studies have been limited by experimental techniques in the repertoire that they have been able to study. As the techniques used required the selection of candidate cytokines it reduces the chance of identifying unanticipated, novel changes in the cytokine expression profile. Many of these studies are contradictory. Animal studies have shown that specific cytokines are involved in aneurysm formation[164, 306, 312, 320]. However, within the human AAA to date no comprehensive description of the cytokine profile of the AAA wall has been given.

The aim of this thesis was to investigate the cytokine expression profile of the AAA wall. In order to achieve this, this study investigated the expression of 42 cytokines within the aneurysm wall and in non-aneurysmal aorta. The key cytokines that were identified by the array were quantified using ELISAs and their localised expression within the aneurysm wall was determined by immunohistochemistry. Finally, the role of chemokine expression in MMP secretion and activation was examined.

To achieve this, the study used a cytokine protein array to measure the expression of 42 different cytokines within homogenised aneurysmal and non-aneurysmal aortic wall. Specimens were obtained from patients undergoing elective open AAA repair (AAA), aorto-coronary bypass graft operations (thoracic aorta) and from cadaveric organ donors (abdominal aorta). The arrays consisted of antibodies against a number of pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines and growth factors. ELISAs were used to measure the expression of IL-6, IL-8, MCP-1 and MCP-2 within all three aortic groups. The chemokines IL-8 and MCP-1 were selected for further study. To localise the expression of these chemokines within the AAA wall immunohistochemistry was performed. The cellular composition of the inflammatory infiltrate was examined and the association between the level of inflammation and chemokine expression was analysed. As IL-8 has been shown to cause neutrophil degranulation of vesicles containing MMP-9[323] the effect of IL-8 on the expression and activation of MMP-2 and MMP-9 was assessed using an AAA explant model.

This study demonstrated that the AAA exhibits a distinct cytokine profile comprising of an elevated expression of pro-inflammatory cytokines and chemokines. Several of these have

been studied within the AAA for the first time. Comparison of the expression profile of the AAA to the profiles of the non-aneurysmal controls revealed that IL-6, IL-8, MCP-1 and MCP-2 were raised within the AAA. This inflammatory cytokine profile of the aneurysm wall may lead to further ECM remodelling through the activation of MMPs and other proteases, inhibition of TIMPs, induction of SMC apoptosis and the chemotaxis of inflammatory cells into the aortic wall[134, 158, 199, 200].

The arrays described the relative expression of IL-6, IL-8, MCP-1 and MCP-2 within the aneurysm compared to the controls. In order to quantify their expression and validate the array's findings ELISAs were performed on these cytokines. The additional benefit of measuring the expression of these cytokine by ELISAs was that larger patient groups could be analysed. The results showed that the expression of IL-6, IL-8 and MCP-1 is significantly increased within the AAA. These cytokines are all involved in inflammation. IL-6 stimulates B-lymphocyte differentiation and antibody production[337]. With the prominence of B-lymphocytes within the aneurysm wall (Chapter 8) the expression of IL-6 may result in the activation of the humoral response. IL-8 and MCP-1 are chemotactic for neutrophils and macrophages, respectively, and may potentially be involved in aneurysm development through the recruitment of inflammatory cells into the aneurysm. Both chemokines are also known to stimulate the release of inflammatory mediators and lytic enzymes[323, 355, 375].

With the relationship between chemokines and inflammation the localised expression of IL-8 and MCP-1 within the aneurysm wall was investigated. The inflammatory infiltrate within the aneurysm specimens was examined to determine its cellular composition. The results of the grading showed that the predominant cell type of the inflammatory infiltrate was the B-lymphocyte. T-lymphocytes were the second major population of the infiltrate, with the occasional macrophage present. Macrophages were predominantly found within the intima. The T-lymphocyte population comprised chief of $CD4^+$ T-lymphocytes with the occasional dispersed $CD8^+$ T-lymphocyte. Analysis of chemokine expression found that IL-8 co-localised with the lymphocyte population within the adventitia, whilst MCP-1 co-localised to the macrophages within the intima and adventitia. Assessment of the effect of increasing IL-8 and MCP-1 expression on the level of inflammation within the aneurysm wall found that there was an association between IL-8 and inflammation, but not between MCP-1 and inflammation. The increasing mural inflammation within the AAA

was associated with an increase in T-lymphocytes within the infiltrate population. The increased expression of IL-8 and MCP-1 shown in the previous chapters along with their expression within the adventitia suggests that these chemokines may have a role in the pathogenesis of the AAA. IL-8 may have a chemotactic role in the development of the inflammatory infiltrate.

The final results chapter of the thesis investigated the potential for IL-8 to induce the secretion and/or activation of MMP-2 and MMP-9 in order to aid cellular migration across the ECM. AAA explants were cultured and treated with increasing concentrations of IL-8. The results demonstrated that MMP-2 was produced constitutively by AAA explants but that MMP-2 expression was not affected by IL-8. The majority of the MMP-2 expressed within the AAA was in the pro-form. Measurement of MMP-9 expression revealed that MMP-9 was expressed by the AAA explants at the start of the culturing period, when recently removed from the patient, but during culture its expression decreased. This suggests that an unknown stimuli within the aneurysm wall is inducing MMP-9 expression, which when the tissue is removed from the patient the stimuli is lost. IL-8 did not affect MMP-9 expression.

10.2 Future Work

Future studies arising from this thesis are discussed below.

This thesis has concerned itself with the expression of cytokines at the protein level. To support these findings future work would involve studying the expression of IL-8, IL-6, MCP-1 and MCP-2 at the genomic level through RT-PCR. With the increased sensitivity of real-time RT-PCR it should be possible to assess the expression of MCP-2 mRNA within the aneurysm compared to non-aneurysmal aorta. As mentioned in *Chapter 6.4 Discussion* homogenising the whole aortic wall, whilst providing an overview, may mask subtle changes within the cytokine profile. Further investigation of cytokine expression within the aneurysm wall would involve the use of laser capture microdissection to isolate specific cell populations, such as the infiltrate, and analyse the local cytokine expression by RT-PCR. As IL-8 may be involved in the process of angiogenesis within the aneurysm wall an investigation into this role could use laser capture microdissection to dissect out a

fixed area around newly formed capillary-like vessels and compare the local cytokine profile to that of vessel-free regions or regions around established vessels within the adventitia.

Immunhistochemistry was used to determine the localised expression of IL-8 and MCP-1. With the dominance of B-lymphocytes within the aneurysm wall and the raised expression of IL-6 this suggests that IL-6 may have a role in B-lymphocyte differentiation within the AAA. Co-localisation studies could be performed to determine whether IL-6 co-localises with the B-lymphocytes. Further work would involve investigating the source of these cytokines through *in situ* hybridisation. Considering the increased expression of IL-8 and other neutrophil activators within the aneurysm wall the presence of neutrophils within the aneurysm wall should have been assessed. Neutrophils are the first inflammatory cells to infiltrate a site of inflammation and are not usually present in chronic inflammation. It therefore seems surprising that so many neutrophil activating chemokines are increased within the aneurysm. Future work would investigate the presence of the neutrophil within the aneurysm and its co-localisation with IL-8.

Receptor expression is a crucial determinant of the spectrum of chemokine activities. Regulation of the expression of chemokine receptors during cellular activation or deactivation is an important stage for controlling the action of specific chemokines. It is equally as important as the regulation of chemokine production and post-translational processing for controlling the inflammatory response. Further extension of this study could investigate the expression of the CXCR1 and CXCR2 receptors for IL-8 and CCR2 for MCP-1 within the aneurysm wall through immunohistochemistry. Identification of the cells within the aneurysm wall that express these receptors could lead to cell culture studies on the isolated cells, either from the aneurysm or through the use of a specific cell line. These studies could investigate the effect of conditions relevant to the aneurysm, such as hypoxia, on receptor expression. Any alterations in the expression of the receptors could be measured through flow cytometry.

As discussed in Chapter 9, further work is required to investigate the effect of IL-8 on the activation of MMP-2 and MMP-9. Alternatively, gelatin zymography could be used to analyse MMP-2 and -9 activity. An extension of this work could investigate the effect of IL-8 and MCP-1 working in synergy on MMP expression within AAA explants. As an

alternative model system, a cell-culture system could be used to determine the effect of IL-8 on the expression of MMPs and a range of inflammatory proteins, including adhesion molecules, by cells that have been shown to express CXCR1 or CXCR2. The affect of IL-8 on cellular activation could also be assessed by measuring the effect on cell-surface markers of activation.

In, conclusion the findings of this thesis are:

1. **The AAA exhibits a distinct cytokine profile comprising of an elevated expression of pro-inflammatory cytokines and chemokines. IL-6, IL-8 and MCP-1 are significantly increased within the AAA.**

2. **IL-8 co-localises with the infiltrating lymphocytes within the adventitia whilst MCP-1 co-localises to macrophages within the intima and adventitia.**

3. **Increasing IL-8 expression is associated with an increase in inflammation. The increasing mural inflammation is associated with a change in the composition of the infiltrate, with an increase in T-lymphocytes.**

4. **MMP-2 is produced constitutively within the aneurysm and the majority of the MMP-2 expressed within the AAA is in the pro-form.**

5. **MMP-9 is expressed within the aneurysm but its expression decreases when removed from unknown stimuli.**

6. **MMP-2 and MMP-9 expression by AAA explants is not affected by IL-8.**

Appendices

APPENDIX I

SOLUTIONS

Eosin

Eosin	10g
Tap water	2L
Formaldehyde	2ml

Explant Culture Medium

DMEM (D5671) without glutamine, 4500mg/ml glucose
4mM Glutamine,
100U/ml Penicillin,
100μg/ml Streptomycin.

10% Formalin

0.15M Sodium chloride,
10% (v/v) of 12.3M Formaldehyde.

Homogenate Buffer, pH 7.6

2M Urea,
50mM Tris-HCl,
20mM Sodium chloride,
3mM EDTA,
0.1% (v/v) Brij 35.

Mayer's Haematoxylin

Haematoxylin	2g
99% IMS	10ml
Distilled water	2L
Potassium alum	100g
Sodium iodate	0.4g
Citric acid	0.4g
Chloral hydrate	100g

MCP-2 Reagent Diluent

1% (w/v) BSA

PBS, pH 7.2

MCP-2 Wash Buffer

0.05% (v/v) Tween® 20

PBS, pH 7.2

Phosphate Buffered Saline, pH 7.2

Potassium dihydrogen orthophosphate	3.402g
Disodium hydrogen orthophosphate	5.706g
Sodium chloride	1.461g
Distilled water	500ml

Phosphate Buffered Saline, pH 7.45

Potassium dihydrogen orthophosphate	0.2g
Disodium hydrogen orthophosphate	1.37g
Sodium chloride	8.0g
Distilled water	1L

Tris Buffered Saline (10X), pH 7.6

Tris base	60.55g
NaCl	85.2g
Distilled water	1L

Used as a 1X solution

APPENDIX II

PROTOCOLS

ELISA Protocols

a) BioSource ELISA Protocol

All steps were performed at RT. The samples and standards (50/100μl) were incubated in microtitre wells containing capture antibodies specific to either IL-6, IL-8, or MCP-1 with 50μl of a biotinylated detection antibody specific to a second epitope on the cytokines. The plates were left for a 1.5 hours (2 hours for the MCP-1 assay). The samples were aspirated and the wells were washed four times (six times for the MCP-1 assay) with wash buffer. The bound cytokines were detected by incubating with 100μl of HRP-SA for 30 minutes. The HRP-SA was removed and the wells were washed as before. 100μl of the chromogen substrate was added to the wells and incubated for 30 minutes in the dark. The reaction was stopped by adding 100μl of stop solution. The resulting colour change was read at 450nm using a microtitre plate spectrophotometer.

b) Amersham Biosciences ELISA Protocol

All steps were performed at RT. 100μl of the standards and samples were incubated in the wells for 2 hours (MMP-2 assay) or 1 hour (MMP-9 assay). The samples/standards were removed and the wells were washed four times. 100μl of the HRP conjugated antibody was pipetted into the wells and incubated for 1 hour (MMP-2 assay) or 2 hours (MMP-9 assay). All wells were washed four times and incubated with 100μl of tetramethylbenzidine for 20 minutes with agitation. The chromogenic reaction was stopped by adding 100μl of 1M sulphuric acid and the plate was read at 450nm.

Antigen Retrieval Methods

All antigen retrieval methods were performed after the slides had been dewaxed and taken to water.

a) Pressure Cooker

The slides were boiled in a pressure cooker for 5.5 minutes in a 10mM citrate buffer, pH 6.0. The slides were transferred to water to cool and transferred to PBS for washing.

b) Microwaving

The slides were microwaved in a 10mM citrate buffer, pH 6.0 for 12 minutes at 850 watts. The slides were transferred to water to cool and transferred to PBS.

c) High pH Microwaving

The slides were microwaved in a high pH Target Retrieval Solution, pH 9.0 (DakoCytomation) for 12 minutes at 850 watts. The slides were transferred to water to cool and transferred to PBS.

d) Trypsin Digestion

The slides were warmed in a water bath at 37°C and transferred to a second water bath containing 1g/L of trypsin, 10mM calcium chloride, pH 7.8. The slides were incubated for 10 minutes. The slides were then placed in running water for 5 minutes to prevent further digestion and transferred to PBS.

Bibliography

1. Lippi D. An aneurysm in the Papyrus of Ebers (108, 3-9). *Med Secoli.* 1990;2:1-4.
2. Erichsen JE. *Observations on aneurism.* London: Syndenham Society; 1844.
3. Cooley DA. Aortic aneurysm operations: past, present, and future. *Ann Thorac Surg.* 1999;67:1959-1962.
4. Dale WA. The beginnings of vascular surgery. *Surgery.* 1974;76:849-866.
5. Friedman SG, Friedman MS. Matas, Antyllus, and endoaneurysmorrhaphy. *Surgery.* 1989;105:761-763.
6. Matas R. Traumatic aneurysm of the left brachial artery. Failure of direct and indirect pressure; ligation of the artery immediately above tumour, return of pulsation on the tenth day; ligation immediately below tumour; failure to arrest pulsation: incision and partial excision of sac; recovery. *Med News New York.* 1888;53:462.
7. UK Small Aneurysm Trial Participants. Mortality results for randomised controlled trial of early elective surgery or ultrasonographic surveillance for small abdominal aortic aneurysms. *Lancet.* 1998;352:1649-1655.
8. Parodi JC, Palmaz JC, Barone HD. Transfemoral intraluminal graft implantation for abdominal aortic aneurysms. *Ann Vasc Surg.* 1991;5:491-499.
9. Endovascular aneurysm repair versus open repair in patients with abdominal aortic aneurysm (EVAR trial 1): randomised controlled trial. *Lancet.* 2005;365:2179-2186.
10. Sterpetti AV, Schultz RD, Feldhaus RJ, Cheng SE, Peetz DJ, Jr. Factors influencing enlargement rate of small abdominal aortic aneurysms. *J Surg Res.* 1987;43:211-219.
11. Collin J. A proposal for a precise definition of abdominal aortic aneurysm. A personal view. *J Cardiovasc Surg (Torino).* 1990;31:168-169.
12. Johnston KW, Rutherford RB, Tilson MD, Shah DM, Hollier L, Stanley JC. Suggested standards for reporting on arterial aneurysms. Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular Surgery. *J Vasc Surg.* 1991;13:452-458.
13. Aneurysms - Peripheral Vascular Disease. In: McLatchie GR, Leaper DJ. ed. *Oxford Handbook of Clinical Surgery.* 2nd ed. Oxford: Oxford University Press; 2002:308-310.

14. Moritz JD, Rotermund S, Keating DP, Oestmann JW. Infrarenal abdominal aortic aneurysms: implications of CT evaluation of size and configuration for placement of endovascular aortic grafts. *Radiology.* 1996;198:463-466.
15. Collin J. Aneurysms: Abdominal aorta. In: Morris P, Wood WC. *Oxford Textbook of Surgery.* Vol 1. 2nd ed. Oxford: Oxford University Press; 2000:833-846.
16. Rochat M. Navigation System for Abdominal Aortic Aneurysm. Last update: 22nd February 2002. Available at http://bigwww.epfl.ch/teaching/projects/abstracts/rochat/index1.html. Accessed 16th January 2006.
17. Thompson MM, Bell PR. ABC of arterial and venous disease. Arterial aneurysms. *BMJ.* 2000;320:1193-1196.
18. Chapter 27. Vascular and Endovascular Surgery. In: Garden OJ, Bradbury AW, Forsythe J. eds. *Principles and Practice of Surgery.* 4th ed. Edinburgh: Churchill Livingstone; 2002:470-473.
19. Dixon AK, Lawrence JP, Mitchell JR. Age-related changes in the abdominal aorta shown by computed tomography. *Clin Radiol.* 1984;35:33-37.
20. Pedersen OM, Aslaksen A, Vik-Mo H. Ultrasound measurement of the luminal diameter of the abdominal aorta and iliac arteries in patients without vascular disease. *J Vasc Surg.* 1993;17:596-601.
21. Pearce WH, Slaughter MS, LeMaire S, Salyapongse AN, Feinglass J, McCarthy WJ, *et. al.* Aortic diameter as a function of age, gender, and body surface area. *Surgery.* 1993;114:691-697.
22. Sonesson B, Hansen F, Stale H, Lanne T. Compliance and diameter in the human abdominal aorta--the influence of age and sex. *Eur J Vasc Surg.* 1993;7:690-697.
23. Wilmink AB, Hubbard CS, Day NE, Quick CR. The incidence of small abdominal aortic aneurysms and the change in normal infrarenal aortic diameter: implications for screening. *Eur J Vasc Endovasc Surg.* 2001;21:165-170.
24. Reed WW, Hallett JW, Jr., Damiano MA, Ballard DJ. Learning from the last ultrasound. A population-based study of patients with abdominal aortic aneurysm. *Arch Intern Med.* 1997;157:2064-2068.
25. Cronenwett JL, Murphy TF, Zelenock GB, Whitehouse WM, Jr., Lindenauer SM, Graham LM, *et. al.* Actuarial analysis of variables associated with rupture of small abdominal aortic aneurysms. *Surgery.* 1985;98:472-483.
26. Brown PM, Zelt DT, Sobolev B. The risk of rupture in untreated aneurysms: the impact of size, gender, and expansion rate. *J Vasc Surg.* 2003;37:280-284.

27. Lederle FA, Johnson GR, Wilson SE, Ballard DJ, Jordan WD, Jr., Blebea J, *et. al.* Rupture rate of large abdominal aortic aneurysms in patients refusing or unfit for elective repair. *JAMA.* 2002;287:2968-2972.

28. Choke E, Cockerill G, Wilson WR, Sayed S, Dawson J, Loftus I, *et. al.* A review of biological factors implicated in abdominal aortic aneurysm rupture. *Eur J Vasc Endovasc Surg.* 2005;30:227-244.

29. Vardulaki KA, Prevost TC, Walker NM, Day NE, Wilmink AB, Quick CR, *et. al.* Growth rates and risk of rupture of abdominal aortic aneurysms. *Br J Surg.* 1998;85:1674-1680.

30. Lederle FA, Wilson SE, Johnson GR, Reinke DB, Littooy FN, Acher CW, *et. al.* Immediate repair compared with surveillance of small abdominal aortic aneurysms. *N Engl J Med.* 2002;346:1437-1444.

31. Szilagyi DE, Smith RF, DeRusso FJ, Elliott JP, Sherrin FW. Contribution of abdominal aortic aneurysmectomy to prolongation of life. *Ann Surg.* 1966;164:678-699.

32. Limet R, Sakalihassan N, Albert A. Determination of the expansion rate and incidence of rupture of abdominal aortic aneurysms. *J Vasc Surg.* 1991;14:540-548.

33. Cronenwett JL. Variables that affect the expansion rate and rupture of abdominal aortic aneurysms. *Ann N Y Acad Sci.* 1996;800:56-67.

34. Nevitt MP, Ballard DJ, Hallett JW, Jr. Prognosis of abdominal aortic aneurysms. A population-based study. *N Engl J Med.* 1989;321:1009-1014.

35. Bengtsson H, Bergqvist D, Ekberg O, Ranstam J. Expansion pattern and risk of rupture of abdominal aortic aneurysms that were not operated on. *Eur J Surg.* 1993;159:461-467.

36. Santilli SM, Littooy FN, Cambria RA, Rapp JH, Tretinyak AS, d'Audiffret AC, *et. al.* Expansion rates and outcomes for the 3.0-cm to the 3.9-cm infrarenal abdominal aortic aneurysm. *J Vasc Surg.* 2002;35:666-671.

37. Fowkes FG, Macintyre CC, Ruckley CV. Increasing incidence of aortic aneurysms in England and Wales. *BMJ.* 1989;298:33-35.

38. Filipovic M, Goldacre MJ, Roberts SE, Yeates D, Duncan ME, Cook-Mozaffari P. Trends in mortality and hospital admission rates for abdominal aortic aneurysm in England and Wales, 1979-1999. *Br J Surg.* 2005;92:968-975.

39. Naylor AR, Webb J, Fowkes FG, Ruckley CV. Trends in abdominal aortic aneurysm surgery in Scotland (1971-1984). *Eur J Vasc Surg.* 1988;2:217-221.

40. Reitsma JB, Pleumeekers HJ, Hoes AW, Kleijnen J, de Groot RM, Jacobs MJ, *et. al.* Increasing incidence of aneurysms of the abdominal aorta in The Netherlands. *Eur J Vasc Endovasc Surg.* 1996;12:446-451.

41. Castleden WM, Mercer JC. Abdominal aortic aneurysms in Western Australia: descriptive epidemiology and patterns of rupture. *Br J Surg.* 1985;72:109-112.

42. Melton LJ, 3rd, Bickerstaff LK, Hollier LH, Van Peenen HJ, Lie JT, Pairolero PC, *et. al.* Changing incidence of abdominal aortic aneurysms: a population-based study. *Am J Epidemiol.* 1984;120:379-386.

43. Best VA, Price JF, Fowkes FG. Persistent increase in the incidence of abdominal aortic aneurysm in Scotland, 1981-2000. *Br J Surg.* 2003;90:1510-1515.

44. Wilmink AB, Quick CR. Epidemiology and potential for prevention of abdominal aortic aneurysm. *Br J Surg.* 1998;85:155-162.

45. Mealy K, Salman A. The true incidence of ruptured abdominal aortic aneurysms. *Eur J Vasc Surg.* 1988;2:405-408.

46. Office of Population Censuses and Surveys. *Mortality Statistics; Cause, England and Wales 1993.* London: HMSO, 1995.

47. Bengtsson H, Bergqvist D, Sternby NH. Increasing prevalence of abdominal aortic aneurysms. A necropsy study. *Eur J Surg.* 1992;158:19-23.

48. McFarlane MJ. The epidemiologic necropsy for abdominal aortic aneurysm. *JAMA.* 1991;265:2085-2088.

49. Turk KA. The post-mortem incidence of abdominal aortic aneurysm. *Proc R Soc Med.* 1965;58:869-870.

50. Holdsworth JD. Screening for abdominal aortic aneurysm in Northumberland. *Br J Surg.* 1994;81:710-712.

51. Morris GE, Hubbard CS, Quick CR. An abdominal aortic aneurysm screening programme for all males over the age of 50 years. *Eur J Vasc Surg.* 1994;8:156-160.

52. Collin J, Araujo L, Walton J, Lindsell D. Oxford screening programme for abdominal aortic aneurysm in men aged 65 to 74 years. *Lancet.* 1988;2:613-615.

53. Lucarotti M, Shaw E, Poskitt K, Heather B. The Gloucestershire Aneurysm Screening Programme: the first 2 years' experience. *Eur J Vasc Surg.* 1993;7:397-401.

54. Smith FC, Grimshaw GM, Paterson IS, Shearman CP, Hamer JD. Ultrasonographic screening for abdominal aortic aneurysm in an urban community. *Br J Surg.* 1993;80:1406-1409.

55. Scott RA, Wilson NM, Ashton HA, Kay DN. Influence of screening on the incidence of ruptured abdominal aortic aneurysm: 5-year results of a randomized controlled study. *Br J Surg.* 1995;82:1066-1070.

56. Pleumeekers HJ, Hoes AW, van der Does E, van Urk H, Hofman A, de Jong PT, *et. al.* Aneurysms of the abdominal aorta in older adults. The Rotterdam Study. *Am J Epidemiol.* 1995;142:1291-1299.

57. Simoni G, Pastorino C, Perrone R, Ardia A, Gianrossi R, Decian F, *et. al.* Screening for abdominal aortic aneurysms and associated risk factors in a general population. *Eur J Vasc Endovasc Surg.* 1995;10:207-210.

58. Bell P, Mantor C, Jacocks MA. Congenital abdominal aortic aneurysm: a case report. *J Vasc Surg.* 2003;38:190-193.

59. Mehall JR, Saltzman DA, Chandler JC, Kidd JN, Wells T, Smith SD. Congenital abdominal aortic aneurysm in the infant: case report and review of the literature. *J Pediatr Surg.* 2001;36:657-658.

60. Dietz HC, Cutting GR, Pyeritz RE, Maslen CL, Sakai LY, Corson GM, *et. al.* Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature.* 1991;352:337-339.

61. Judge DP, Dietz HC. Marfan's syndrome. *Lancet.* 2005;366:1965-1976.

62. Baxter BT. Heritable diseases of the blood vessels. *Cardiovasc Pathol.* 2005;14:185-188.

63. Pereira L, Lee SY, Gayraud B, Andrikopoulos K, Shapiro SD, Bunton T, *et. al.* Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1. *Proc Natl Acad Sci U S A.* 1999;96:3819-3823.

64. Kronzon I. Diagnosis and treatment of iatrogenic femoral artery pseudoaneurysm: a review. *J Am Soc Echocardiogr.* 1997;10:236-245.

65. Parkhurst GF, Dekcer JP. Bacterial aortitis and mycotic aneurysm of the aorta; a report of twelve cases. *Am J Pathol.* 1955;31:821-835.

66. Sommerville RL, Allen EV, Edwards JE. Bland and infected arteriosclerotic abdominal aortic aneurysms: a clinicopathologic study. *Medicine (Baltimore).* 1959;38:207-221.

67. Klontz KC. Frequency of infected aneurysms among patients in Department of Veterans Affairs hospitals, 1986-1990: the role of Salmonella. *Mil Med.* 1997;162:766-768.

68. Chan FY, Crawford ES, Coselli JS, Safi HJ, Williams TW, Jr. In situ prosthetic graft replacement for mycotic aneurysm of the aorta. *Ann Thorac Surg.* 1989;47:193-203.

69. Muller BT, Wegener OR, Grabitz K, Pillny M, Thomas L, Sandmann W. Mycotic aneurysms of the thoracic and abdominal aorta and iliac arteries: experience with anatomic and extra-anatomic repair in 33 cases. *J Vasc Surg.* 2001;33:106-113.

70. Gomes MN, Choyke PL, Wallace RB. Infected aortic aneurysms. A changing entity. *Ann Surg.* 1992;215:435-442.

71. Miller DV, Oderich GS, Aubry MC, Panneton JM, Edwards WD. Surgical pathology of infected aneurysms of the descending thoracic and abdominal aorta: clinicopathologic correlations in 29 cases (1976 to 1999). *Hum Pathol.* 2004;35:1112-1120.

72. Juvonen J, Juvonen T, Laurila A, Alakarppa H, Lounatmaa K, Surcel HM, *et. al.* Demonstration of Chlamydia pneumoniae in the walls of abdominal aortic aneurysms. *J Vasc Surg.* 1997;25:499-505.

73. Karlsson L, Gnarpe J, Naas J, Olsson G, Lindholm J, Steen B, *et. al.* Detection of viable Chlamydia pneumoniae in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg.* 2000;19:630-635.

74. Choudhary SK, Bhan A, Talwar S, Goyal M, Sharma S, Venugopal P. Tubercular pseudoaneurysms of aorta. *Ann Thorac Surg.* 2001;72:1239-1244.

75. Railo M, Isoluoma M, Keto P, Salo JA. Surgical treatment of inflammatory abdominal aortic aneurysms: a long-term follow-up of 19 patients. *Ann Vasc Surg.* 2005;19:361-366.

76. Walker DI, Bloor K, Williams G, Gillie I. Inflammatory aneurysms of the abdominal aorta. *Br J Surg.* 1972;59:609-614.

77. Rasmussen TE, Hallett JW, Jr. Inflammatory aortic aneurysms. A clinical review with new perspectives in pathogenesis. *Ann Surg.* 1997;225:155-164.

78. Rose AG, Dent DM. Inflammatory variant of abdominal atherosclerotic aneurysm. *Arch Pathol Lab Med.* 1981;105:409-413.

79. Clifton MA. Familial abdominal aortic aneurysms. *Br J Surg.* 1977;64:765-766.

80. Kuivaniemi H, Shibamura H, Arthur C, Berguer R, Cole CW, Juvonen T, *et. al.* Familial abdominal aortic aneurysms: collection of 233 multiplex families. *J Vasc Surg.* 2003;37:340-345.

81. Pierce GE. *Abdominal Aortic Aneurysms.* London: Saunders; 1989.

82. Shteinberg D, Halak M, Shapiro S, Kinarty A, Sobol E, Lahat N, *et. al.* Abdominal aortic aneurysm and aortic occlusive disease: a comparison of risk factors and inflammatory response. *Eur J Vasc Endovasc Surg.* 2000;20:462-465.

83. Armstrong PJ, Johanning JM, Calton WC, Jr., Delatore JR, Franklin DP, Han DC, *et. al.* Differential gene expression in human abdominal aorta: aneurysmal versus occlusive disease. *J Vasc Surg.* 2002;35:346-355.

84. Davis VA, Persidskaia RN, Baca-Regen LM, Fiotti N, Halloran BG, Baxter BT. Cytokine pattern in aneurysmal and occlusive disease of the aorta. *J Surg Res.* 2001;101:152-156.

85. McMillan WD, Patterson BK, Keen RR, Pearce WH. In situ localization and quantification of seventy-two-kilodalton type IV collagenase in aneurysmal, occlusive, and normal aorta. *J Vasc Surg.* 1995;22:295-305.

86. McMillan WD, Patterson BK, Keen RR, Shively VP, Cipollone M, Pearce WH. In situ localization and quantification of mRNA for 92-kD type IV collagenase and its inhibitor in aneurysmal, occlusive, and normal aorta. *Arterioscler Thromb Vasc Biol.* 1995;15:1139-1144.

87. Reilly JM, Sicard GA, Lucore CL. Abnormal expression of plasminogen activators in aortic aneurysmal and occlusive disease. *J Vasc Surg.* 1994;19:865-872.

88. Cornuz J, Sidoti Pinto C, Tevaearai H, Egger M. Risk factors for asymptomatic abdominal aortic aneurysm: systematic review and meta-analysis of population-based screening studies. *Eur J Public Health.* 2004;14:343-349.

89. Lederle FA, Johnson GR, Wilson SE, Chute EP, Littooy FN, Bandyk D, *et. al.* Prevalence and associations of abdominal aortic aneurysm detected through screening. Aneurysm Detection and Management (ADAM) Veterans Affairs Cooperative Study Group. *Ann Intern Med.* 1997;126:441-449.

90. Vega de Ceniga M, Gomez R, Estallo L, Rodriguez L, Baquer M, Barba A. Growth Rate and Associated Factors in Small Abdominal Aortic Aneurysms. *Eur J Vasc Endovasc Surg.* 2005.

91. Vardulaki KA, Prevost TC, Walker NM, Day NE, Wilmink AB, Quick CR, *et. al.* Incidence among men of asymptomatic abdominal aortic aneurysms: estimates from 500 screen detected cases. *J Med Screen.* 1999;6:50-54.

92. Scott RA, Bridgewater SG, Ashton HA. Randomized clinical trial of screening for abdominal aortic aneurysm in women. *Br J Surg.* 2002;89:283-285.

93. Vardulaki KA, Walker NM, Day NE, Duffy SW, Ashton HA, Scott RA. Quantifying the risks of hypertension, age, sex and smoking in patients with abdominal aortic aneurysm. *Br J Surg.* 2000;87:195-200.

94. Office for National Statistics. Mortality Statistics: Cause. London: HMSO, 1995.

95. Semmens JB, Norman PE, Lawrence-Brown MM, Holman CD. Influence of gender on outcome from ruptured abdominal aortic aneurysm. *Br J Surg.* 2000;87:191-194.

96. Baird PA, Sadovnick AD, Yee IM, Cole CW, Cole L. Sibling risks of abdominal aortic aneurysm. *Lancet.* 1995;346:601-604.

97. Salo JA, Soisalon-Soininen S, Bondestam S, Mattila PS. Familial occurrence of abdominal aortic aneurysm. *Ann Intern Med.* 1999;130:637-642.

98. Wanhainen A, Bergqvist D, Boman K, Nilsson TK, Rutegard J, Bjorck M. Risk factors associated with abdominal aortic aneurysm: a population-based study with historical and current data. *J Vasc Surg.* 2005;41:390-396.

99. Lindholt JS, Henneberg EW, Fasting H, Juul S. Hospital based screening of 65-73 year old men for abdominal aortic aneurysms in the county of Viborg, Denmark. *J Med Screen.* 1996;3:43-46.

100. Krohn CD, Kullmann G, Kvernebo K, Rosen L, Kroese A. Ultrasonographic screening for abdominal aortic aneurysm. *Eur J Surg.* 1992;158:527-530.

101. Grimshaw GM, Thompson JM, Hamer JD. Prevalence of abdominal aortic aneurysm associated with hypertension in an urban population. *J Med Screen.* 1994;1:226-228.

102. Dobrin PB. Pathophysiology and pathogenesis of aortic aneurysms. Current concepts. *Surg Clin North Am.* 1989;69:687-703.

103. Bengtsson H, Bergqvist D, Ekberg O, Janzon L. A population based screening of abdominal aortic aneurysms (AAA). *Eur J Vasc Surg.* 1991;5:53-57.

104. Hammond EC, Garfinkel L. Coronary heart disease, stroke, and aortic aneurysm. *Arch Environ Health.* 1969;19:167-182.

105. MacSweeney ST, Ellis M, Worrell PC, Greenhalgh RM, Powell JT. Smoking and growth rate of small abdominal aortic aneurysms. *Lancet.* 1994;344:651-652.

106. Madaric J, Vulev I, Bartunek J, Mistrik A, Verhamme K, De Bruyne B, *et. al.* Frequency of abdominal aortic aneurysm in patients >60 years of age with coronary artery disease. *Am J Cardiol.* 2005;96:1214-1216.

107. Singh K, Bonaa KH, Jacobsen BK, Bjork L, Solberg S. Prevalence of and risk factors for abdominal aortic aneurysms in a population-based study : The Tromso Study. *Am J Epidemiol.* 2001;154:236-244.

108. Reed D, Reed C, Stemmermann G, Hayashi T. Are aortic aneurysms caused by atherosclerosis? *Circulation.* 1992;85:205-211.

109. Blanchard JF, Armenian HK, Friesen PP. Risk factors for abdominal aortic aneurysm: results of a case-control study. *Am J Epidemiol.* 2000;151:575-583.

110. Ashton HA, Buxton MJ, Day NE, Kim LG, Marteau TM, Scott RA, *et. al.* The Multicentre Aneurysm Screening Study (MASS) into the effect of abdominal aortic aneurysm screening on mortality in men: a randomised controlled trial. *Lancet.* 2002;360:1531-1539.

111. Ernst CB. Abdominal aortic aneurysm. *N Engl J Med.* 1993;328:1167-1172.

112. Crow P, Shaw E, Earnshaw JJ, Poskitt KR, Whyman MR, Heather BP. A single normal ultrasonographic scan at age 65 years rules out significant aneurysm disease for life in men. *Br J Surg.* 2001;88:941-944.

113. Endovascular aneurysm repair and outcome in patients unfit for open repair of abdominal aortic aneurysm (EVAR trial 2): randomised controlled trial. *Lancet.* 2005;365:2187-2192.

114. Aune S, Amundsen SR, Evjensvold J, Trippestad A. Operative mortality and long-term relative survival of patients operated on for asymptomatic abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg.* 1995;9:293-298.

115. Koskas F, Kieffer E. Long-term survival after elective repair of infrarenal abdominal aortic aneurysm: results of a prospective multicentric study. Association for Academic Research in Vascular Surgery (AURC). *Ann Vasc Surg.* 1997;11:473-481.

116. Johnston KW. Nonruptured abdominal aortic aneurysm: six-year follow-up results from the multicenter prospective Canadian aneurysm study. Canadian Society for Vascular Surgery Aneurysm Study Group. *J Vasc Surg.* 1994;20:163-170.

117. Gadowski GR, Pilcher DB, Ricci MA. Abdominal aortic aneurysm expansion rate: effect of size and beta-adrenergic blockade. *J Vasc Surg.* 1994;19:727-731.

118. Slaiby JM, Ricci MA, Gadowski GR, Hendley ED, Pilcher DB. Expansion of aortic aneurysms is reduced by propranolol in a hypertensive rat model. *J Vasc Surg.* 1994;20:178-183.

119. Brophy C, Tilson JE, Tilson MD. Propranolol delays the formation of aneurysms in the male blotchy mouse. *J Surg Res.* 1988;44:687-689.

120. Lindholt JS, Henneberg EW, Juul S, Fasting H. Impaired results of a randomised double blinded clinical trial of propranolol versus placebo on the expansion rate of small abdominal aortic aneurysms. *Int Angiol.* 1999;18:52-57.

121. Propranolol for small abdominal aortic aneurysms: results of a randomized trial. *J Vasc Surg.* 2002;35:72-79.

122. Miralles M, Wester W, Sicard GA, Thompson R, Reilly JM. Indomethacin inhibits expansion of experimental aortic aneurysms via inhibition of the cox2 isoform of cyclooxygenase. *J Vasc Surg.* 1999;29:884-892.

123. Holmes DR, Petrinec D, Wester W, Thompson RW, Reilly JM. Indomethacin prevents elastase-induced abdominal aortic aneurysms in the rat. *J Surg Res.* 1996;63:305-309.

124. Armstrong PJ, Franklin DP, Carey DJ, Elmore JR. Suppression of experimental aortic aneurysms: comparison of inducible nitric oxide synthase and cyclooxygenase inhibitors. *Ann Vasc Surg.* 2005;19:248-257.

125. Thompson RW, Baxter BT. MMP inhibition in abdominal aortic aneurysms. Rationale for a prospective randomized clinical trial. *Ann N Y Acad Sci.* 1999;878:159-178.

126. Kaito K, Urayama H, Watanabe G. Doxycycline treatment in a model of early abdominal aortic aneurysm. *Surg Today.* 2003;33:426-433.

127. Baxter BT, Pearce WH, Waltke EA, Littooy FN, Hallett JW, Jr., Kent KC, *et. al.* Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (Phase II) multicenter study. *J Vasc Surg.* 2002;36:1-12.

128. Snell RS. Chapter 5 The Abdomen: Part II - The Abdominal Cavity. In: *Clinical Anatomy.* 7th ed. Baltimore: Lippincott, Williams & Wilkins; 2003: 291-292.

129. Tortora GJ, Grabowski SR. Chapter 21. The Cardiovascular System: Blood Vessels and Haemodynamics. In: *Principles of Anatomy and Physiology*. 9th ed. New York: John Wiley & Sons, Inc.; 2000:670-671.

130. MacSweeney ST, Powell JT, Greenhalgh RM. Pathogenesis of abdominal aortic aneurysm. *Br J Surg.* 1994;81:935-941.

131. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Chapter 19. Cell junctions, cell adhesion, and the extracellular matrix. In: *Molecular Biology of the Cell.* 4th ed. New York: Garland Science; 2002:1090-1103 .

132. Jacob MP, Badier-Commander C, Fontaine V, Benazzoug Y, Feldman L, Michel JB. Extracellular matrix remodeling in the vascular wall. *Pathol Biol (Paris).* 2001;49:326-332.

133. Cattell MA, Anderson JC, Hasleton PS. Age-related changes in amounts and concentrations of collagen and elastin in normotensive human thoracic aorta. *Clin Chim Acta.* 1996;245:73-84.

134. Henderson EL, Geng YJ, Sukhova GK, Whittemore AD, Knox J, Libby P. Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms. *Circulation.* 1999;99:96-104.

135. Sumner DS, Hokanson DE, Strandness DE, Jr. Stress-strain characteristics and collagen-elastin content of abdominal aortic aneurysms. *Surg Gynecol Obstet.* 1970;130:459-466.

136. Campa JS, Greenhalgh RM, Powell JT. Elastin degradation in abdominal aortic aneurysms. *Atherosclerosis.* 1987;65:13-21.

137. Rizzo RJ, McCarthy WJ, Dixit SN, Lilly MP, Shively VP, Flinn WR, *et. al.* Collagen types and matrix protein content in human abdominal aortic aneurysms. *J Vasc Surg.* 1989;10:365-373.

138. Gandhi RH, Irizarry E, Cantor JO, Keller S, Nackman GB, Halpern VJ, *et. al.* Analysis of elastin cross-linking and the connective tissue matrix of abdominal aortic aneurysms. *Surgery.* 1994;115:617-620.

139. Rijbroek A, Moll FL, von Dijk HA, Meijer R, Jansen JW. Inflammation of the abdominal aortic aneurysm wall. *Eur J Vasc Surg.* 1994;8:41-46.

140. Carmo M, Colombo L, Bruno A, Corsi FR, Roncoroni L, Cuttin MS, *et. al.* Alteration of elastin, collagen and their cross-links in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg.* 2002;23:543-549.

141. White JV, Haas K, Phillips S, Comerota AJ. Adventitial elastolysis is a primary event in aneurysm formation. *J Vasc Surg.* 1993;17:371-380.

142. Sakalihasan N, Heyeres A, Nusgens BV, Limet R, Lapiere CM. Modifications of the extracellular matrix of aneurysmal abdominal aortas as a function of their size. *Eur J Vasc Surg.* 1993;7:633-637.

143. Dobrin PB, Baker WH, Gley WC. Elastolytic and collagenolytic studies of arteries. Implications for the mechanical properties of aneurysms. *Arch Surg.* 1984;119:405-409.

144. Dobrin PB, Mrkvicka R. Failure of elastin or collagen as possible critical connective tissue alterations underlying aneurysmal dilatation. *Cardiovasc Surg.* 1994;2:484-488.

145. Gargiulo M, Stella A, Spina M, Faggioli G, Cenacchi G, Degani A, *et. al.* Content and turnover of extracellular matrix protein in human "nonspecific" and inflammatory abdominal aortic aneurysms. *Eur J Vasc Surg.* 1993;7:546-553.

146. Menashi S, Campa JS, Greenhalgh RM, Powell JT. Collagen in abdominal aortic aneurysm: typing, content, and degradation. *J Vasc Surg.* 1987;6:578-582.

147. Bode MK, Soini Y, Melkko J, Satta J, Risteli L, Risteli J. Increased amount of type III pN-collagen in human abdominal aortic aneurysms: evidence for impaired type III collagen fibrillogenesis. *J Vasc Surg.* 2000;32:1201-1207.

148. Mesh CL, Baxter BT, Pearce WH, Chisholm RL, McGee GS, Yao JS. Collagen and elastin gene expression in aortic aneurysms. *Surgery.* 1992;112:256-261.

149. McGee GS, Baxter BT, Shively VP, Chisholm R, McCarthy WJ, Flinn WR, *et. al.* Aneurysm or occlusive disease--factors determining the clinical course of atherosclerosis of the infrarenal aorta. *Surgery.* 1991;110:370-375.

150. Leung DY, Glagov S, Mathews MB. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. *Science.* 1976;191:475-477.

151. Lopez-Candales A, Holmes DR, Liao S, Scott MJ, Wickline SA, Thompson RW. Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms. *Am J Pathol.* 1997;150:993-1007.

152. Liao S, Curci JA, Kelley BJ, Sicard GA, Thompson RW. Accelerated replicative senescence of medial smooth muscle cells derived from abdominal aortic aneurysms compared to the adjacent inferior mesenteric artery. *J Surg Res.* 2000;92:85-95.

153. Patel MI, Melrose J, Ghosh P, Appleberg M. Increased synthesis of matrix metalloproteinases by aortic smooth muscle cells is implicated in the etiopathogenesis of abdominal aortic aneurysms. *J Vasc Surg.* 1996;24:82-92.

154. Crowther M, Goodall S, Jones JL, Bell PR, Thompson MM. Increased matrix metalloproteinase 2 expression in vascular smooth muscle cells cultured from abdominal aortic aneurysms. *J Vasc Surg.* 2000;32:575-583.

155. Allaire E, Muscatelli-Groux B, Mandet C, Guinault AM, Bruneval P, Desgranges P, *et. al.* Paracrine effect of vascular smooth muscle cells in the prevention of aortic aneurysm formation. *J Vasc Surg.* 2002;36:1018-1026.

156. Koch AE, Haines GK, Rizzo RJ, Radosevich JA, Pope RM, Robinson PG, *et. al.* Human abdominal aortic aneurysms. Immunophenotypic analysis suggesting an immune-mediated response. *Am J Pathol.* 1990;137:1199-1213.

157. Bobryshev YV, Lord RS. Vascular-associated lymphoid tissue (VALT) involvement in aortic aneurysm. *Atherosclerosis.* 2001;154:15-21.

158. Cohen JR, Keegan L, Sarfati I, Danna D, Ilardi C, Wise L. Neutrophil chemotaxis and neutrophil elastase in the aortic wall in patients with abdominal aortic aneurysms. *J Invest Surg.* 1991;4:423-430.

159. Bobryshev YV, Lord RS, Parsson H. Immunophenotypic analysis of the aortic aneurysm wall suggests that vascular dendritic cells are involved in immune responses. *Cardiovasc Surg.* 1998;6:240-249.

160. Ocana E, Bohorquez JC, Perez-Requena J, Brieva JA, Rodriguez C. Characterisation of T and B lymphocytes infiltrating abdominal aortic aneurysms. *Atherosclerosis.* 2003;170:39-48.

161. Galle C, Schandene L, Stordeur P, Peignois Y, Ferreira J, Wautrecht JC, *et. al.* Predominance of type 1 CD4+ T cells in human abdominal aortic aneurysm. *Clin Exp Immunol.* 2005;142:519-527.

162. Gregory AK, Yin NX, Capella J, Xia S, Newman KM, Tilson MD. Features of autoimmunity in the abdominal aortic aneurysm. *Arch Surg.* 1996;131:85-88.

163. Halme S, Juvonen T, Laurila A, Juvonen J, Mosorin M, Saikku P, *et. al.* Chlamydia pneumoniae reactive T lymphocytes in the walls of abdominal aortic aneurysms. *Eur J Clin Invest.* 1999;29:546-552.

164. Xiong W, Zhao Y, Prall A, Greiner TC, Baxter BT. Key roles of CD4+ T cells and IFN-gamma in the development of abdominal aortic aneurysms in a murine model. *J Immunol.* 2004;172:2607-2612.

165. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, *et. al.* Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. *Circulation.* 2005;112:232-240.

166. Ailawadi G, Eliason JL, Upchurch GR, Jr. Current concepts in the pathogenesis of abdominal aortic aneurysm. *J Vasc Surg.* 2003;38:584-588.

167. Cawston T. Matrix metalloproteinases and TIMPs: properties and implications for the rheumatic diseases. *Mol Med Today.* 1998;4:130-137.

168. Powell WC, Matrisian LM. Complex roles of matrix metalloproteinases in tumor progression. *Curr Top Microbiol Immunol.* 1996;213:1-21.

169. Cockett MI, Murphy G, Birch ML, O'Connell JP, Crabbe T, Millican AT, *et. al.* Matrix metalloproteinases and metastatic cancer. *Biochem Soc Symp.* 1998;63:295-313.

170. Carrell TW, Burnand KG, Wells GM, Clements JM, Smith A. Stromelysin-1 (matrix metalloproteinase-3) and tissue inhibitor of metalloproteinase-3 are overexpressed in the wall of abdominal aortic aneurysms. *Circulation.* 2002;105:477-482.

171. Annabi B, Shedid D, Ghosn P, Kenigsberg RL, Desrosiers RR, Bojanowski MW, *et. al.* Differential regulation of matrix metalloproteinase activities in abdominal aortic aneurysms. *J Vasc Surg.* 2002;35:539-546.

172. Curci JA, Liao S, Huffman MD, Shapiro SD, Thompson RW. Expression and localization of macrophage elastase (matrix metalloproteinase-12) in abdominal aortic aneurysms. *J Clin Invest.* 1998;102:1900-1910.

173. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest.* 2002;110:625-632.

174. Petersen E, Wagberg F, Angquist KA. Proteolysis of the abdominal aortic aneurysm wall and the association with rupture. *Eur J Vasc Endovasc Surg.* 2002;23:153-157.

175. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT. Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol.* 1995;15:1145-1151.

176. Pyo R, Lee JK, Shipley JM, Curci JA, Mao D, Ziporin SJ, *et. al.* Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J Clin Invest.* 2000;105:1641-1649.

177. Sakalihasan N, Delvenne P, Nusgens BV, Limet R, Lapiere CM. Activated forms of MMP2 and MMP9 in abdominal aortic aneurysms. *J Vasc Surg.* 1996;24:127-133.

178. Elmore JR, Keister BF, Franklin DP, Youkey JR, Carey DJ. Expression of matrix metalloproteinases and TIMPs in human abdominal aortic aneurysms. *Ann Vasc Surg.* 1998;12:221-228.

179. Eskandari MK, Vijungco JD, Flores A, Borensztajn J, Shively V, Pearce WH. Enhanced abdominal aortic aneurysm in TIMP-1-deficient mice. *J Surg Res.* 2005;123:289-293.

180. Alexander JJ. The pathobiology of aortic aneurysms. *J Surg Res.* 2004;117:163-175.

181. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, *et. al.* Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet.* 1997;17:439-444.

182. Allaire E, Hasenstab D, Kenagy RD, Starcher B, Clowes MM, Clowes AW. Prevention of aneurysm development and rupture by local overexpression of plasminogen activator inhibitor-1. *Circulation.* 1998;98:249-255.

183. Louwrens HD, Kwaan HC, Pearce WH, Yao JS, Verrusio E. Plasminogen activator and plasminogen activator inhibitor expression by normal and aneurysmal human aortic smooth muscle cells in culture. *Eur J Vasc Endovasc Surg.* 1995;10:289-293.

184. Saito S, Zempo N, Yamashita A, Takenaka H, Fujioka K, Esato K. Matrix metalloproteinase expressions in arteriosclerotic aneurysmal disease. *Vasc Endovascular Surg.* 2002;36:1-7.

185. Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P. Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. *J Clin Invest.* 1998;102:576-583.

186. Eriksson P, Jones KG, Brown LC, Greenhalgh RM, Hamsten A, Powell JT. Genetic approach to the role of cysteine proteases in the expansion of abdominal aortic aneurysms. *Br J Surg.* 2004;91:86-89.

187. Tung WS, Lee JK, Thompson RW. Simultaneous analysis of 1176 gene products in normal human aorta and abdominal aortic aneurysms using a membrane-based complementary DNA expression array. *J Vasc Surg.* 2001;34:143-150.

188. Gacko M, Glowinski S. Cathepsin D and cathepsin L activities in aortic aneurysm wall and parietal thrombus. *Clin Chem Lab Med.* 1998;36:449-452.

189. Sukhova GK, Wang B, Libby P, Pan JH, Zhang Y, Grubb A, *et. al.* Cystatin C deficiency increases elastic lamina degradation and aortic dilatation in apolipoprotein E-null mice. *Circ Res.* 2005;96:368-375.

190. Lindholt JS, Erlandsen EJ, Henneberg EW. Cystatin C deficiency is associated with the progression of small abdominal aortic aneurysms. *Br J Surg.* 2001;88:1472-1475.

191. Hance KA, Tataria M, Ziporin SJ, Lee JK, Thompson RW. Monocyte chemotactic activity in human abdominal aortic aneurysms: role of elastin degradation peptides and the 67-kD cell surface elastin receptor. *J Vasc Surg.* 2002;35:254-261.

192. Haug ES, Skomsvoll JF, Jacobsen G, Halvorsen TB, Saether OD, Myhre HO. Inflammatory aortic aneurysm is associated with increased incidence of autoimmune disease. *J Vasc Surg.* 2003;38:492-497.

193. Walton LJ, Powell JT, Parums DV. Unrestricted usage of immunoglobulin heavy chain genes in B cells infiltrating the wall of atherosclerotic abdominal aortic aneurysms. *Atherosclerosis.* 1997;135:65-71.

194. Yen HC, Lee FY, Chau LY. Analysis of the T cell receptor V beta repertoire in human aortic aneurysms. *Atherosclerosis.* 1997;135:29-36.

195. Lindholt JS, Ostergard L, Henneberg EW, Fasting H, Andersen P. Failure to demonstrate Chlamydia pneumoniae in symptomatic abdominal aortic aneurysms by a nested polymerase chain reaction (PCR). *Eur J Vasc Endovasc Surg.* 1998;15:161-164.

196. Sodeck G, Domanovits H, Khanakah G, Schillinger M, Thalmann M, Bayegan K, *et. al.* The role of Chlamydia pneumoniae in human aortic disease-a hypothesis revisited. *Eur J Vasc Endovasc Surg.* 2004;28:547-552.

197. Petersen E, Boman J, Wagberg F, Angquist KA. Presence of Chlamydia pneumoniae in abdominal aortic aneurysms is not associated with increased activity of matrix metalloproteinases. *Eur J Vasc Endovasc Surg.* 2002;24:365-369.

198. Anidjar S, Dobrin PB, Eichorst M, Graham GP, Chejfec G. Correlation of inflammatory infiltrate with the enlargement of experimental aortic aneurysms. *J Vasc Surg.* 1992;16:139-147.

199. Keen RR, Nolan KD, Cipollone M, Scott E, Shively VP, Yao JS, *et. al.* Interleukin-1 beta induces differential gene expression in aortic smooth muscle cells. *J Vasc Surg.* 1994;20:774-784.

200. Newman KM, Jean-Claude J, Li H, Ramey WG, Tilson MD. Cytokines that activate proteolysis are increased in abdominal aortic aneurysms. *Circulation.* 1994;90:II224-227.

201. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J Clin Invest.* 1995;96:2304-2310.

202. Miller FJ, Jr., Sharp WJ, Fang X, Oberley LW, Oberley TD, Weintraub NL. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurysmal remodeling. *Arterioscler Thromb Vasc Biol.* 2002;22:560-565.

203. Yajima N, Masuda M, Miyazaki M, Nakajima N, Chien S, Shyy JY. Oxidative stress is involved in the development of experimental abdominal aortic aneurysm: a study of the transcription profile with complementary DNA microarray. *J Vasc Surg.* 2002;36:379-385.

204. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. *J Clin Invest.* 1996;98:2572-2579.

205. Peppin GJ, Weiss SJ. Activation of the endogenous metalloproteinase, gelatinase, by triggered human neutrophils. *Proc Natl Acad Sci U S A.* 1986;83:4322-4326.

206. Li PF, Dietz R, von Harsdorf R. Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *FEBS Lett.* 1997;404:249-252.

207. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* 2000;105:1605-1612.

208. Wang YX, Martin-McNulty B, Freay AD, Sukovich DA, Halks-Miller M, Li WW, *et. al.* Angiotensin II increases urokinase-type plasminogen activator expression and induces aneurysm in the abdominal aorta of apolipoprotein E-deficient mice. *Am J Pathol.* 2001;159:1455-1464.

209. Liao S, Miralles M, Kelley BJ, Curci JA, Borhani M, Thompson RW. Suppression of experimental abdominal aortic aneurysms in the rat by treatment with angiotensin-converting enzyme inhibitors. *J Vasc Surg.* 2001;33:1057-1064.

210. van Keulen CJ, van de Akker E, Pals G, Rauwerda JA. The role of type III collagen in the development of familial abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg.* 1999;18:65-70.

211. Powell J, Greenhalgh RM. Cellular, enzymatic, and genetic factors in the pathogenesis of abdominal aortic aneurysms. *J Vasc Surg.* 1989;9:297-304.

212. Kontusaari S, Tromp G, Kuivaniemi H, Romanic AM, Prockop DJ. A mutation in the gene for type III procollagen (COL3A1) in a family with aortic aneurysms. *J Clin Invest.* 1990;86:1465-1473.

213. Tromp G, Wu Y, Prockop DJ, Madhatheri SL, Kleinert C, Earley JJ, *et. al.* Sequencing of cDNA from 50 unrelated patients reveals that mutations in the triple-helical domain of type III procollagen are an infrequent cause of aortic aneurysms. *J Clin Invest.* 1993;91:2539-2545.

214. Anderson DW, Edwards TK, Ricketts MH, Kuivaniemi H, Tromp G, Stolle CA, *et. al.* Multiple defects in type III collagen synthesis are associated with the pathogenesis of abdominal aortic aneurysms. *Ann N Y Acad Sci.* 1996;800:216-228.

215. Liu X, Wu H, Byrne M, Krane S, Jaenisch R. Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A.* 1997;94:1852-1856.

216. Pereira L, Andrikopoulos K, Tian J, Lee SY, Keene DR, Ono R, *et. al.* Targetting of the gene encoding fibrillin-1 recapitulates the vascular aspect of Marfan syndrome. *Nat Genet.* 1997;17:218-222.

217. Francke U, Berg MA, Tynan K, Brenn T, Liu W, Aoyama T, *et. al.* A Gly1127Ser mutation in an EGF-like domain of the fibrillin-1 gene is a risk factor for ascending aortic aneurysm and dissection. *Am J Hum Genet.* 1995;56:1287-1296.

218. Eriksson P, Jormsjo-Pettersson S, Brady AR, Deguchi H, Hamsten A, Powell JT. Genotype-phenotype relationships in an investigation of the role of proteases in abdominal aortic aneurysm expansion. *Br J Surg.* 2005;92:1372-1376.

219. Hinterseher I, Bergert H, Kuhlisch E, Bloomenthal A, Pilarsky C, Ockert D, *et. al.* Matrix Metalloproteinase 2 Polymorphisms in a Caucasian Population with Abdominal Aortic Aneurysm. *J Surg Res.* 2006.

220. Jones GT, Phillips VL, Harris EL, Rossaak JI, van Rij AM. Functional matrix metalloproteinase-9 polymorphism (C-1562T) associated with abdominal aortic aneurysm. *J Vasc Surg.* 2003;38:1363-1367.

221. Wang X, Tromp G, Cole CW, Verloes A, Sakalihasan N, Yoon S, *et. al.* Analysis of coding sequences for tissue inhibitor of metalloproteinases 1 (TIMP1) and 2 (TIMP2) in patients with aneurysms. *Matrix Biol.* 1999;18:121-124.

222. Tilson MD, Reilly JM, Brophy CM, Webster EL, Barnett TR. Expression and sequence of the gene for tissue inhibitor of metalloproteinases in patients with abdominal aortic aneurysms. *J Vasc Surg.* 1993;18:266-270.

223. Ogata T, Shibamura H, Tromp G, Sinha M, Goddard KA, Sakalihasan N, *et. al.* Genetic analysis of polymorphisms in biologically relevant candidate genes in patients with abdominal aortic aneurysms. *J Vasc Surg.* 2005;41:1036-1042.

224. Rossaak JI, Van Rij AM, Jones GT, Harris EL. Association of the 4G/5G polymorphism in the promoter region of plasminogen activator inhibitor-1 with abdominal aortic aneurysms. *J Vasc Surg.* 2000;31:1026-1032.

225. Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci U S A.* 1995;92:1851-1855.

226. Jones K, Powell J, Brown L, Greenhalgh R, Jormsjo S, Eriksson P. The influence of 4G/5G polymorphism in the plasminogen activator inhibitor-1 gene promoter on the incidence, growth and operative risk of abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg.* 2002;23:421-425.

227. Jones KG, Brull DJ, Brown LC, Sian M, Greenhalgh RM, Humphries SE, *et. al.* Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms. *Circulation.* 2001;103:2260-2265.

228. Bown MJ, Burton PR, Horsburgh T, Nicholson ML, Bell PR, Sayers RD. The role of cytokine gene polymorphisms in the pathogenesis of abdominal aortic aneurysms: a case-control study. *J Vasc Surg.* 2003;37:999-1005.

229. Tilson MD, Ozsvath KJ, Hirose H, Xia S. A genetic basis for autoimmune manifestations in the abdominal aortic aneurysm resides in the MHC class II locus DR-beta-1. *Ann N Y Acad Sci.* 1996;800:208-215.

230. Rasmussen TE, Hallett JW, Jr., Schulte S, Harmsen WS, O'Fallon WM, Weyand CM. Genetic similarity in inflammatory and degenerative abdominal aortic aneurysms: a study of human leukocyte antigen class II disease risk genes. *J Vasc Surg.* 2001;34:84-89.

231. Hirose H, Tilson MD. Negative genetic risk factor for abdominal aortic aneurysm: HLA-DQ3, a Japanese study. *J Vasc Surg.* 1999;30:959-960.

232. Hirose H, Takagi M, Miyagawa N, Hashiyada H, Noguchi M, Tada S, *et. al.* Genetic risk factor for abdominal aortic aneurysm: HLA-DR2(15), a Japanese study. *J Vasc Surg.* 1998;27:500-503.

233. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, *et. al.* Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation.* 1995;92:1387-1388.

234. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-1346.

235. Fatini C, Pratesi G, Sofi F, Gensini F, Sticchi E, Lari B, *et. al.* ACE DD genotype: a predisposing factor for abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg.* 2005;29:227-232.

236. Pola R, Gaetani E, Santoliquido A, Gerardino L, Cattani P, Serricchio M, *et. al.* Abdominal aortic aneurysm in normotensive patients: association with angiotensin-converting enzyme gene polymorphism. *Eur J Vasc Endovasc Surg.* 2001;21:445-449.

237. Yeung JM, Heeley M, Gray S, Lingam MK, Manning G, Nash JR, *et. al.* Does the angiotensin-converting enzyme (ACE) gene polymorphism affect rate of abdominal aortic aneurysm expansion? *Eur J Vasc Endovasc Surg.* 2002;24:69-71.

238. Hamano K, Ohishi M, Ueda M, Fujioka K, Katoh T, Zempo N, *et. al.* Deletion polymorphism in the gene for angiotensin-converting enzyme is not a risk factor predisposing to abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg.* 1999;18:158-161.

239. Sho E, Sho M, Nanjo H, Kawamura K, Masuda H, Dalman RL. Hemodynamic regulation of CD34+ cell localization and differentiation in experimental aneurysms. *Arterioscler Thromb Vasc Biol.* 2004;24:1916-1921.

240. De Keulenaer GW, Chappell DC, Ishizaka N, Nerem RM, Alexander RW, Griendling KK. Oscillatory and steady laminar shear stress differentially affect human endothelial redox state: role of a superoxide-producing NADH oxidase. *Circ Res.* 1998;82:1094-1101.

241. Masuda H, Zhuang YJ, Singh TM, Kawamura K, Murakami M, Zarins CK, *et. al.* Adaptive remodeling of internal elastic lamina and endothelial lining during flow-induced arterial enlargement. *Arterioscler Thromb Vasc Biol.* 1999;19:2298-2307.

242. Hoshina K, Sho E, Sho M, Nakahashi TK, Dalman RL. Wall shear stress and strain modulate experimental aneurysm cellularity. *J Vasc Surg.* 2003;37:1067-1074.

243. Sho E, Sho M, Hoshina K, Kimura H, Nakahashi TK, Dalman RL. Hemodynamic forces regulate mural macrophage infiltration in experimental aortic aneurysms. *Exp Mol Pathol.* 2004;76:108-116.

244. Vorp DA, Wang DH, Webster MW, Federspiel WJ. Effect of intraluminal thrombus thickness and bulge diameter on the oxygen diffusion in abdominal aortic aneurysm. *J Biomech Eng.* 1998;120:579-583.

245. Benjamin HB, Becker AB. Etiologic incidence of thoracic and abdominal aneurysms. *Surg Gynecol Obstet.* 1967;125:1307-1310.

246. Heistad DD, Marcus ML, Larsen GE, Armstrong ML. Role of vasa vasorum in nourishment of the aortic wall. *Am J Physiol.* 1981;240:781-787.

247. Vorp DA, Lee PC, Wang DH, Makaroun MS, Nemoto EM, Ogawa S, *et. al.* Association of intraluminal thrombus in abdominal aortic aneurysm with local hypoxia and wall weakening. *J Vasc Surg.* 2001;34:291-299.

248. Fillinger MF, Raghavan ML, Marra SP, Cronenwett JL, Kennedy FE. In vivo analysis of mechanical wall stress and abdominal aortic aneurysm rupture risk. *J Vasc Surg.* 2002;36:589-597.

249. Callard RE, Gearing AJH. *The Cytokine FactsBook.* London: Academic Press Ltd; 1994.

250. Sporn MB, Roberts AB. Autocrine, paracrine and endocrine mechanisms of growth control. *Cancer Surv.* 1985;4:627-632.

251. Ozaki K, Leonard WJ. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem.* 2002;277:29355-29358.

252. Gouwy M, Struyf S, Proost P, Van Damme J. Synergy in cytokine and chemokine networks amplifies the inflammatory response. *Cytokine Growth Factor Rev.* 2005;16:561-580.

253. Goldsby RA, Kindt TJ, Osborne BA. *Kuby Immunology.* 4th ed. New York: W. H. Freeman and Company; 2000.

254. The Cytokines Web. 3-D Structures of Cytokines, Evolutionary and Topological Relations. Available at: *http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/cyt_strucs/index.html.* Accessed 22nd March 2006.

255. Gillis S, Mizel SB. T-Cell lymphoma model for the analysis of interleukin 1-mediated T-cell activation. *Proc Natl Acad Sci U S A.* 1981;78:1133-1137.

256. Xie J, Gallagher G. The ability of transforming growth factor-beta 1 to preferentially inhibit the induction of cytotoxicity in human T cells is determined by the nature of the activating signals. *Anticancer Res.* 1994;14:1595-1598.

257. Boom WH, Liano D, Abbas AK. Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin 4- and interleukin 2-producing T cell clones on resting B lymphocytes. *J Exp Med.* 1988;167:1350-1363.

258. Banchereau J, Briere F, Liu YJ, Rousset F. Molecular control of B lymphocyte growth and differentiation. *Stem Cells.* 1994;12:278-288.

259. Chen-Kiang S. Regulation of terminal differentiation of human B-cells by IL-6. *Curr Top Microbiol Immunol.* 1995;194:189-198.

260. Nathan CF, Murray HW, Wiebe ME, Rubin BY. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med.* 1983;158:670-689.

261. Cao H, Wolff RG, Meltzer MS, Crawford RM. Differential regulation of class II MHC determinants on macrophages by IFN-gamma and IL-4. *J Immunol.* 1989;143:3524-3531.

262. Oswald IP, Wynn TA, Sher A, James SL. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. *Proc Natl Acad Sci U S A.* 1992;89:8676-8680.

263. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med.* 1991;174:1209-1220.

264. Doherty TM, Kastelein R, Menon S, Andrade S, Coffman RL. Modulation of murine macrophage function by IL-13. *J Immunol.* 1993;151:7151-7160.

265. Standiford TJ, Kunkel SL, Kasahara K, Milia MJ, Rolfe MW, Strieter RM. Interleukin-8 gene expression from human alveolar macrophages: the role of adherence. *Am J Respir Cell Mol Biol.* 1991;5:579-585.

266. Tabbara IA, Robinson BE. Hematopoietic growth factors. *Anticancer Res.* 1991;11:81-90.

267. Carding SR, West J, Woods A, Bottomly K. Differential activation of cytokine genes in normal CD4-bearing T cells is stimulus dependent. *Eur J Immunol.* 1989;19:231-238.

268. Street NE, Mosmann TR. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J.* 1991;5:171-177.

269. Sieling PA, Wang XH, Gately MK, Oliveros JL, McHugh T, Barnes PF, *et. al.* IL-12 regulates T helper type 1 cytokine responses in human infectious disease. *J Immunol.* 1994;153:3639-3647.

270. Oriss TB, McCarthy SA, Morel BF, Campana MA, Morel PA. Crossregulation between T helper cell (Th)1 and Th2: inhibition of Th2 proliferation by IFN-gamma involves interference with IL-1. *J Immunol.* 1997;158:3666-3672.

271. Kasama T, Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy.* 2005;4:273-279.

272. Szekanecz Z, Shah MR, Pearce WH, Koch AE. Intercellular adhesion molecule-1 (ICAM-1) expression and soluble ICAM-1 (sICAM-1) production by cytokine-activated human aortic endothelial cells: a possible role for ICAM-1 and sICAM-1 in atherosclerotic aortic aneurysms. *Clin Exp Immunol.* 1994;98:337-343.

273. Faccioli LH, Souza GE, Cunha FQ, Poole S, Ferreira SH. Recombinant interleukin-1 and tumor necrosis factor induce neutrophil migration "in vivo" by indirect mechanisms. *Agents Actions.* 1990;30:344-349.

274. Fujiwara N, Kobayashi K. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy.* 2005;4:281-286.

275. Fontana A, Constam DB, Frei K, Malipiero U, Pfister HW. Modulation of the immune response by transforming growth factor beta. *Int Arch Allergy Immunol.* 1992;99:1-7.

276. Shah M, Revis D, Herrick S, Baillie R, Thorgeirson S, Ferguson M, *et. al.* Role of elevated plasma transforming growth factor-beta1 levels in wound healing. *Am J Pathol.* 1999;154:1115-1124.

277. Theiss AL, Simmons JG, Jobin C, Lund PK. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. *J Biol Chem.* 2005;280:36099-36109.

278. Denis M, Bisson D. Antigen-induced alveolitis: cytokine production in a mouse model. *Inflammation.* 1995;19:157-177.

279. Kiefer R, Kieseier BC, Stoll G, Hartung HP. The role of macrophages in immune-mediated damage to the peripheral nervous system. *Prog Neurobiol.* 2001;64:109-127.

280. Kirkiles-Smith NC, Tereb DA, Kim RW, McNiff JM, Schechner JS, Lorber MI, *et. al.* Human TNF can induce nonspecific inflammatory and human immune-mediated microvascular injury of pig skin xenografts in immunodeficient mouse hosts. *J Immunol.* 2000;164:6601-6609.

281. Johnson DR, Pober JS. HLA class I heavy-chain gene promoter elements mediating synergy between tumor necrosis factor and interferons. *Mol Cell Biol.* 1994;14:1322-1332.

282. Ernestam S, Af Klint E, Catrina AI, Sundberg E, Engstrom M, Klareskog L, *et. al.* Synovial expression of IL-15 in rheumatoid arthritis is not influenced by blockade of tumour necrosis factor. *Arthritis Res Ther.* 2005;8:R18.

283. Joosten LA, Netea MG, Kim SH, Yoon DY, Oppers-Walgreen B, Radstake TR, *et. al.* IL-32, a proinflammatory cytokine in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* 2006;103:3298-3303.

284. Santiago B, Baleux F, Palao G, Gutierrez-Canas I, Ramirez JC, Arenzana-Seisdedos F, *et. al.* CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans. *Arthritis Res Ther.* 2006;8:R43.

285. Danese S, Sans M, Scaldaferri F, Sgambato A, Rutella S, Cittadini A, *et. al.* TNF-alpha blockade down-regulates the CD40/CD40L pathway in the mucosal microcirculation: a novel anti-inflammatory mechanism of infliximab in Crohn's disease. *J Immunol.* 2006;176:2617-2624.

286. Chinen T, Kobayashi T, Ogata H, Takaesu G, Takaki H, Hashimoto M, *et. al.* Suppressor of cytokine signaling-1 regulates inflammatory bowel disease in which both IFNgamma and IL-4 are involved. *Gastroenterology.* 2006;130:373-388.

287. Kitamura K, Nakamoto Y, Kaneko S, Mukaida N. Pivotal roles of interleukin-6 in transmural inflammation in murine T cell transfer colitis. *J Leukoc Biol.* 2004;76:1111-1117.

288. Kiener PA, Marek F, Rodgers G, Lin PF, Warr G, Desiderio J. Induction of tumor necrosis factor, IFN-gamma, and acute lethality in mice by toxic and non-toxic forms of lipid A. *J Immunol.* 1988;141:870-874.

289. Evans GF, Snyder YM, Butler LD, Zuckerman SH. Differential expression of interleukin-1 and tumor necrosis factor in murine septic shock models. *Circ Shock.* 1989;29:279-290.

290. Wang Y, Yang J, Gao Y, Du Y, Bao L, Niu W, *et. al.* Regulatory effect of e2, IL-6 and IL-8 on the growth of epithelial ovarian cancer cells. *Cell Mol Immunol.* 2005;2:365-372.

291. Szlosarek PW, Grimshaw MJ, Kulbe H, Wilson JL, Wilbanks GD, Burke F, *et. al.* Expression and regulation of tumor necrosis factor alpha in normal and malignant ovarian epithelium. *Mol Cancer Ther.* 2006;5:382-390.

292. Haura EB, Livingston S, Coppola D. Autocrine interleukin-6/interleukin-6 receptor stimulation in non-small-cell lung cancer. *Clin Lung Cancer.* 2006;7:273-275.

293. Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, *et. al.* Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis.* 2005;180:11-17.

294. Hulthe J, McPheat W, Samnegard A, Tornvall P, Hamsten A, Eriksson P. Plasma interleukin (IL)-18 concentrations is elevated in patients with previous myocardial infarction and related to severity of coronary atherosclerosis independently of C-reactive protein and IL-6. *Atherosclerosis.* 2006; 188:450-454.

295. Schieffer B, Selle T, Hilfiker A, Hilfiker-Kleiner D, Grote K, Tietge UJ, *et. al.* Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation.* 2004;110:3493-3500.

296. Pearce WH, Sweis I, Yao JS, McCarthy WJ, Koch AE. Interleukin-1 beta and tumor necrosis factor-alpha release in normal and diseased human infrarenal aortas. *J Vasc Surg.* 1992;16:784-789.

297. Satoh H, Nakamura M, Satoh M, Nakajima T, Izumoto H, Maesawa C, *et. al.* Expression and localization of tumour necrosis factor-alpha and its converting enzyme in human abdominal aortic aneurysm. *Clin Sci (Lond).* 2004;106:301-306.

298. Reilly JM, Miralles M, Wester WN, Sicard GA. Differential expression of prostaglandin E2 and interleukin-6 in occlusive and aneurysmal aortic disease. *Surgery.* 1999;126:624-627.

299. Szekanecz Z, Shah MR, Pearce WH, Koch AE. Human atherosclerotic abdominal aortic aneurysms produce interleukin (IL)-6 and interferon-gamma but not IL-2 and IL-4: the possible role for IL-6 and interferon-gamma in vascular inflammation. *Agents Actions.* 1994;42:159-162.

300. Juvonen J, Surcel HM, Satta J, Teppo AM, Bloigu A, Syrjala H, *et. al.* Elevated circulating levels of inflammatory cytokines in patients with abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol.* 1997;17:2843-2847.

301. Chan WL, Pejnovic N, Liew TV, Hamilton H. Predominance of Th2 response in human abdominal aortic aneurysm: mistaken identity for IL-4-producing NK and NKT cells? *Cell Immunol.* 2005;233:109-114.

302. Schonbeck U, Sukhova GK, Gerdes N, Libby P. T(H)2 predominant immune responses prevail in human abdominal aortic aneurysm. *Am J Pathol.* 2002;161:499-506.

303. Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest.* 1992;90:1479-1485.

304. Lacraz S, Nicod L, Galve-de Rochemonteix B, Baumberger C, Dayer JM, Welgus HG. Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. *J Clin Invest.* 1992;90:382-388.

305. Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med.* 1991;174:1549-1555.

306. Shimizu K, Shichiri M, Libby P, Lee RT, Mitchell RN. Th2-predominant inflammation and blockade of IFN-gamma signaling induce aneurysms in allografted aortas. *J Clin Invest.* 2004;114:300-308.

307. Damme JV, Mantovani A. From cytokines to chemokines. *Cytokine Growth Factor Rev.* 2005;16:549-551.

308. Van den Steen PE, Proost P, Wuyts A, Van Damme J, Opdenakker G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood.* 2000;96:2673-2681.

309. Male D, Cooke A, Owen M, Trowsdale J, Champion B. Cell traffic and inflammation. In: *Advanced Immunology.* 3rd ed. London: Mosby; 1996:14.11-13.

310. Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, *et. al.* Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms. *Am J Pathol.* 1993;142:1423-1431.

311. Treska V, Topolcan O, Pecen L. Cytokines as plasma markers of abdominal aortic aneurysm. *Clin Chem Lab Med.* 2000;38:1161-1164.

312. Colonnello JS, Hance KA, Shames ML, Wyble CW, Ziporin SJ, Leidenfrost JE, *et. al.* Transient exposure to elastase induces mouse aortic wall smooth muscle cell

production of MCP-1 and RANTES during development of experimental aortic aneurysm. *J Vasc Surg.* 2003;38:138-146.

313. Ghilardi G, Biondi ML, Battaglioli L, Zambon A, Guagnellini E, Scorza R. Genetic risk factor characterizes abdominal aortic aneurysm from arterial occlusive disease in human beings: CCR5 Delta 32 deletion. *J Vasc Surg.* 2004;40:995-1000.
314. Schecter AD, Calderon TM, Berman AB, McManus CM, Fallon JT, Rossikhina M, *et. al.* Human vascular smooth muscle cells possess functional CCR5. *J Biol Chem.* 2000;275:5466-5471.
315. Thompson MM, Jones L, Nasim A, Sayers RD, Bell PR. Angiogenesis in abdominal aortic aneurysms. *Eur J Vasc Endovasc Surg.* 1996;11:464-469.
316. Paik DC, Fu C, Bhattacharya J, Tilson MD. Ongoing angiogenesis in blood vessels of the abdominal aortic aneurysm. *Exp Mol Med.* 2004;36:524-533.
317. Kobayashi M, Matsubara J, Matsushita M, Nishikimi N, Sakurai T, Nimura Y. Expression of angiogenesis and angiogenic factors in human aortic vascular disease. *J Surg Res.* 2002;106:239-245.
318. Fukui D, Miyagawa S, Soeda J, Tanaka K, Urayama H, Kawasaki S. Overexpression of transforming growth factor beta1 in smooth muscle cells of human abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg.* 2003;25:540-545.
319. Bobik A, Agrotis A, Kanellakis P, Dilley R, Krushinsky A, Smirnov V, *et. al.* Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. *Circulation.* 1999;99:2883-2891.
320. Losy F, Dai J, Pages C, Ginat M, Muscatelli-Groux B, Guinault AM, *et. al.* Paracrine secretion of transforming growth factor-beta1 in aneurysm healing and stabilization with endovascular smooth muscle cell therapy. *J Vasc Surg.* 2003;37:1301-1309.
321. Dai J, Losy F, Guinault AM, Pages C, Anegon I, Desgranges P, *et. al.* Overexpression of transforming growth factor-beta1 stabilizes already-formed aortic aneurysms: a first approach to induction of functional healing by endovascular gene therapy. *Circulation.* 2005;112:1008-1015.
322. Hingorani A, Ascher E, Scheinman M, Yorkovich W, DePippo P, Ladoulis CT, *et. al.* The effect of tumor necrosis factor binding protein and interleukin-1 receptor antagonist on the development of abdominal aortic aneurysms in a rat model. *J Vasc Surg.* 1998;28:522-526.

323. Chakrabarti S, Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. *J Leukoc Biol.* 2005;78:279-288.

324. Kodali R, Hajjou M, Berman AB, Bansal MB, Zhang S, Pan JJ, *et. al.* Chemokines induce matrix metalloproteinase-2 through activation of epidermal growth factor receptor in arterial smooth muscle cells. *Cardiovasc Res.* 2006;69:706-715.

325. Son KN, Hwang J, Kwon BS, Kim J. Human CC chemokine CCL23 enhances expression of matrix metalloproteinase-2 and invasion of vascular endothelial cells. *Biochem Biophys Res Commun.* 2006;340:498-504.

326. Townend J. Chapter 13.6 Non-parametric Tests - Kruskal-Wallis Test. In: *Practical Statistics for Environmental and Biological Scientists*. Chichester: John Wiley & Sons Ltd; 2002:195-198.

327. Evans CH, Georgescu HI, Lin CW, Mendelow D, Steed DL, Webster MW. Inducible synthesis of collagenase and other neutral metalloproteinases by cells of aortic origin. *J Surg Res.* 1991;51:399-404.

328. Bian ZM, Elner SG, Strieter RM, Kunkel SL, Lukacs NW, Elner VM. IL-4 potentiates IL-1beta- and TNF-alpha-stimulated IL-8 and MCP-1 protein production in human retinal pigment epithelial cells. *Curr Eye Res.* 1999;18:349-357.

329. Brown Z, Gerritsen ME, Carley WW, Strieter RM, Kunkel SL, Westwick J. Chemokine gene expression and secretion by cytokine-activated human microvascular endothelial cells. Differential regulation of monocyte chemoattractant protein-1 and interleukin-8 in response to interferon-gamma. *Am J Pathol.* 1994;145:913-921.

330. Modur V, Feldhaus MJ, Weyrich AS, Jicha DL, Prescott SM, Zimmerman GA, *et. al.* Oncostatin M is a proinflammatory mediator. In vivo effects correlate with endothelial cell expression of inflammatory cytokines and adhesion molecules. *J Clin Invest.* 1997;100:158-168.

331. Ertel W, Keel M, Steckholzer U, Ungethum U, Trentz O. Interleukin-10 attenuates the release of proinflammatory cytokines but depresses splenocyte functions in murine endotoxemia. *Arch Surg.* 1996;131:51-56.

332. Tham DM, Martin-McNulty B, Wang YX, Wilson DW, Vergona R, Sullivan ME, *et. al.* Angiotensin II is associated with activation of NF-kappaB-mediated genes and downregulation of PPARs. *Physiol Genomics.* 2002;11:21-30.

333. Tilson MD, Fu C, Xia SX, Syn D, Yoon Y, McCaffrey T. Expression of molecular messages for angiogenesis by fibroblasts from aneurysmal abdominal aorta versus dermal fibroblasts. *Int J Surg Investig.* 2000;1:453-457.

334. Lugering N, Kucharzik T, Maaser C, Kraft M, Domschke W. Interleukin-15 strongly inhibits interleukin-8 and monocyte chemoattractant protein-1 production in human colonic epithelial cells. *Immunology.* 1999;98:504-509.

335. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J.* 1998;334:297-314.

336. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol.* 2003;149:1-38.

337. Kishimoto T, Hirano T. Molecular regulation of B lymphocyte response. *Annu Rev Immunol.* 1988;6:485-512.

338. Rampart M, Van Damme J, Zonnekeyn L, Herman AG. Granulocyte chemotactic protein/interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. *Am J Pathol.* 1989;135:21-25.

339. Huber AR, Kunkel SL, Todd RF, 3rd, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science.* 1991;254:99-102.

340. Smith WB, Gamble JR, Clark-Lewis I, Vadas MA. Chemotactic desensitization of neutrophils demonstrates interleukin-8 (IL-8)-dependent and IL-8-independent mechanisms of transmigration through cytokine-activated endothelium. *Immunology.* 1993;78:491-497.

341. Derevianko A, D'Amico R, Graeber T, Keeping H, Simms HH. Endogenous PMN-derived reactive oxygen intermediates provide feedback regulation on respiratory burst signal transduction. *J Leukoc Biol.* 1997;62:268-276.

342. Dewald B, Thelen M, Baggiolini M. Two transduction sequences are necessary for neutrophil activation by receptor agonists. *J Biol Chem.* 1988;263:16179-16184.

343. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, *et. al.* Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science.* 1992;258:1798-1801.

344. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med.* 1998;338:436-445.

345. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol.* 2000;18:217-242.

346. Terkeltaub R, Boisvert WA, Curtiss LK. Chemokines and atherosclerosis. *Curr Opin Lipidol.* 1998;9:397-405.

347. Van Coillie E, Van Damme J, Opdenakker G. The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev.* 1999;10:61-86.

348. Rohde LE, Arroyo LH, Rifai N, Creager MA, Libby P, Ridker PM, *et. al.* Plasma concentrations of interleukin-6 and abdominal aortic diameter among subjects without aortic dilatation. *Arterioscler Thromb Vasc Biol.* 1999;19:1695-1699.

349. Yan SF, Tritto I, Pinsky D, Liao H, Huang J, Fuller G, *et. al.* Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. *J Biol Chem.* 1995;270:11463-11471.

350. Yamagishi M, Higashikata T, Ishibashi-Ueda H, Sasaki H, Ogino H, Iihara K, *et. al.* Sustained upregulation of inflammatory chemokine and its receptor in aneurysmal and occlusive atherosclerotic disease: results form tissue analysis with cDNA macroarray and real-time reverse transcriptional polymerase chain reaction methods. *Circ J.* 2005;69:1490-1495.

351. Szekanecz Z, Shah MR, Harlow LA, Pearce WH, Koch AE. Interleukin-8 and tumor necrosis factor-alpha are involved in human aortic endothelial cell migration. The possible role of these cytokines in human aortic aneurysmal blood vessel growth. *Pathobiology.* 1994;62:134-139.

352. Manna SK, Ramesh GT. Interleukin-8 induces nuclear transcription factor-kappaB through a TRAF6-dependent pathway. *J Biol Chem.* 2005;280:7010-7021.

353. Ishibashi M, Egashira K, Zhao Q, Hiasa K, Ohtani K, Ihara Y, *et. al.* Bone marrow-derived monocyte chemoattractant protein-1 receptor CCR2 is critical in angiotensin II-induced acceleration of atherosclerosis and aneurysm formation in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol.* 2004;24:e174-178.

354. Gouwy M, Struyf S, Catusse J, Proost P, Van Damme J. Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. *J Leukoc Biol.* 2004;76:185-194.

355. Jiang Y, Beller DI, Frendl G, Graves DT. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol.* 1992;148:2423-2428.

356. Vaddi K, Newton RC. Regulation of monocyte integrin expression by beta-family chemokines. *J Immunol.* 1994;153:4721-4732.

357. Zhang QY, Hammerberg C, Baldassare JJ, Henderson PA, Burns D, Ceska M, *et. al.* Retinoic acid and phorbol ester synergistically up-regulate IL-8 expression and specifically modulate protein kinase C-epsilon in human skin fibroblasts. *J Immunol.* 1992;149:1402-1408.

358. Lisignoli G, Pozzi C, Toneguzzi S, Tomassetti M, Monaco MC, Facchini A. Different pattern of cytokine production and mRNA expression by lymphoid and non-lymphoid cells isolated from human palatine tonsil. *Int J Clin Lab Res.* 1998;28:23-28.

359. Koch AE, Kunkel SL, Burrows JC, Evanoff HL, Haines GK, Pope RM, *et. al.* Synovial tissue macrophage as a source of the chemotactic cytokine IL-8. *J Immunol.* 1991;147:2187-2195.

360. de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol.* 2000;190:619-626.

361. Wang JM, Sica A, Peri G, Walter S, Padura IM, Libby P, *et. al.* Expression of monocyte chemotactic protein and interleukin-8 by cytokine-activated human vascular smooth muscle cells. *Arterioscler Thromb.* 1991;11:1166-1174.

362. Van Damme J, Proost P, Put W, Arens S, Lenaerts JP, Conings R, *et. al.* Induction of monocyte chemotactic proteins MCP-1 and MCP-2 in human fibroblasts and leukocytes by cytokines and cytokine inducers. Chemical synthesis of MCP-2 and development of a specific RIA. *J Immunol.* 1994;152:5495-5502.

363. Colotta F, Sciacca FL, Sironi M, Luini W, Rabiet MJ, Mantovani A. Expression of monocyte chemotactic protein-1 by monocytes and endothelial cells exposed to thrombin. *Am J Pathol.* 1994;144:975-985.

364. He CM, Roach MR. The composition and mechanical properties of abdominal aortic aneurysms. *J Vasc Surg.* 1994;20:6-13.

365. Satta J, Laurila A, Paakko P, Haukipuro K, Sormunen R, Parkkila S, *et. al.* Chronic inflammation and elastin degradation in abdominal aortic aneurysm disease: an immunohistochemical and electron microscopic study. *Eur J Vasc Endovasc Surg.* 1998;15:313-319.

366. Stella A, Gargiulo M, Pasquinelli G, Preda P, Faggioli GL, Cenacchi G, *et. al.* The cellular component in the parietal infiltrate of inflammatory abdominal aortic aneurysms (IAAA). *Eur J Vasc Surg.* 1991;5:65-70.

367. Brophy CM, Reilly JM, Smith GJ, Tilson MD. The role of inflammation in nonspecific abdominal aortic aneurysm disease. *Ann Vasc Surg.* 1991;5:229-233.

368. Parums DV, Dunn DC, Dixon AK, Mitchinson MJ. Characterization of inflammatory cells in a patient with chronic periaortitis. *Am J Cardiovasc Pathol.* 1990;3:121-129.

369. Treska V, Kocova J, Boudova L, Neprasova P, Topolcan O, Pecen L, *et. al.* Inflammation in the wall of abdominal aortic aneurysm and its role in the symptomatology of aneurysm. *Cytokines Cell Mol Ther.* 2002;7:91-97.

370. Satta J, Soini Y, Mosorin M, Juvonen T. Angiogenesis is associated with mononuclear inflammatory cells in abdominal aortic aneurysms. *Ann Chir Gynaecol.* 1998;87:40-42.

371. Goodall S, Porter KE, Bell PR, Thompson MM. Enhanced invasive properties exhibited by smooth muscle cells are associated with elevated production of MMP-2 in patients with aortic aneurysms. *Eur J Vasc Endovasc Surg.* 2002;24:72-80.

372. Franklin IJ, Walton LJ, Greenhalgh RM, Powell JT. The influence of indomethacin on the metabolism and cytokine secretion of human aneurysmal aorta. *Eur J Vasc Endovasc Surg.* 1999;18:35-42.

373. Davis V, Persidskaia R, Baca-Regen L, Itoh Y, Nagase H, Persidsky Y, *et. al.* Matrix metalloproteinase-2 production and its binding to the matrix are increased in abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol.* 1998;18:1625-1633.

374. Li A, Varney ML, Valasek J, Godfrey M, Dave BJ, Singh RK. Autocrine role of interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. *Angiogenesis.* 2005;8:63-71.

375. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol.* 2003;170:3369-3376.