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

WRW Wilson, July 2006.

"Between my finger and my thumb

The squat pen rests.

I'll dig with it."

Professor Seamus Heaney

Born 1933 in Co. Londonderry, Northern Ireland.

Noble Prize for Literature, 1998.

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SYNOPSIS

The rupture of an abdominal aortic aneurysm is associated with a mortality of 60-70% and accounts for approximately 8000 deaths per year in men over 60 years of age in the United Kingdom. Aneurysm formation is clinically silent until, with increasing diameter, rupture occurs. At a cellular level, aneurysm formation is associated with an atherosclerotic or inflammatory trigger. An initial loss of elastin and smooth muscle cells from the aneurysm wall causes early aortic dilatation. Continued expansion and rupture is thought to be due to the loss of collagen mediated via either a global or local up-regulation of extracellular collagenase activity. A putative collagenase has not been identified for this process.

Matrix metalloproteinases (MMP's) represent the main physiological regulators of the extracellular matrix, and any imbalance between the level of MMP's and their inhibitors could cause increased matrix degradation. Indeed there is sound evidence to suggest that MMP-2 and –9 are intimately involved in the degradation of elastin as part of aneurysm formation.

The hypothesis of this study was that a potent collagenase, MMP-8, exists in the aneurysm wall and its expression is elevated over that found in the normal aorta. The action of this collagenase is increased further in the rupture process and is associated with an increase in inflammatory cell infiltration. The aim of this study was to quantify MMP-8 in normal aorta, to quantify other MMP's, TIMP's and inflammatory cell subtypes in abdominal aortic aneurysms and to correlate this with the clinical presentation of the aneurysm either non-ruptured or ruptured.

Work from this thesis has been published as follows:

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WRW Wilson, J Evans, PRF Bell, MM Thompson. HMG Co-A reductase inhibitors reduce metalloproteinase expression in abdominal aortic aneurysms. European Journal of Vascular and Endovascular Surgery 2005; 30(3): 259-262.

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East Midlands Surgical Meeting, April 2004.

East Midlands Surgical Meeting, May 2005.

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ABBREVIATIONS

AAA	Abdominal Aortic Aneurysm
AAA-st	Aneurysm biopsy of pre-operative statin user
AAA-nst	Aneurysm biopsy of pre-operative non-statin user
Anti-FITC	anti-Fluoro Immuno Target Complex
α-SMA	alpha-smooth muscle actin
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DPX	Diphosphoxylene
ELISA	Enzyme Linked Immunosorbent Assay
EVAR	Endovascular Aneurysm Repair
EVG	Elastin Van Geison
H&E	Hematoxylin and Eosin
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A (or Statin)
HRQL	Health Related Quality of Life
IHC	Immunohistochemistry
IMS	Industrial Methylated Spirits
ISH	*In Situ* hybridisation
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic Acid
MT1-MMP	Membrane-Bound-1 Matrix Metalloproteinase
NA	Non-aneurysmal / normal aorta
NR-AAA	Non-Ruptured Abdominal Aortic Aneurysm
PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
RAAA	Ruptured Abdominal Aortic Aneurysm
RAS	Rupture Anterior Sac Biopsy
RE	Ruptured Edge Biopsy
SMC	Smooth Muscle Cell
SSC	Standard Saline Citrate
TBS	Tris Buffer Saline
TIMP	Tissue Inhibitor of Matrix Metalloproteinase

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CHAPTER 1

INTRODUCTION

THE NORMAL AORTA

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1.1 Gross Anatomy

The aorta is the largest artery in the body and carries oxygenated blood from the left ventricle of the heart, to the body. The aorta can be sub-divided into four regions, the ascending thoracic aorta, aortic arch, descending thoracic aorta and abdominal aorta. The ascending thoracic aorta begins at the aortic root above the aortic valve. From the ascending aorta the aortic arch passes over the heart then continues posteriorly downwards as the descending thoracic aorta. The primary branches of the aortic arch are the brachiocephalic artery, the left common carotid artery and the right subclavian artery. The brachiocephalic artery divides to form the right common carotid and subclavian arteries. Smaller tributaries pass from the descending thoracic aorta as it passes through the diaphragm to become the abdominal aorta. Important branches of the abdominal aorta include the coeliac, superior mesenteric, renal, inferior mesenteric and lumbar arteries. The abdominal aorta terminates at its bifurcation to form the right and left common iliac arteries.

1.2 Histology

The aorta is constructed of three layers: the tunica intima, tunica media, and tunica adventitia (Figure 1.1). The intima is the inner most layer consisting of an endothelial cell layer, a basement membrane, a internal elastic lamina and a subendothelial smooth muscle cell layer. The tunica media consists of a collagen and elastin network with smooth muscle cells and an outer layer of elastin called the external elastic lamina. Beyond the external elastic lamina is the tunica adventitia. This outermost layer is composed principally of collagen and fibroblasts, but also contains elastin and small blood vessels called the vasa vasorum. As the tunica adventitia is too far from the aortic lumen to allow diffusion, the vasa vasorum form a vascular network of tiny vessels to sustain this layer.

The aorta's physical properties of distensibility and tension are attributed to the structural proteins, elastin and collagen. These proteins originate from the smooth muscle cell population with collagens synthesized throughout life but elastin primarily produced during gestation. Elastin forms an amorphous mesh characterized by multiple amino acid cross links rendering it relatively resistant to degradation and capable of stretching to over 50% of its original size (Mesh et al., 1992; Baxter et al., 1992). Thus elastin provides for distention and compliance against systolic blood pressure (Dobrin et al., 1994). Microfibrillar proteins make up a small proportion of the elastin component but are crucial in maintaining the integrity of the aorta (Dobrin et al., 1994).

There are three principal collagens within the aorta – types I, III and IV. Type IV collagen constitutes the basement membrane but does not impart physical strength. The fibrillar collagens types I and III, are composed of tightly wound helical amino acid chains. This formation is facilitated by the smallest amino acid - glycine, at every third position (Burton et al., 1954). In the normal abdominal aorta there are 2 to 3 times more type I than type III collagen (Menashi et al., 1987; Rizzo et al., 1989). Fibrillar collagens stretched to between 2 and 4% thus impart tensile strength to the aorta (Burton et al., 1954).

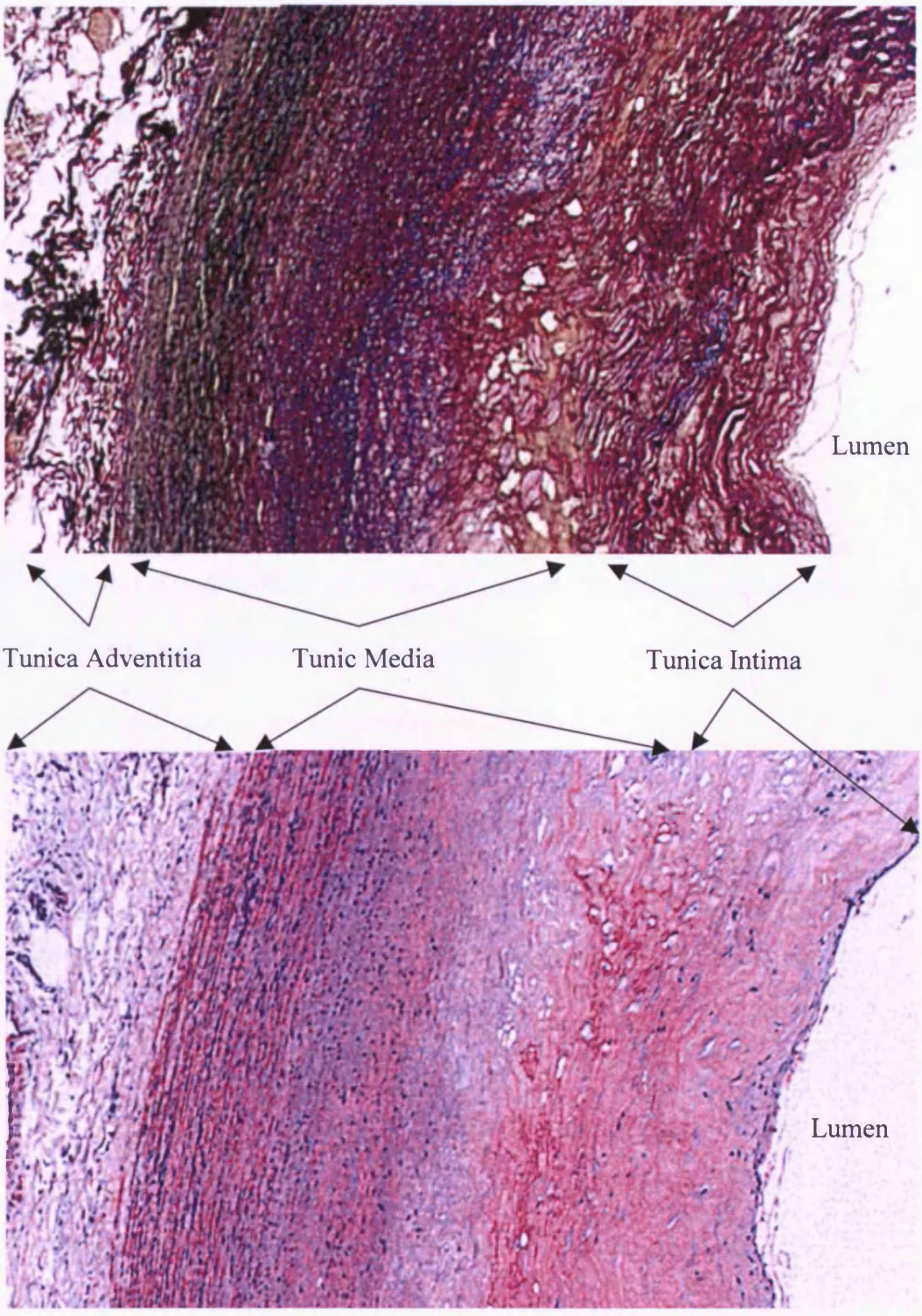

Figure 1.1

The abdominal aorta from a 68 yr old man.
Staining haematoxylin and eosin , magnification x100.

1.3 Aortic Wall Dynamics

Elastin provides the aorta with distensibility whereas collagen provides the aorta with tensile strength. Since the elastin content of the aorta decreases along its length, and the collagen content increases, the thoracic aorta is more distensible and the abdominal aorta is less distensible (Nichols et al., 1990; Clark et al., 1985). At low and medium pressures the distending force is borne by the elastin fibers. At high pressure the aorta becomes less compliant as the distensile load is borne by the collagen fibers of the tunica adventitia (Dobrin et al., 1984; Dobrin et al., 1988).

The biophysical principle expressed by the "Law of Laplace" states that wall stress is directly proportional to aortic diameter and blood pressure.

$$\sigma = (P*r)/t$$

where

σ = Circumferential wall stress

P = Intraluminal pressure

r = vessel radius

t = Thickness of wall vessel

However, it is known that the stress distribution in the aorta does not conform to the approximations of the Law of Laplace. The Law of Laplace yields only an estimate of the average stresses generated in thin wall cylinders. This equation is derived from assumptions that render it incompatible with the complex geometrical structure of the aorta.

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2.1 Epidemiology and Incidence

Current knowledge suggests the incidence of an abdominal aortic aneurysm, with an aortic diameter ≥ 3cm, is around 4 to 5% (Ashton et al., 2002; Lawrence-Brown et al., 2001; Lederle et al., 2001) and is 1-3% for abdominal aortic aneurysms of diameter ≥ 4cm (Pleumeekers et al., 1995; Scott et al., 1991; Bengtsson et al., 1993). The incidence of an abdominal aortic aneurysm in males is 4 to 6 times higher than in females (Lederle et al., 2001; Vardulaki et al., 2000). The incidence increases with age; the incidence aneurysms ≥ 4cm in men over 60 years of age is 1%, reaching a maximum of around 10% in men over 80 years (Lawrence-Brown et al., 2001; Scott et al., 1995). As well as female gender, black race is associated with a lower aneurysm incidence (Pleumeekers et al., 1995; Lederle et al., 1997a).

Progressive expansion of an aneurysm ultimately results in rupture, which can be catastrophic for the patient. The principal of aneurysm treatment is surgical repair, either open or more recently by endovascular means, excluding the aneurysm from the systemic circulation and prevent rupture. The commitment to elective surgery is not without risk. Thirty-day mortality following elective aneurysm repair ranges from 2 to 6% (Ashton et al., 2002; Bradbury et al., 1998; Jones et al., 1998; Lederle et al., 2002; UK Small Aneurysm Trial Participants, 1998). The timing of surgical intervention, defined as the point where the risk of death from rupture exceeds the risk of death from elective repair, is the critical determinant. The rational for non-intervention in asymptomatic aneurysms 5.5cm or less, has been established by two independent large randomized trials based on similar criteria - the UK Small Aneurysm Trial (UK Small Aneurysm Trial Participants 1998) and the US Aneurysm

Diameter and Management Trial (Lederle et al., 2002). The balance favouring surgical intervention in patients with larger aneurysms was clear soon after the inception of aneurysmectomy (Szilagi et al., 1966), however, the lack of advantage for the operative repair of larger aneurysms, presenting with poor physiological status, was only recently rationalised (EVAR-2 Trial Participants, 2005).

Vascular surgeons must estimate of the risk of rupture in larger aneurysms in order to recommend elective repair in the context of poor physiological status. If aneurysms likely to rupture could be accurately predicted, outcome advantage may be gained. A survey of vascular surgeons assessed the perceived risk of rupture risk based on aneurysm diameter alone. This demonstrated widely ranging opinion, with median estimates for annual rupture risk being more than twice that of published observational studies (Lederle et al., 1996). Ambiguities aside, the mortality associated with rupture of an abdominal aortic aneurysm is most certainly high. The pre-operative mortality in ruptured aneurysms is upwards of 50% (Heikkinen et al., 2002; Ingoldby et al., 1986; Semmens et al., 2000). Thirty-day mortality following operation remains between 35% and 65% (Ashton et al., 2002; Heikkinen et al., 2002; Basnyat et al., 1999). The overall mortality, both pre- and post–operative, following aneurysm rupture is dismal - 75 to 95% (Basnyatt et al., 1999; Choksy et al., 1999; Heikkinen et al., 2002; Semmens et al., 2000).

2.2 Risk Factors For Previously Unrecognised Non-Ruptured Abdominal Aortic Aneurysms

The risk factors associated with previously unrecognized non-ruptured aneurysms include, smoking, chronic obstructive airways disease, hypertension, other cardiovascular diseases, familial predisposition, but not diabetes.

Smoking and COPD

Smoking was the factor most closely associated with an abdominal aortic aneurysm by the Aneurysm Diameter And Management trial participants (Lederle et al., 1997). Smoking as a cause of cardiovascular disease was reported in multivariate analyses in the two previous case-control studies (Strachan et al., 1991; Reed et al., 1992) and in univariate analyses in several other large prospective studies (Doll et al., 1994; Hammond et al., 1969). If the association observed by Lederle et al., between smoking and aneurysms is assumed to be causal, then 75% aneurysms $\geq$ 4.0 cm discovered in the Aneurysm Diameter And Management trial could be attributed to smoking (Lederle et al., 1997). This study failed to observe an association between duration of smoking and aneurysm diameter. This could imply that smoking is more important in genesis than the growth of aneurysms, but other researchers have found continued smoking to be associated with an increased growth rate (MacSweeney et al., 1994).

Several studies observe a univariable association between abdominal aortic aneurysms and chronic obstructive pulmonary disease. It is suggested an abnormality of elastin

or elastin breakdown is the possible link (Bengtsson et al., 1991; Smith et al., 1993). Interestingly, Lederle's multivariable models indicated that the univariate association of chronic obstructive pulmonary disease with the abdominal aortic aneurysm is mediated through heavy smoking rather than through a pre-existing biochemical abnormality such as alpha-1-antitypsin deficiency (Lederle et al., 1997).

Hypertension

The association between hypertension and the abdominal aortic aneurysm is marginally significant. Most screening studies describe no univariate association between aneurysms and hypertension (Collin et al., 1990; Bengtsson et al., 1991; Scott et al., 1991), however, one study does (Lederle et al., 1997). In contrast case - control studies describe hypertension as an independent risk factor for aneurysms (Reed et al., 1997; Strachan et al., 1991).

Associated Cardiovascular Vascular Disease

Coronary artery disease is significantly associated with the abdominal aortic aneurysm, as is "any atherosclerosis," but the associations are considerably weaker than the association with smoking (Lederle et al., 1997). An independent association between high serum cholesterol levels and aneurysms has been determined in large studies (Lederle et al., 1997) but was not determined in other smaller multivariate analyses (Strachan et al., 1991; Reed et al., 1997).

Familial / Genetic Predisposition

Familial clustering of the abdominal aortic aneurysm was first described in 1977 (Clifton et al., 1977). Subsequently many investigators have shown that the risk of an abdominal aortic aneurysm is greater in sibling relatives of patients with the disorder, than in the same age group of the general population (Baird et al., 1995; Powell et al., 1987; Darling et al., 1989). Frydman et al., determined an overall 30% incidence in siblings of patients with aneurysms. The incidence was 45% in brothers of female patients, 42% in brothers of male patients, 23% in sisters of female patients, and 14% in sisters of male patients (Frydman et al., 2003). Salo et al., examined first-degree relatives of patients having surgery for abdominal aortic aneurysms (Salo et al., 1999). Aging brothers of patients with a known abdominal aortic aneurysm had the highest risk for developing the disease at 18%. This compares to a 1.5% incidence in males with no affected siblings. Age of onset of the abdominal aortic aneurysm is younger in patients reporting familial traits than those not (Verloes et al., 1995; Baird et al., 1995; Johansen et al., 1986).

No single inheritance mode explains the occurrence of abdominal aortic aneurysms, however, first-degree family members and male relatives, are at increased risk (Kuivaniemi et al., 2003). Some studies suggest that there is probably an X-linked and autosomal dominant inheritance in this disease (Tilson et al., 1984; Verloes et al., 1995).

Diabetes

A negative association is reported between abdominal aortic aneurysms and diabetes (Lederle et al., 1997). This finding may be an artifact caused by limiting screening data to previously undiagnosed aneurysms. For example diabetic patients may be more likely than those who do not have diabetes to have died with an aneurysm. However, the two case control studies did not report an association between diabetes and dying with or from an aneurysm (Reed et al., 1997; Strachan et al., 1991). Other studies support a negative association between aneurysms and diabetes, and a non-significant reduction is observed in the frequency of diabetes in patients with an aneurysm (Pleumeekers et al., 1995; Smith et al., 1993).

Diabetes does appear to have an effect on large arteries, causing increased aortic stiffness, and on peripheral arteries, causing medial calcification (Salomaa et al., 1995). These changes may stabilize the aorta, inhibiting aneurysmal formation (Pillari et al., 1988). However, increased aortic wall stiffness is described in patients with aneurysms (MacSweeney et al., 1992), and no reciprocal association is been described between calcification and the expansion rate of aneurysms (Wolf et al., 1994). Consequently, the reason for a negative association between abdominal aortic aneurysms and diabetes is undefined.

2.3 Risk Factors For Abdominal Aortic Aneurysm Rupture

The absolute risk factors for rupture, determined in 1969 by Foster et al., of aneurysm size and associated diseases (Foster et al., 1969), have slowly been elaborated upon and include expansion rate, intraluminal thrombus, hypertension, smoking and obstructive pulmonary disease.

Aneurysm Diameter

Small Aneurysms

Of historical interest are several studies that report the natural history of aneurysms 3-3.9cm and 4-4.9cm in diameter. The pervading trends are of low rupture rates in aneurysm less than 5cm; increasing rupture rate with aneurysm diameter; the majority of aneurysms terminating in rupture, enlarge to over 5cm before rupturing. More recently the United Kingdom Small Aneurysm Trial and an equivalent study from the United States represent the gold standard in small aneurysm management (UK Small Aneurysm Trial Participants, 1998; Lederle et al., 2002).

Limet et al., classified the incidence of aneurysm rupture as zero in aneurysms 3-3.9cm, 12% in aneurysms 4-4.9cm, and 22% in aneurysms 5cm or greater (Limet et al., 1991). Nichols et al., measured ruptured aneurysms immediately prior to surgery with 10% of ruptured aneurysms 5cm or less (Nicholls et al., 1998). However, the use of post-rupture diameter measurements to reliably indicate aneurysm diameter during the period preceding rupture is undetermined. Furthermore, estimation of rupture risk

based on initial aneurysm diameter at entry to follow up may misleadingly overestimate the rupture rate at any given size.

Studies of aneurysms initially less than 5cm but also reporting diameters closest to the point of rupture, report rupture only in aneurysms attaining diameters >5cm (Brown et al., 1996; Glimaker et al., 1991). Brown et al., reported the prospective study of 895 patients with aneurysms less than 5cm in which CT scans were obtained every 6 months. Twelve percent died during the study though none died from proven or suspected aneurysm rupture. Surgery was performed in 250 patients with expansion, or symtomatology but no patient suffered rupture (Brown et al., 2003).

The UK Small Aneurysm Trial studied 1090 patients (mean duration of follow-up 8 years); 563 patients were randomised to early surgical repair with 452 having operation within 5 months; 527 patients were randomised to ultrasound surveillance with delay repair with expansion $\geq$1cm/yr, expansion to 5.5cm or symptomatology. Overall survival was not significantly different between the early surgery and surveillance groups (UK Small Aneurysm Trial Participants, 1998). Annual rupture rates were determined as 0.3% for aneurysms <4cm, 1.5% for aneurysms 4-4.9cm, and 6.5% for aneurysms 5-5.9cm.

Aneurysm Diameter And Management Trial examined aneurysm 5-5.4cm in diameter, followed up for a mean of 4.9 years; 569 were randomised to immediate repair; 567 were randomised to active surveillance with 61.6% repaired at trial completion. As with the UK Small Aneurysm Trial, the rate of aneurysm related deaths was not reduced by early surgical repair. Aneurysm rupture occurred in 11 patients in the

surveillance group, a rate of 0.6 per year. A further 20 patients under surveillance were repaired when symptoms heralded a rupture. Two patients in the immediate repair group ruptured – one incidentally determined at elective repair, the other a contained rupture proximal to a previous open repair (Lederle et al., 2002).

The selective repair of small aneurysms with rapid expansion, and thus greater rupture risk, bias the low rate of rupture in both these studies but serve to demonstrate an important point. In practice the rupture rate in small aneurysms may be negligible if sequential follow-up is maintained and prompt repair undertaken in aneurysms enlarging to greater than 5.5cm or rapidly expanding at 1cm per year or more.

Large Aneurysms

The natural history of large aneurysms (greater than 5.5 cm) is difficult to determine since most are promptly repaired if the patient is physically fit. Therefore, a detailed knowledge of the natural history of these aneurysms beyond the point of detection and repair is impossible to obtain and of no practical value. The point of operative intervention in patients with large aneurysms but high surgical risk is less clear. Co-morbid factors contraindicating surgical repair in patients with larger aneurysms include cardiac, cerebrovascular, pulmonary and renal impairment, advanced malignancy, advanced age and patient wishes. Longitudinal studies have been complicated by the uncertainty regarding definite outcome endpoints. Imaging, surgery and autopsy all permit an accurate diagnosis of rupture but occurs in only the minority of patients. In the majority of patients the diagnosis of aneurysm rupture is unconfirmed by these modalities and relies on the certifying officer, hospitals records

or eyewitness reports and should be viewed with less confidence. In this context three recent studies have reported varying results yet generally similar patterns of disease history in aneurysms greater than 5.5cm in diameter (Conway et al., 2001; Tanquilut et al., 2002; Lederle et al., 2002).

Conway et al., determined the outcome of 106 patients with aneurysms greater than 5.5cm in diameter unfit for open repair with a median time to death of 9 months. Thirty-nine (51%) patients were certified as having died of non-rupture causes related to medical co-morbidities, and 37 (49%) were certified as dying from aneurysm rupture. In aneurysms measuring 5.5 to 5.9 cm, there was little difference between the survival free period in patients who died from rupture and patients who died from non-rupture causes. However, for patients with an aneurysm greater than 6.0 cm, the risk or hazard of dying from a ruptured aneurysm, as opposed to a non-rupture cause, became apparent as early as 6 months (Conway et al., 2001).

Tanquilut et al., reported 72 patients with aneurysm diameters greater than 5.5cm unfit for open repair. Fifty-three patients ultimately underwent repair of their aneurysm secondary to rapid enlargement or onset of symptoms. Operative repair was open in 33 patients and endovascular in 20 patients. Six patients deceased within 30 days of surgery. Of the 19 patients managed non-operatively, 7 (37%) were still alive after a mean of 4 years, 9 (47%) died from medical co-morbidities after less than 2 years and 3 (16%) died from aneurysm rupture after a mean of 3 years. Overall aneurysm related mortality was 12.5% (6 operative related mortalities, 3 rupture related mortalities). Therefore, selective delayed surgical or endovascular

intervention was an important aspect of care and appeared to reduce rupture in this cohort (Tanquilut et al., 2002).

Lederle et al., reported the prospective observation of 198 patients, with aneurysm diameter greater than 5.5cm. Rate of rupture was based on both initial and attained aneurysm diameter. Ruptures were reported as definitive – ruptures confirmed by surgery, CT scan or autopsy; possible – definite and probable ruptures plus patients with an unconfirmed symptomatic aneurysm or patients with sudden death. The 1 year rupture rates (definite versus possible) based on initial aneurysm diameter were 5.5% versus 9.4% for aneurysms 5.5-5.9cm, 7.5% versus 10.2% for aneurysms 6-6.9cm, and 27.9% versus 34% for aneurysms greater than 7cm. The 1 year rupture rates (definite versus possible) based on attained diameter were 6.4% versus 12% in aneurysms 5.5-5.9cm, 3.8% versus 7.4% in aneurysms 6-6.9cm, and 23.4% versus 30.9% in aneurysms greater than 7cm. Fundamental patterns emerge from this study; possible rupture rates are persistently 4-6% higher than definitive rupture rates; rupture rates increase three-fold in aneurysms of diameter > 7cm (Lederle et al., 2002).

It is of interest to note that the physiological contraindications to repair in larger aneurysms such as cardiac or respiratory impairment are also associated with increased risk of rupture and is discussed in the following sections. Indeed up to two-thirds of such patients die from associated medical co-morbidities rather than aneurysm rupture (Jones et al., 1998).

Aneurysm Expansion

Aneurysm expansion is defined as the difference between the first and last examination measurement expressed as a factor of time. Most authors do find a correlation between initial aneurysm size and subsequent expansion (Limet et al., 1991; Brown et al., 1996). However, some studies indicated that size does not correlate with the expansion rate (Reed et al., 1997; Cronenwett et al., 1990). The aneurysm expansion rate in patients prospectively monitored shows that the mean expansion rate is significantly greater in patients who rupture than those who do not (Schewe et al., 1994; Gloviczki et al., 1992).

Limet et al., considered exponential growth values and demonstrated no significant change in rate over different size ranges. This confirmed that the rate of change in aneurysm size was dependent on time, as well as initial size (Limet et al., 1991). Exponential aneurysm expansion is estimated at 6 to 10% (Limet et al., 1991; Bernstein et al., 1984; Hallin et al., 2001). Only a part of the variation on the individual expansion rate can be explained by initial aneurysm size and expansion clearly depends on several factors. Other factors including respiratory and cardiac disease are associated with a faster rate of aneurysm expansion. Women had faster expansion rates than men. Beta-Blocking medication tended to non-significantly lower expansion rates (Bengtsson et al., 1993; Cronenwett et al., 1990).

Relative Anatomical Parameters

Estimates of rupture risk based on aneurysm diameter with respect to other morphological parameters have been attempted but their poor uptake in clinical practice suggests they are of poor predicative value. Ouriel et al., considered standardising aortic aneurysm diameter to measures of patient size and normal supra-renal aortic diameter, however, the improvement in predictive accuracy aneurysm rupture was minimal (Ouriel et al., 1992). Hatakeyama et al., demonstrated that a combination of aneurysm diameter, length and diastolic blood pressure predicted aneurysm rupture. In two aneurysms with the same transverse diameter, the more longitudinally stretched aneurysm may have a higher risk of rupture (Hatakeyama et al., 2001).

The law of Laplace dictates that dilation can decrease wall resistance thus a localised dilation and may precipitate rupture. Faggioli et al., demonstrated the diameter of ruptured aneurysms corresponded with the presence of a saccular out pouching in the aneurysm wall (Faggioli et al., 1994). Hunter et al also reported saccular out pouchings in aneurysms ranging from 5 to 30mm on CT scan that were unrelated to aneurysm size. Histologically these areas represented marked thinning of the medial elastin (Hunter et al., 1996).

Aneurysm Wall Stress

Abdominal aortic aneurysm rupture is believed to occur when the mechanical stress acting on the wall exceeds the strength of the aortic wall. Evaluation of the stresses acting on the intact aneurysm wall could be useful in predicting risk of rupture. Computer generated finite element analysis allows the non-invasive estimation of wall stress at any point on the aneurysm. Modelling predicts that asymmetric bulges substantially increase localised wall stress (Vorp et al., 1998). Finite element analysis determines peak wall stress to be significantly elevated in ruptured and symptomatic aneurysms in comparison to asymptomatic aneurysms at a given aneurysm diameter (Fillinger et al., 2002). Analysis of non-ruptured aneurysm CT scans shows peak wall stress to be a significantly better predictor of emergency surgery than aneurysm diameter. Further, the site of aneurysm rupture, determined at open repair or by imaging, correlates with the site of highest wall stress (Fillinger et al., 2003). This suggests that the estimation of aneurysm wall stress based on finite element analysis may better predict the risk of rupture than aneurysm diameter.

Intra Mural Thrombus

The role of intraluminal thrombus in mediating aneurysm development is not clearly defined. Retrospective reviews of serial CT scans at least six months apart in 80 patients, showed that increased thrombus load correlated with increased rate of aneurysm expansion. However, thrombus thickness was smaller in size matched ruptured aneurysms than non-ruptured aneurysms (Wolf et al., 1994). A possible

explanation may be that the increase in thrombus volume keeps pace with aneurysm growth below 7cm diameter. Above 7cm diameter, the exponential nature of aneurysm expansion overtook thrombus generation (Pillari et al., 1988).

Two theories are proposed for the destabilisation of the aneurysm wall by intraluminal thrombus. Firstly, the thrombus may act as a pool for plasmin generation, which is free to leech into the aneurysm wall and up-regulate proteolytic activity, thus potentiating further growth (Jean-Claude et al., 1994). Alternatively, the presence of a luminal arc of thrombus probably prevents adequate diffusion, rendering the inner media anoxic. The adventitia and outer media gain oxygen from the vasa vasora, so the inner media is susceptible to a fall in luminal diffusion (Vorp et al., 1996).

Other reports suggest intraluminal thrombus is protective and correlates with a lowering of stress forces in the aneurysm wall. Such reports relate only to computer generated finite element analysis. Inzoli et al., described a reduction in peak wall stress of up to 30% in the presence intraluminal thrombus (Inzoli et al., 1993). Mower et al., and Di Martino et al, also reported a reduction in wall stress attributed to the presence of thrombus (Mower et al., 1997; Di Martino et al., 1998).

Smoking and Lung Function

Smoking is associated with increased expansion rate and increased risk of rupture of abdominal aortic aneurysms (UK Small Aneurysm Trial Participants, 2000). In a study of male civil servants in England the risk of all types of fatal aneurysm was

substantially increased for current smokers (Strachan et al., 1991). The relative risk of death from aneurysm rupture increased 4.6-fold for cigarette smokers, 2.4-fold for cigar smokers and 14.6-fold for smokers of hand-rolled cigarettes (Strachan et al., 1991).

Patients often poorly declare an accurate smoking history. Thus whilst the level of risk for aneurysm rupture increase significantly with plasma cotinine (a metabolite of nicotine), observed increase in ruptures among self-reported current smokers was of borderline significance. Higher aneurysm growth rates are also observed in patients who continue to smoke than those who stop (MacSweeney et al., 1994).

A very significant negative correlation between current smoking habit and lung function has been described and both are associated with accelerated aneurysm growth and aneurysm rupture (Strachan et al., 1991; Brown et al., 1999). The degree of obstructive pulmonary disease correlates positively with risk of rupture (Cronenwett et al., 1985). Furthermore, the forced expiratory volume in one minute has been shown to be the most powerful predictor of long-term survival in aneurysm patients (Brown et al., 1999).

Hypertension

Diastolic hypertension has consistently proved a highly significant independent predictor of increased aneurysm expansions and rupture across a number of studies (Cronenwett et al., 1985; Hatakeyama et al., 2001; Sterpetti et al., 1991; Schewe et al.,

1994). An elevated systolic blood pressure has been shown to correlate with rupture risk in univariate but not multivariate analysis (Schewe et al., 1994). The UK Small Aneurysm Trial reported higher mean blood pressure to be independently associated with aneurysm rupture risk (Brown et al., 1999), though this factor is significantly weighted by the diastolic pressure component. The importance of diastolic blood pressure as a risk factor for aneurysm rupture cannot be overemphasised given that it is one of the few factors that can be fully managed by pharmacotherapy.

Familial Predisposition

As well as an increased occurrence in first-degree relatives, patients with a familial trait for abdominal aortic aneurysms have different rupture risk profile. Verloes et al., compared male patients reporting familial history of abdominal aortic aneurysms with males without a family history: the ages at rupture were 65 years (familial) and 75 years (sporadic) ($p < 0.001$), and the rupture rate was 32.4% (familial) and 8.7% (sporadic, $p < 0.001$), respectively (Verloes et al., 1995). Darling et al., reported similar results in a 9-year prospective study of 542 consecutive patients undergoing operation for abdominal aortic aneurysms. Patients with abdominal aortic aneurysms and a positive family history of the disease had a greater risk of rupture, especially if female (Darling et al., 1989).

Gender

Females are three times more likely to experience aneurysm rupture than males over the period of a surveillance programme, however, since males represent the larger group of non-ruptured aneurysms they also represent the larger proportion of ruptured aneurysms (Fillinger et al., 2003; Brown et al., 1999). Indeed female sex represents a significant independent risk for aneurysm rupture with the risk of fatal rupture being significantly higher in females compared to males (UK Small Aneurysm Trial Participants, 2002; Brown et al., 1999). The mean aneurysm diameter at rupture is smaller in females than males - 5 cm in women and 6 cm in men. Women also do worse following open repair in both the elective and rupture scenario, with a higher mortality than males (Semmems et al., 2000; UK Small Aneurysm Trial Participants, 2002; Pearce et al., 1999).

The differences in presentation and outcome for females with abdominal aortic aneurysm may arise from structural differences in the aorta between the sexes. Women have a smaller diameter, more compliant aorta than men but degenerative changes appear later in females than males (Sonesson et al., 1993).

Other Risk Factors

The following cardiovascular risk factors are perhaps paradoxically not associated with an increased risk of aneurysm rupture: patient age and height, body mass index, serum or plasma cholesterol levels, low-density and high-density lipoproteins profiles,

glucose intolerance, angina, intermittent claudication and abnormal ankle/brachial pressure index (Brown et al., 1999; Schewe et al., 1994; Cronenwett et al., 1985; Strachan et al., 1991).

A single study reported history of smoking, myocardial infarction and coronary artery heart bypass, were unexpectedly protective of aneurysm rupture. This is certainly an artefact secondary to the higher mortality associated with these co-morbidities such that these patients die before their aneurysms rupture (Lederle et al., 2002).

2.4 Contemporary Management Of Non-Ruptured Abdominal Aortic Aneurysms

Aneurysm Screening

The Multicentre Aneurysm Screening Study (MASS) was a randomised controlled trial investigating the effect of serial ultrasound screening on abdominal aortic aneurysm mortality in men aged 65-74 years (Ashton et al., 2002). Of the 33400 men invited for scanning, there was an uptake rate of 80% and an aneurysm detection rate of 5% of which 12% were ≥ 5.5cm. Surgery was undertaken with aneurysm diameter ≥5.5cm, expansion ≥1cm per annum, or symptomatology. The control group was not invited for screening but was of similar population size to the screened cohort. Mean follow-up was 4.1 years, primary outcome being aneurysm-related mortality, with secondary outcomes including thirty-day all-cause mortality and health related quality of life.

Aneurysm detection through screening decreased the rate of emergency repair by 50% and increased elective repair. Initial mortality in the screened group exceeded the non-screened group, because of the associated aneurysm-related mortality from elective repair. However, over the trial period aneurysm-related mortality was lower in the screened group (Odds ratio 0.58 (0.42-0.78), p=0.0002) and the risk of dying from an aneurysm was reduced from 3.3 per 1000 to 1.9 per 1000. This equated to a 42% reduction in risk in the screened group as a whole, but a 53% reduction in patients who complied with screening. Thirty-day mortality was 6% after elective repair and 37% after emergency repair. Since aneurysm related mortality contributed

only 3% to total all-cause mortality, over the trial period there is no benefit between screened and non-screened cohorts with respect to all-cause mortality.

The cost effectiveness data from the MASS trial showed the mean additional cost of the screening was £63 per patient. The mean survival time free from aneurysm-related mortality was greater in the intervention group than the control group, giving a cost effectiveness ratio at 4 years of £28 400 per life year gained, at the margins of acceptability. Over a longer period of time the cost effectiveness will improve and projected cost per life year gained after 10 years should approach £8000, well within the perceived threshold values (Multicentre Aneurysm Screening Study Group, 2002).

Aneurysm screening does not incur any long-term adverse psychological effect. In the short term the points of aneurysm detection and repair are associated with a lower health related score (Ashton et al., 2002). MASS and other studies suggest that ultrasound can accurately visualise the aorta in 99% of subjects (Ashton et al., 2002; Scott et al., 1991). Screening females is of little benefit as the much lower incidence of abdominal aortic aneurysms in females fails to result in a reduction in rupture rate (Scott et al., 2002). Similarly re-screening men with normal aortic diameter is of little benefit due to their very low rupture risk (Ashton et al., 2002). A potential limitation of the screening trials is the determination of participants' cause of death. Though it was not thought to bias results, 8% of patients certified as dying of an abdominal aortic aneurysm in the MASS trial, when independently reviewed, were considered to have another cause of death.

The MASS trial demonstrated that a 50% reduction in rupture risk and a 50% reduction in aneurysm-related mortality risk could be achieved with ultrasound screening. With the cost effectiveness at four years approaching acceptability, and projections of cost effectiveness well within acceptability by 10 years. These clinical and economic facts provide strong evidence supporting the introduction of aneurysm screening of sixty-year-old men across the United Kingdom.

Parameters of Aneurysm Repair

The abdominal aortic aneurysm should undergo elective repair when the risk of rupture is sufficiently high to justify the risk of surgery i.e. the risk of death from rupture exceeds the risk of death from elective repair. The annual rupture rate of an abdominal aortic aneurysm (Table 2.1) varies according to aneurysm diameter such that the risk of rupture of an aneurysm <5.5cm is no more than 1% per year (Lederle et al., 2002; UK Small Aneurysm Trial Participants, 2002). The rupture rate in aneurysms of diameter ≥5.5 cm increases to 10%, but reaches 30% in aneurysms >7cm (Lederle et al., 2002). Clearly 5.5cm represents a threshold point for rupture risk. However should aneurysms undergo repair before they reach 5.5cm or after this point?

Initial AAA diameter (cm)	Annual risk of rupture	
3.0	0.2-0.4%	Vardulaki et al 1998
4.0	0.8-1.1%	Vardulaki et al 1998
4.0 – 5.5	0.6-1.0%	ADAM study Group 2002, UK Small aneurysm trial 1998
5.5-5.9	9.4%	Lederle et al., 2002
6.0-6.9	10.2%	Lederle et al., 2002
<7.0	30.5-32.5%	Lederle et al., 2002

Table 2.1: Annual rupture rates of abdominal aortic aneurysms according to size (based on pooled available data)

This question was answered by two trials, The UK Small Aneurysm Trial (UK Small Aneurysm Trial Participants, 1998) and a similar study in the USA (Lederle et al., 2002). Both studies recruited over one thousand subjects with small aneurysms (4-5.5cm) and randomised to early surgery or ultrasound surveillance. The UK study had a preponderance of male (83% male, mean age 68 years); the USA study was almost exclusively men (98.5% male, mean age 69 years). Ninety-six percent of aneurysms in the early surgery cohorts of both trials were repaired. Within the surveillance cohorts of both trials, 62% of aneurysms were repaired, as a result of expansion ≥1cm per annum, expansion to ≥5.5cm or onset of symptomatology over 5 years.

Comparing early surgery with surveillance (Table 2.2), the thirty-day post-operative all-cause mortality was similar within each study (USA Trial 2.1% versus 1.8%, $p>0.05$; UK Trial 5.8% versus 7.1 %, $p>0.05$). However, the thirty-day mortality was much lower in both arms of the US trial than in the UK trial. Survival was not affected by patient age, gender, or aneurysm size in either trial. Early and

intermediate survival was not affected by early surgical repair thus the delay in repair associated with surveillance did not effect survival. Intermediate results from both trials demonstrated near identical all cause mortality at 4 years with early surgery or surveillance.

Eight-year results were only reported by the UK study. The hazard ratio for all cause mortality pointed towards a benefit to early repair (0.83 (0.69-1), p=0.05) (UK Small Aneurysm Trial Participants, 2002). During the first 6 months of randomisation the death rate after early surgery was two-and-a-half that of the surveillance group. Among those that survived 6 months, the death rate in the early surgery group dropped to three-quarters (7.8% lower) that of the surveillance group. It was suggested that this was due to smoking, since the cessation of smoking was significantly higher in the early surgery group, but it may well have been due to an adverse biological influence mediated by the non-operated aneurysm. Whatever the reasons, in younger patients with a longer post-operative survival, a non-significant benefit from early surgery was suggested.

	Early Surgery vs.	**Surveillance**	**p-value**
30 day mortality			
USA ADAM	2.1%	1.8%	>0.05
UK SAT	5.8%	7.1%	>0.05
4 yr all cause mortality (Early Surgery vs Surveillance)			
USA ADAM (Odds ration)	1.21 (0.95 - 1.54)		>0.05
UK SAT (Odds ratio)	0.94 (0.75 - 1.17)		>0.05

Table 2.2: Outcome data from transatlantic small aneurysm studies.

Overall the evidence relating to aneurysm size at repair is not as clear as one might initially observe. One should note that the UK Small Aneurysm Trial and the USA study defined simply that open surgery brought no short or intermediate advantage for aneurysms ≤5.5cm in diameter. Neither trial determined a definitive aneurysm size that should be operated on. Furthermore, 62% of the surveillance patients in both trials ended up having an operation. Given that most aneurysms ultimately require repair and there is a long-term advantage to early surgery, the repair of aneurysms ≤5.5cm is justified particularly in younger male smokers. In addition subsequent studies have demonstrated and increased risk of rupture in females with a 5 cm aneurysm in a female equivalent to a 6 cm aneurysm in males.

Endovascular Repair

Transabdominal (open) repair with an inlay graft has become the standard surgical management of abdominal aortic aneurysms over the last 50 years. Virtually all infra-renal aneurysms are suitable for transabdominal inlay graft. By contrast endovascular repair is dependent upon and limited by aneurysm morphology. The primary success of the endovascular repair, to exclude an aneurysm from the circulation, relies on the ability of the deployed endograft to form a secure seal both proximal and distal to the aneurysm. The integrity of the seal is dependant upon the radial force of the endograft. Thus planning for endosurgery involves the detailed measurement of aneurysm anatomy and suitable endograft selection. Figure 2.1 displays the anatomical parameters important for endograft planning.

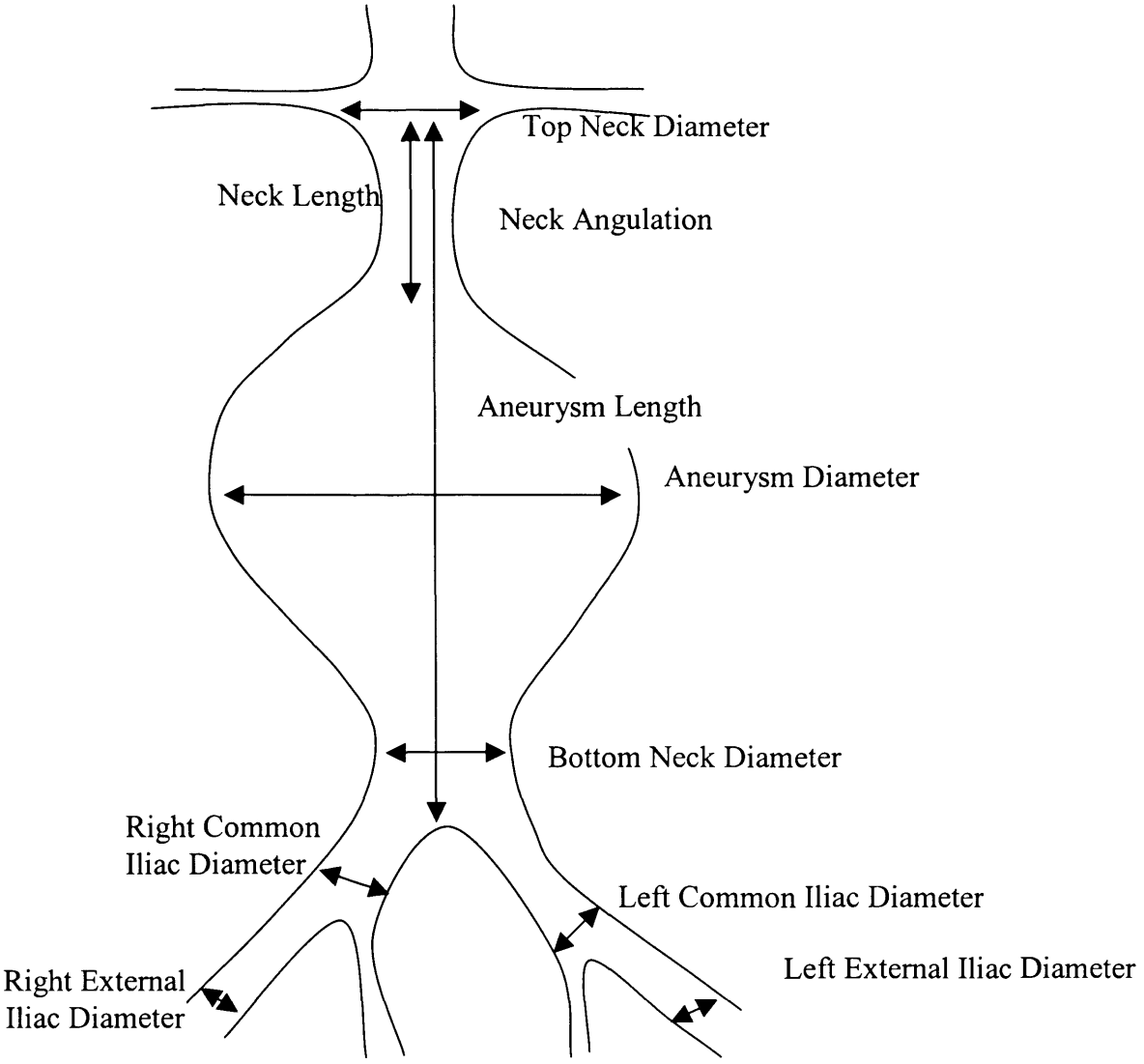


Figure 3.3. Anatomical parameters required for the planning endovascular aneurysm repair.

The pre-eminent factor in determining an aneurysm's suitability for endovascular repair is the morphology of the proximal neck of the aorta. Generally a diameter less than 30mm, length greater than 10mm and angulation less than 60° is favourable for commercial endografts. The proportion of aneurysms suitable for endovascular repair varies between studies from 30% to 70%, however, with increasing acceptance and familiarity the percentage tends to increase (Wilson et al., 2004; Wolf et al., 2000; Woodburn et al., 2001). Large neck diameter, short proximal neck length and extremes of neck angulation, create a hostile morphology and predispose to "endoleak". An endoleak represents the abnormal flow of blood outside the endograft and within the residual aneurysm sac. Five types of endoleak exist (Table 2.3).

Initial AAA diameter (cm)	**Annual risk of rupture**
Type I	Incomplete seal
Type II	Patent aortic branch(es)
Type III	Stent-graft structural failure
Type IV	Graft porosity
Type V	Endotension

Table 2.3: Types of endoleak

Open Versus Endovascular Repair

The Endovascular Aneurysm Repair (EVAR) Trials scrutinize the advantage of endovascular aneurysm repair over open repair (Greenhalgh et al., 2004; EVAR-1 Trial Participants, 2005). The EVAR trials recruited patients over 60 years of age

with aneurysms of diameter ≥5.5cm and morphology suitable for endovascular repair. EVAR-1 recruited just over 1000 patients anaesthetically fit for open repair and randomised to either open or endovascular repair (Greenhalgh et al., 2004). EVAR-2 recruited 340 patients considered anaesthetically unfit for open repair and randomised to either endovascular or non-surgical best medical therapy (EVAR-2 Trial Participants, 2005). The trials reported thirty-day mortality; all-cause and aneurysm-related mortality at 4 years; post-operative complication and re-intervention rates; health related quality of life and cost. The results are summarised in Table 2.4.

The results from EVAR-1 demonstrated the thirty-day operative mortality following endovascular repair was one third of that observed with open repair (1.7% versus 4.7%, p=0.009) (Greenhalgh et al., 2004). The thirty-day secondary re-intervention rates were higher following endovascular repair compared to open repair (9.8 versus 5.8%, p=0.002). Four-year follow-up failed so show a significant benefit to endovascular repair for all cause mortality, however, the 3% advantage in aneurysm related mortality to endovascular repair was maintained (EVAR-1 Trial Participants, 2005). Within the first six months of repair, aneurysm related mortality was lower in the endovascular group but after six months there was no difference. Thus the 4 year aneurysm-related mortality advantage of 3% following endovascular repair could be attributed to the decrease in initial thirty-day mortality. This persistent, all-be-it, diminishing advantage towards endovascular aneurysm repair was particularly noteworthy given the higher complication and re-intervention rates attributed to endovascular repair. Furthermore, during the course of the EVAR trials (September 1999 to December 2003) endograft design and user experience evolved such that the complication and re-intervention rates were likely to be much lower year-on-year.

Interestingly, the higher re-intervention and complication rates with endovascular repair did not adversely affect health related quality of life, which was in fact higher in the first 3 months following endovascular repair but equivalent to open repair thereafter. Endovascular repair was slightly more costly but this is likely to decrease as follow up becomes more defined, and complication rates lessen.

EVAR-2 randomised 340 patients unfit for open repair into endovascular repair or best medical therapy (EVAR-2 Trial Participants, 2005). There was no difference in four-year aneurysm-related mortality, all-cause mortality, nor health related quality of life between cohorts. Indeed deaths from aneurysm rupture in the medical therapy group matched the rupture and operative deaths in the endovascular group. The cost of endovascular repair was significantly greater than medical therapy alone. There was no difference in the health related quality of life score between the two groups at any time. The lack of difference between the study cohorts may relate to the lower rate of rupture in the medical therapy group – approximately half that expected, with only 21 ruptures over 4 years from 172 patients. However, 30 patients initially randomised to best medical therapy subsequently had endovascular or open aneurysm repair for symptomatology, rapid enlargement or no stated reason. Though the authors suggested that this crossover did not bias the trial findings, the observed number of ruptures would have been higher without this. Compared with EVAR-1, the thirty-day mortality within the endovascular cohort of EVAR-2 was much greater (9% versus 1.7%, $p<0.0001$) but complication and re-intervention rates were comparable (EVAR-1 Trial Participants, 2005; EVAR-2 Trial Participants, 2005). As with the UK Small Aneurysm Trial, EVAR-1 and -2 failed to show significant associations for mortality with age, gender, aneurysm size or creatinine concentration

(EVAR-1 Trial Participants, 2005; EVAR-2 Trial Participants, 2005; UK Small Aneurysm Trial Participants, 2002).

The Dutch (DREAM) Trial was of analogous concept to the EVAR-1 trial but recruited only 345 subjects with aneurysms of diameter ≥5cm (Prinssen et al., 2004). Thirty-day mortality was lower in the endovascular cohort relative to the open group (1.2% versus 4.6%, p=0.07). Two-year all-cause mortalities were similar (10.4% versus 10.3%) but aneurysm related mortality was lower in the endovascular group (2.1% versus 5.7%) (Blankensteijn et al., 2005). Re-interventions in DREAM were three times higher in the first 9 months following endovascular repair than open (p=0.03), but after this period rates were similar. Complications were stratified on severity with the rate of survival free of a severe event being comparable at two years. Numerically mortality figures mirrored EVAR-1 results, however, the lower recruitment by DREAM resulted in an under powering and the trial failed to prove statistical significance (EVAR-1 Trial Participants, 2005; Prinssen et al., 2004; Blankensteijn et al., 2005).

The rationale for observation of small aneurysms should be revisited with endovascular surgery and there are trials currently ongoing to investigate this (Cao et al., 2005). The UK and US small aneurysm trials were both associated with a relatively high thirty-day mortality following open surgery (Lederle et al., 2002; United Kingdom Small Aneurysm Trial Participants, 1998) but thirty-day mortality was lower in the endovascular arm of EVAR-1 (Greenhalgh et al., 2004). If thirty-day mortality is reduced by endovascular surgery then it becomes beneficial to operate on aneurysms of diameter below 5.5cm. Indeed matched subgroup

comparisons from the ultrasound surveillance arm of the UK Small Aneurysm Trial (UK Small Aneurysm Trial Participants, 1998) with the endovascular arm of the AneuRx trial (Zarins et al., 2001) (endovascular repair of aneurysms ≤5.5cm), advocates a survival advantage following endovascular repair of small aneurysms. The comparison demonstrated a significant reduction in fatal aneurysm rupture and aneurysm-related death, with improved overall patient survival following endovascular repair compared to ultrasound surveillance and selective open surgical repair (Zarins et al., 2005).

The NCEPOD report from 2005 suggests that all patients who are anatomically suitable for endovascular repair should in fact receive this (Gray et al., 2005). The results of the EVAR and DREAM Trials confirm that for fit patients, endovascular aneurysm repair reduces thirty-day mortality by one-third. Despite a higher re-intervention and complication rate, a 3% aneurysm-related mortality advantage persisted over 4 years follow-up. All-cause mortality is the same between open and endovascular groups, but this is also true of the MASS trial and what we should be looking at is aneurysm related mortality only. Endovascular aneurysm repair in unfit patients currently shows no clear advantage over best medical therapy.

	Open Repair	Endovascular Repair	Best Medical Therapy	Hazard Ratio (95% CI)	p-value
EVAR-1					
Patient numbers	539	543			
Mortality					
30-day	4.7%	1.7%		0.35 (0.16-0.77)	0.009
All Cause (4yr)	26%	29%		0.90 (0.69-1.18)	NS
AAA Related (4yr)	7%	4%		0.55 (0.31-0.96)	0.04
Complications (4yr)	9%	41%		4.9 (3.5-6.8)	<0.0001
Re-interventions (4yr)	6%	20%		2.7 (1.8-4.1)	<0.0001
HRQL (2yr)	Initial advantage to endovascular; after 3 months equivalence				NS
Cost (4yr)	£10,000	£13,300			
EVAR-2					
Patient numbers		166	172		
Mortality					
30-day		9%			
All Cause (4yr)		66%	62%	1.21 (0.87-1.69)	NS
AAA Related (4yr)		14%	19%	1.01 (0.55-1.84)	NS
Complications (4yr)		43%	18%	5.3 (2.8-10.0)	<0.0001
Re-interventions (4yr)		26%	4%	5.8 (2.4-14)	<0.0001
HRQL (2yr)					NS
Cost (4yr)		£13,500	£5000		
DREAM					
Patient numbers	178	173			
Mortality					
30-day	4.6%	1.2%		3.9 (0.9-32.9)	0.07
All Cause (2yr)	10.4%	10.3%			NS
AAA Related (2yr)	5.7%	2.1%			0.05

Table 2.4: Early and Intermediate Results from EVAR and DREAM Trials. (NS – non-significant, HRQL – health related quality of life)

Medical Optimisation

The treatment of any disease includes risk factor modification. Smokers have higher aneurysm incidence, expansion rates and risk of rupture than non-smokers (Lederle et al., 1997; MacSweeney et al., 1994). If the association is assumed to be causal, then 75% of aneurysms ≥4.0cm diameter, may be attributed to smoking (Lederle et al., 1997). A univariate association between abdominal aortic aneurysms and chronic obstructive pulmonary disease is described. Whilst an abnormality of elastin or collagen breakdown has been suggested (Bengtsson et al., 1991; Smith et al., 1993) multivariate models indicate the association to be mediated through heavy smoking rather than a pre-existing biochemical abnormality (Lederle et al., 1997). An independent association between high serum cholesterol levels and the presence of an abdominal aortic aneurysm has been determined in large studies (Lederle et al., 1997) but has not been shown in smaller studies (Reed et al., 1997; Strachan et al 1991). The association with hypertension is of marginal significance with most studies failing to show an association (Bengtsson et al., 1991; Collin et al., 1990; Scott et al, 1991) but case control studies describing an independent risk for abdominal aneurysms (Reed et al., 1997; Strachan et al., 1991). Cardiovascular risk modification is considered suboptimal in aneurysm patients (Gray et al., 2005; EVAR-2 Trial Participants, 2005) with possibly only 60% of patients taking an anti-platelet agent and 40% a statin, β-blocker or ACE inhibitor (Lloyd et al., 2004).

Cardiovascular complications are the most important cause of peri-operative morbidity and mortality among patients undergoing vascular surgery. The use of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) may reduce peri-

operative risk of death following vascular surgery through stabilization of coronary plaques (Lindenauer et al., 2004; Durazzo et al., 2004; Wallace et al., 1998). Retrospective studies report a significantly lower incidence of all-cause mortality, cardiovascular mortality, myocardial infarction and congestive cardiac failure in statin users than in non-statin (Kertai et al., 2004; O'Neill-Callahan et al., 2005). Moreover, a prospective randomized trial of short-term treatment with atorvastatin reported a three times lower incidence of major adverse cardiac events with atorvastatin than with placebo (8.0% vs. 26.0%, p=0.031) after vascular surgery (Durazzo et al., 2004). Similar evidence is reported with peri-operative β-blocker use. β-blockers attenuate endogenous sympathetic activity, decrease heart rate and improve subendocardial perfusion. A prospective randomised trial of peri-operative bisoprolol administered to patients at high risk for coronary artery disease and undergoing major non-cardiac vascular surgery, significantly reduced the incidence of death from cardiac causes and nonfatal myocardial infarction (Poldermans et al., 1999).

Molecular Targets For Disease Modification

The pathobiology of aneurysm formation and expansion is characterised by the infiltration of inflammatory cells and loss of smooth muscle cells from the aneurysm wall. These cellular changes are associated with an up-regulation of matrix metalloproteinases (MMP's), which in turn cause the initial loss of elastin (Sakalihasan et al., 1993). The critical role of MMP's in AAA formation is supported by gene knock-out studies (Longo et al., 2002). Clearly MMP's represent a therapeutic target and there down regulation may cause cessation or regression of the

aneurysm. The primary role of a statin is lipid lowering; however, secondary actions mediated through mesenchymal cell inhibition (Bellosta et al., 1998; Guijarro et al., 1998) are described and include the *in vitro* suppression of MMP production in abdominal aortic aneurysms (Nagashima et al., 2002). Recently, a novel stress-activated proteinase, Jun N-terminal kinase has been found to be critical in the development of abdominal aortic aneurysms in a mouse model. Indeed a Jun N-terminal kinase inhibitor suppresses MMP's, prevents the development and causes the regression of aneurysms in the mouse model (Yoshimura et al., 2005). In the next 5 years, pharmacotherapy aimed at retarding aneurysm expansion is likely to become better defined and instituted into clinical practice. This will be particularly important if screening is introduced as the majority of screen-detected aneurysms are below the threshold for intervention.

2.5 Contemporary Management Of Ruptured Abdominal Aortic Aneurysms

Transabdominal repair with an inlay graft has become the standard surgical management of abdominal aortic aneurysms over the last 50 years. The major technical advancement in abdominal aortic aneurysm surgery in the last decade has been endovascular repair (Parodi et al., 1991). In the elective situation, endovascular repair is associated with improvements in cardiac, respiratory, renal, gastrointestinal, and metabolic function (Baxendale et al., 1996; Boyle et al., 1997; Boyle et al., 2000). In the emergency setting, mortality following open repair of ruptured abdominal aortic aneurysms is between 40% and 60% (Basnyat et al., 1999; Gloviczki et al 1992). Initial experience following the application of endovascular repair to ruptured aneurysms suggests a survival advantage in carefully selected patient groups (Ohki et al., 2000; Orend et al., 2002; Peppelenbosch et al., 2003; Scharrer-Palmer et al., 2003; Yilmaz et al., 2002).

Instituting an endovascular program for ruptured aneurysms presents several challenges, including pre-operative imaging in the after-hours setting, having an appropriate endograft inventory in the hospital, and anatomical challenges related to rupture morphology. To address these problems we undertook a study to define the morphology of ruptured and non-ruptured aneurysms presenting to a single center; estimated the percentage of aneurysms suitable for endovascular repair within each cohort; identified the range of endografts required to support an endovascular treatment program for ruptured aneurysms.

The morphology of ruptured and non-ruptured infra-renal abdominal aortic aneurysms was compared by retrospective review of computed tomographic scans from 51 patients (47 male, mean age 76 years) with ruptured infrarenal abdominal aortic aneurysms and 50 (37 males, mean age 74 years) with non-ruptured infrarenal abdominal aortic aneurysms. Three experienced clinicians reviewed the scans for endovascular suitability based on a generic trimodular endograft with supra-renal fixation.

Morphology of Ruptured Versus Non-Ruptured AAA

Analysis of the morphological features of ruptured and non-ruptured aneurysms (Table 2.5) found a greater mean aneurysm diameter in ruptured aneurysms (75 +/- 15 mm) than in non-ruptured aneurysms (63 +/- 9 mm, $p<0.001$). In contrast, the mean neck length observed in ruptured aneurysms (17 +/- 12 mm) was significantly shorter than that of non-ruptured aneurysms (22 +/- 11, $p=0.031$). Correction for multiple hypothesis testing determined that only maximal aneurysm diameter remained different between the groups.

Measurements (cm)	**Ruptured AAA**	**Non-ruptured AAA**	**p-value***
Neck Diameter	26+/-9 (17-75)	26+/-4 (20-41)	0.897
Neck length	17+/-12 (0-45)	22+/-11 (6-50)	0.031
AAA length	119+/-14 (68-160)	115+/-12 (83-145)	0.264
AAA diameter	75+/-15 (42-110)	63+/-9 (45-82)	<0.001
Common Iliac Diameter	15+/-5 (10-42)	16+/-7 (8-42)	0.366

*Table 2.5: Anatomical characteristics of ruptured and non-ruptured abdominal aortic aneurysms. (Mean +/- standard deviation (range), *independent t-test).*

There were 2 significant correlations for ruptured aneurysms. It is interesting to note that as the neck length decreased, the neck diameter increased, as did the overall aneurysm diameter (Figure 2.2 A and B). However, neither the correlation of neck length with neck diameter (r= -0.34, p=0.018) nor aneurysm diameter (r=-0.33, p=0.020) was particularly strong. There were no significant correlations within the non-ruptured aneurysm cohort (neck length to neck diameter, r=0.06, p=0.691; neck length to aneurysm diameter, r=0.044, p=0.770).

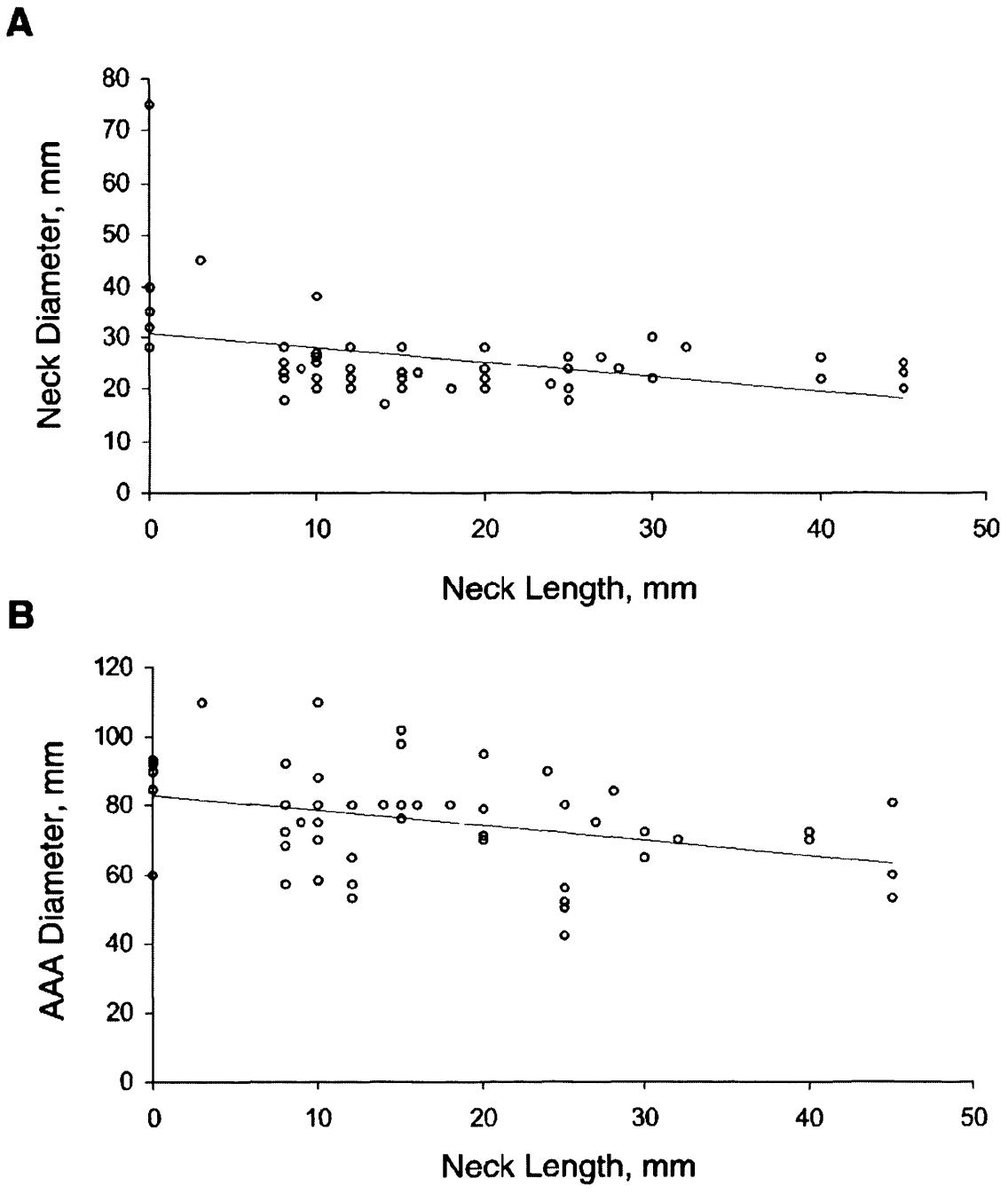


Figure 2.2: A - Correlation between neck length and neck diameter in ruptured aneurysms. B – Correlation between neck length and aneurysm diameter in ruptured aneurysm.

Proportion of Ruptured / Non-Ruptured AAA Suitable for Endovascular Repair

Not quite half (41%, 21/51) of the ruptured aneurysms were suitable for endovascular repair, while 68% (34/50) of non-ruptured aneurysms were deemed appropriate for endovascular repair (p=0.009). The main factor preventing endovascular repair in each aneurysm (Table 2.6) was hostile neck morphology (encompassing extremes of neck length, diameter, and angulation) for both ruptured (51%) and non-ruptured (26%) aneurysms. A combination of these factors frequently co-existed, so the upper limits of neck diameter suitable for endovascular repair was 28 mm in ruptured AAAs and 30 mm in non-ruptured aneurysms, while the minimum neck lengths were 12 and 10 mm, respectively. Agreement between the 3 clinicians in assessing aneurysm suitability for endovascular repair was high (κ =0.75, 0.77, and 0.83; p<0.001 for each comparison).

	Ruptured AAA	**Non-ruptured AAA**	**p-value***
No contraindication	41%	68%	0.009
Neck length †	29%	10%	0.020
Neck angulation	12%	10%	1.0
Neck diameter ‡	10%	6%	0.715
Common iliac diameter	6%	4%	1.0
Iliac occlusive disease	2%	2%	1.0

Table 2.6: Factors contraindicating endovascular repair in abdominal aortic aneurysms. Fisher's Exact test. †neck length in ruptures <1.2cm, non-ruptures <1.0cm; ‡ neck diameter in ruptures >28mm, non-ruptures >30mm.

The Endograft Inventory

The range of generic trimodular endograft components required to treat 41% of ruptured aneurysms is displayed in Table 2.7. While no specific pattern emerged, a full range of endografts would be required. The most frequently chosen aortic component was 24 mm in diameter, and the most frequently chosen iliac component was 16 mm.

Iliac Component	**Aortic Component Diameter**			
Diameter	24mm	28mm	32mm	Totals
12mm	2	1	-	3
14mm	2	1	-	3
16mm	2	4	2	8
18mm	3	2	1	6
20mm	-	-	-	0
22mm	1	-	-	1
Totals	10	8	3	

Table 2.7: Range of trimodular endografts required for endovascular repair of ruptures aneurysms. Dimensions are the aortic component diameter versus the larger of the 2 iliac component diameters required for each aneurysm.

Discussion

Although the concept of endovascular repair for non-ruptured aneurysms has gained widespread acceptance, the ultimate success of the procedure for elective aneurysm repair will depend on the long-term durability of the endograft in excluding an aneurysm from the systemic circulation. However, the application of endovascular repair to a ruptured aneurysm, first reported by Yusuf et al., in 1994, is geared toward rapidly halting blood loss (Yusuf et al., 1994).

An endovascular program for ruptured aneurysms has some identifiable challenges. Firstly, patients require pre-operative imaging (CT or angiography) to confirm the presence of a ruptured aneurysm and to determine suitability for endovascular repair, which introduces delays both at the time of image capture and during transfer between departments. Secondly, a range of endografts must be maintained to accommodate aneurysm morphology. Thirdly, ruptured aneurysms may represent separate technical challenges not encountered in the treatment of non-ruptured aneurysms.

The reported percentage of patients with a ruptured aneurysm suitable for endovascular repair varies considerably. Disparities among studies are confounded by variations in patient's selection criteria, the haemodynamic threshold acceptable for endovascular repair (Yilmaz et al., 2002; Lachat et al., 2002), evolving endograft designs (Ohki et al., 2000; Orend et al., 2002; Yilmaz et al., 2002; Lachat et al., 2002; Willmann et al., 2001) availability of experienced staff, and other practical aspects (Yilmaz et al., 2002). Despite these irregularities and the disconcerting report of a 45% mortality rate from one center's initial experience (Hinchliffe et al., 2001), an

undeniable trend is emerging. Lower mortality, in the range of 10% to 20%, can be achieved in carefully selected patients with ruptured aneurysms (Ohki et al., 2000; Lachat et al., 2002; Yilmaz et al., 2002), which is far better than the 40% to 60% operative mortality for transabdominal repair (Basnyat et al., 1999; Gloviczki et al., 1992).

This study found approximate 40% of ruptured abdominal aortic aneurysms were suitability for endovascular repair and this concurs with other retrospective and prospective "intention-to-treat" studies (Lachat et al., 2002; Willmann et al., 2001; Reichart et al., 2003). It is at odds with 2 reports (Ohki et al., 2000; Peppelenbosch et al., 2003) quoting up to 80% suitability. The proportion of non-ruptured aneurysms suitable for endovascular repair in our cohort was 68%, which is at the higher end of the reported 30% to 70% range (Wolf et al., 2000; Woodburn et al., 2001).

The aorto-mono-iliac device facilitates rapid exclusion of ruptured aneurysms from the circulation and obviates the need for contralateral limb deployment (Reichart et al., 2003). However, there has been a move toward bifurcated endografts in ruptured aneurysms, which may have been driven by the increasing experience with this graft configuration in the elective setting. Favourable results have been observed (Scharrer-Palmer et al., 2003; Lachat et al., 2002), and comparable operating times are reported (Orend et al., 2002; Scharrer-Palmer et al., 2003). The bifurcated endograft preserves native vessel anatomy, is associated with a reduced incidence of type I endoleak, and avoids the need for iliac occlusion and an extra-anatomical femoro-femoral conduit (Moore et al., 2001; Chuter et al., 1999). The use of an aorto-mono-iliac graft has been reported to increase the proportion of ruptured aneurysms suitable

for endovascular repair by overcoming unfavourable iliac anatomy (Ohki et al., 2000; Reichart et al., 2003). The iliac component dimensions of the generic endografts considered in this study facilitated the inclusion of patients with common iliac artery diameters up to 20 mm. Given this specification, an aorto-mono-iliac device may not have increased acceptance in our study cohorts.

The principal factor contraindicating endovascular repair in both non-ruptured and ruptured aneurysm groups was unacceptable neck morphology, which has been reported previously (Peppelenbosch et al., 2003; Hinchliffe et al., 2003). The consideration of endografts with proximal diameters >32 mm would not increase the proportion of ruptured aneurysms appropriate for endovascular repair in our study. Indeed, the reported deployment of endografts in aneurysms with challenging neck morphology points to an increased risk of acute proximal type I endoleak (Reichart et al., 2003), which may necessitate conversion to open repair (Hinchliffe et al., 2001; Sternbergh et al., 2002).

The application of our findings may be limited for the following reasons. The series of CT scans of ruptured aneurysms examined retrospectively in this study were collected over an 8-year period, and the CT quality and slice depth were not uniform throughout the series. If anything, this may have contributed to underestimating the number of ruptured aneurysms suitable for endovascular repair. A patient's pre-operative haemodynamic status was not considered in this study, and extreme hypotension is a recognized contraindication to CT scanning (Peppelenbosch et al., 2003; Reichart et al., 2003). This study was carried out in a single center with an established endovascular program for non-ruptured and ruptured aneurysms. This

experience may have biased the results toward higher levels of inter-observer agreement and acceptability for endovascular repair in both ruptured and non-ruptured cohorts. Most significantly, our ruptured group represents only a 20% subset of patients with ruptured aneurysms seen in our unit. The reason patients in this study underwent CT scanning was primarily diagnostic uncertainty, but one could conclude that this group represents an ideal starting point for the initiation of a ruptured endovascular program. Whether this self-selection had a biased effect on the aneurysm morphology is indeterminate.

Summary

This study demonstrates that approximately 40% of ruptured aneurysms are suitable for endovascular repair. When initiating an endovascular program for ruptured aneurysms, we recommend having an inventory of endografts with aortic components of 24, 28, and 32 mm and a duplicate set of iliac components ranging from 12 to 22 mm.

2.6 Conclusion

Reducing the mortality from abdominal aortic aneurysms requires an active programme aimed at decreasing the number of aneurysms rupturing. Population ultrasound screening to detect aneurysms at the quiescent stage will increase the frequency of elective aneurysm repair while decreasing aneurysm rupture by up to 50%. Aneurysms less than 5.5cm can normally be safely observed. Observation should be accompanied by lifestyle and medical risk adjustment including the cessation of smoking and the treatment of hypertension and hypercholesterolemia. Aneurysm repair is indicated if the maximal diameter exceeds 5.5cm or at a lesser diameter with expansion exceeding 1cm per year or the development of symptoms. Endovascular repair is preferable in patients surgically fit for open repair. With co-morbidities prohibitive of open repair, best practice dictates surgical non-intervention but medical optimisation until such times as the patient is fit. The mortality from ruptured abdominal aortic aneurysms has decreased slowly over 20 years; the application of endovascular repair to ruptured AAA may decrease this further. The pursuit of medical therapy for small aneurysms is ongoing and can potentially impact on the mortality of this disease process.

CHAPTER 3

INTRODUCTION

BIOLOGICAL CHANGES IN THE WALL OF THE ABDOMINAL AORTIC ANEURYSM

3.1 Cellular Changes in Abdominal Aortic Aneurysms

Smooth Muscle Cells
Fibroblasts
Vascular Endothelial Cells
Macrophage
Lymphocytes
Neutrophils

3.2 Proteolytic Changes in Abdominal Aortic Aneurysms

3.3 Matrix Metalloproteinases in Abdominal Aortic Aneurysms

The Elastases
The Collagenases
Other Proteolytic Effectors
Tissue Inhibitors of MMP'

3.4 Other Proteolytic Activators in Abdominal Aortic Aneurysms

Cysteine Proteases
Plasminogen Activators

3.5 Matrix Metalloproteinases in Abdominal Aortic Aneurysm Rupture

3.6 Rational For Thesis

The structural integrity of the abdominal aortic wall is dependent on extracellular matrix proteins. In turn extracellular proteins are dependant for their synthesis upon the mesenchymal cells found within the aortic media and adventitia. Two groups of cells and extracellular matrix proteins predominate – smooth muscle and fibroblast cell types, and elastin and collagen proteins (Baxter et al., 1992)(Rizzo et al., 1989). Elastins are an amorphous group of proteins located in the media that provide extensile properties to the aorta (Baxter et al., 1992). The fibrillar collagens type I and III impart tensile strength to the aorta and are largely distributed in the adventitia (Davidson et al., 1985).

Aneurysmal dilatation is associated with a chronic inflammatory response (Newman et al., 1994), a decrease in medial smooth muscle cells (Annabi et al., 2002)(Lopez-Candales et al., 1997), and a disproportionate degradation of elastins and collagens (Cohen et al., 1987)(Evans et al., 1991). Animal models and human data suggest that loss of elastin is responsible for early aortic expansion and loss of recoil, whilst late expansion and rupture are modulated by collagen breakdown (Dobrin et al., 1984). To understanding the process of elastin and collagen loss is to understand the mechanism of aneurysm formation itself.

3.1 Cellular Changes In Abdominal Aortic Aneurysms

It is evident that the extensive matrix remodelling within the abdominal aortic aneurysm wall involves degradation of the diverse structural matrix proteins, progressive involvement of multiple cell types, and many different enzymes. The normal aorta is almost entirely devoid of inflammatory cells (Rizzo et al., 1989). By contrast, the aneurysmal aorta demonstrates a prominent inflammatory infiltrate, comprising largely lymphocytes and macrophages (Brophy et al., 1991)(Freestone et al., 1995). Good evidence exists for the active expression of various elastin- and collagen-degrading MMP's by myofibroblasts, vascular endothelium and inflammatory cells. How these different cell types and their proteolytic products might interact during the various stages of aneurysm evolution remains to be delineated.

Smooth Muscle Cells

Smooth muscle cells maintain matrix protein levels within the aortic wall but are otherwise considered quiescent under normal physiological conditions (Chamley-Campbell et al., 1981). Following physical or chemical insult, these cells are capable of a phenotypic de-differentiation and adoption of a synthetic potential required for injury repair (Casscells et al., 1992). Interestingly, smooth muscle cell de-differentiation is associated with decreased alpha-isoactin expression, whilst cyto-differentiation is associated with cessation of synthetic conditions, induction of alpha-isoactin production and return to the quiescent state (Owen et al., 1986). Levels of alpha-isoactin expression are reported to decrease by 90% in aneurysmal aortic tissue

compared to normal (Annabi et al., 2002) with levels of other markers remaining constant.

Up-regulation of MMP's in aneurysmal aortic wall is well documented (McMillan et al., 1997; Freestone et al., 1997). The tissue synthesis of many MMP's has been co-localised to smooth muscle or mesenchymal cells by immunohistochemistry and *in situ* hybridisation. Smooth muscle cell culture models of aneurysmal and non-aneurysmal aortic origin reinforce this association, though *in vitro* models may not accurately reflect what is happening *in vivo* (McMillan et al., 1995). Smooth muscle cells are implicated in the expression of many MMP's, most notably MMP-2 and the TIMP's (McMillan et al., 1997; Freestone et al., 1997).

An increase in the apoptotic rate of smooth muscle cells is reported in the aneurysmal wall (Henderson et al., 1999; Rowe et al., 2000). Indeed smooth muscle cell death is not compensated for by proliferation, and overall cell density in aneurysms is reduced (Fukui et al., 2003). The recognition of cell apoptosis and phagocytosis by macrophages or adjacent smooth muscle cells, prevents release of intracellular pro-inflammatory mediators (Bennett et al., 1995). Failure of phagocytosis to keep pace with apoptosis may contribute to the release of oxidising molecules and cationic proteins precipitating chronic inflammation.

Fibroblasts

Fibroblasts are present in the adventitia of normal and aneurysmal aorta. Whilst the role of other mesenchymal cells, most notably smooth muscle cells, have been extensively investigated within the aortic media, the potential influence of the fibroblast has been overlooked. Fibroblast production of MMP's in response to stimuli are documented in tumour invasion, particularly MMP-2 (Aimes et al., 1995; Okada et al., 1995). Induction of MMP expression by fibroblasts in the outer layers of the aneurysm wall might act to rapidly destabilize the aneurysm wall, accelerating aneurysm expansion and lead to rupture, however, to date only one paper describes an immunohistochemical link (Curci et al., 1998).

Vascular Endothelial Cells

The luminal surface of an abdominal aortic aneurysm is represented by thrombus and is devoid of endothelial cells and recognizable intima, however, endothelial cells remain in the vasa vasorum. These vessels show increased density in comparison to normal or atherosclerotic aorta and have a strong spatial correlation to elastin disruption and chronic inflammatory infiltrates (Freestone et al., 1995; McMillan et al., 1995; Thompson et al., 1995). Increased cellular density from neovascularisation suggests the adoption of a proliferative and secretory phenotype associated with MMP-1 production (Herron et al., 1991). The association between vasa vasorum and inflammatory infiltrates suggests an association between endothelial cells and tissue remodelling.

Macrophage

The infiltration of monocyte/macrophage cells is characteristic of the histological changes observed in the aneurysmal aortic wall (Brophy et al., 1991). Macrophages are localised primarily to the medial-adventitial junction (Thompson et al., 1995; Davis et al., 1998) and have also been described adjacent to the peri-adventitial vasa vasorum (McMillan et al., 1995). A correlation between elastase breakdown and mononuclear cell infiltration is observed (Allaire et al., 1997). Aneurysm-infiltrating macrophages are likely to directly mediate extracellular matrix breakdown by expressing the protease MMP-9 and possibly MMP-2 (Thompson et al., 1995; McMillan et al., 1995). Furthermore, an indirect mechanism for extracellular matrix destruction is suggested via the interleukin-1 paracrine mediated up-regulation of MMP production by cultured smooth muscle cells (Lee et al., 1995).

Lymphocytes

The aneurysm wall is noted for its chronic inflammatory lymphocytic infiltrate with T and B cells (Freestone et al., 1995). T and B lymphocytes are normally activated by the presentation of processed antigen by HLR-DR positive macrophages, endothelial cells, vascular smooth muscle cells or CD4+ T-helper cells. Histological analysis reveals that much of the infiltrate is in the adventitia (Koch et al., 1990). The correlation between the extent of inflammatory infiltrate, loss of smooth muscle cells, elastin breakdown and increased number of vasa vasorum suggest a role for the lymphocyte in the aneurysm process (Anidjar et al., 1991; Thompson et al., 1996). T-

lymphocytes can produce MMP-2 following their adhesion to endothelial cells (Romanic et al., 1994), however, no histological study has localised MMP expression to aortic lymphocytes *in situ*. The mechanism of smooth muscle cell apoptosis may be triggered by T lymphocyte production of Fas protein. Fas is a protein recognised to induce apoptosis through a ligand-receptor interaction and up-regulation of caspase proteases (Geng et al., 1995). Smooth muscle cell induced apoptosis is described *in vitro* following priming with cytokines such as interferon-γ, tumour necrosis factor and interleukin-1 (Geng et al., 1996). The observation that Fas protein exists in aneurysms but not in normal aorta further supports this pathway (Henderson et al., 1999).

A conflicting role for the lymphocyte in the aneurysm pathology is also proposed. Production by T-lymphocytes of interferon-gamma or interleukin-1 has been demonstrated to reduce monocyte-macrophage synthesis of the MMP's and increase TIMP-1 levels (Lacraz et al., 1994; Shapiro et al., 1990; Romanic et al., 1994). This dichotomous interpretation suggests that the lymphocyte might reduce MMP production as part of a protective measure.

Neutrophils

The neutrophil was initially implicated in aneurysmal pathogenesis following the description of neutrophil elastase in the aneurysm wall (Cohen et al., 1987). However, neutrophils were not detected in the aortic media, except for an occasional neutrophil caught within the vasa vasorum (Cohen et al., 1991) and a single

description in the adventitia of elastase induced aneurysms in mice (Pyo et al., 2000). Therefore, little interest was proportioned to this cell type and its associated proteases until recently, whereupon production of neutrophil elastase and neutrophil collagenase was attributed to the macrophage and other atheroma associated cell groups (Herman et al., 2001; Dollery et al., 2003).

3.2 Proteolytic Changes In Abdominal Aortic Aneurysms

White et al., reported a loss of elastin fibers of between 81% and 86% in small abdominal aortic aneurysms compared to control aorta. The widely quoted conclusion from White et al., states that elastin is lost early in aneurysm development and does not contribute to further growth. This was based on the failure to detect further elastin loss in medium sized or large aneurysms (White et al., 1993). Sakalihasan et al., confirmed White's results noting a highly significant reduction in elastin concentration with aneurysm formation but a progressive but non-significant reduction with increasing diameter and rupture (Sakalihasan et al., 1993).

The experiments published in 1984 by Dobrin et al., demonstrated that in vitro perfusion of canine carotid artery with elastase caused aneurysmal dilatation whilst perfusion with a collagenase caused less dilatation but resulted in rupture in every case. When conducted on human iliac artery, less dilation was recorded following elastase perfusion (Dobrin et al., 1984). Perfusion of human iliac artery with a collagenase produced a greater dilatation then rupture in all human vessels (Dobrin et al., 1984). These findings suggest initial aneurysm formation in humans is associated with elastin loss, but continued aneurysm growth and ultimately rupture requires breakdown of collagen.

Two studies indicated the infusion of murine aorta with an ultra-pure pancreatic elastase was insufficient to induce aneurysm formation, in spite of evidence of elastin degradation (Curci et al., 1999; Carsten et al., 2001). Indeed comparing elastases of varying purity, the most highly purified enzyme produced the smallest aortic dilation.

In contrast, the elastase preparation with the greatest aneurysm formation was the least pure with the highest contaminated with other polypeptides. There was no correlation between aneurysm size and the residual elastin content. The additional polypeptide or protease contaminant within less pure pancreatic elastase was required to initiate inflammation and aneurysm formation (Curci et al., 1999; Carsten et al., 2001). Importantly the degree of elastase purity was not recorded in Dobrin's studies (Dobrin et al., 1984).

Busuttil et al., first described collagenolytic activity in aortic aneurysm wall biopsies in 1980. The study compared 11 aneurysmal aortas to 5 aorto-occlusive specimens. Despite being unable to extract a collagenase, collagenolytic activity was determined by immunoblotting in the aneurysmal wall but not the occlusive aortas. The group reportedly found collagenolytic activity to correlate positively with aneurysm diameter (Busuttil et al., 1980). The difficulty in quantifying collagenase activity is well documented since their extraction from tissue requires their dissolution from endogenous substrate and inhibitors, for which collagenases have a high affinity (Murphy et al., 1985; Weeks et al., 1976).

Zarins et al., in 1986 described a cynomolgus monkey model, surgically establishing either mild or severe mid-thoracic aortic stenosis. After six months, monkeys with mild stenosis had no post stenotic dilatation and a significant decrease in collagenase activity compared with the same aortic region in control monkeys lacking stenosis. In contrast monkeys with severe stenosis had minimally discernable post-dilatation but a two-fold increase in collagenase activity at three months. The monkeys with severe aortic stenosis went on to develop pronounced post-stenotic dilatations at six months.

One of two conclusions can be drawn from this work. Firstly, increased collagenase activity in the aorta distal to a severe stenosis predates and may cause significant aortic dilatation or increased collagenase activity and dilatation are secondary to altered heamodynamics (Zarins et al., 1986).

Menashi et al., 1987 demonstrated collagenase activity in the aneurysmal aortic wall but only after activation of latent collagenase with trypsin, and the abstraction of endogenous inhibitor. Collagenase activity was raised in ruptured aneurysms compared to non-ruptured aneurysms, suggesting increased collagen breakdown associated with but not conclusively pre-empting the rupture event (Menashi et al., 1987). However, Herron et al., failed to demonstrate any collagenolytic activity within control or aneurysmal aortic tissue following sodium dodecyl-sulphate substrate gel electrophoresis and immunoprecipitation (Herron et al., 1991).

Vine and Powell confirmed detection of an activated collagenase by immunoblotting in all homogenates of aneurysmal aorta, in just under half of athero-occlusive aorta but not in normal aorta (Vine and Powel, 1991). Evans et al., measured collagenase levels in an aortic explant model of rabbit abdominal aorta. Collagenase activity was detected in explant cultures stimulated with interleukin-1 or phorbol myristate acetate when fully activated with para-aminophenylmercuric acetate (Evans et al., 1991).

Powell and Greenhalgh showed increased collagenase activity in biopsies of five ruptured abdominal aortic aneurysms and comparatively little activity in non-ruptured aneurysms. Collagenase activity in non-ruptured aortic explants was increased six-fold following the addition of the medium from an established aortic macrophage

culture. This increase fell someway short of the collagen activity in unstimulated ruptured aortas. Interestingly, a comparatively small increase in collagenase activity was observed with ruptured aortic explants stimulated by the same macrophage products. Histological examination of the ruptured aneurysm wall confirmed an increased prevalence of inflammatory cells. This suggested that inflammatory cells, in particular macrophages, may be either the source of collagenase activity or produce the paracrine trigger for its production from another cell source (Powell et al., 1989).

Various endogenous proteases have been investigated for collagenase and elastase activity including plasminogen activators (Carmeliet et al., 1997; Reilly et al., 1994), serine and cysteine proteases (Cohen et al., 1992; Shi et al., 1999), and matrix metalloproteinases (Newman et al., 1994; Freestone et al., 1995; Thompson et al., 1995). Following a decade of study, matrix metalloproteinases have emerged as the foremost group of enzymes involved in this process.

3.3 Matrix Metalloproteinases in Abdominal Aortic Aneurysms

The matrix metalloproteinases (MMP's) are a family of zinc dependent endopeptidases that share sequence homologue and a common multi-domain organisation. These enzymes are capable of degrading all the components of the extracellular matrix. Currently, 23 vertebrate MMP's have been identified and are organised into five main classes - collagenases MMP-1, MMP-8, MMP-13, MMP-18; gelatinases MMP-2, MMP-9; stromelysins MMP-3, MMP-10, MMP-11, MMP-19, MMP-20); membrane type MMP-14, MMP-15, MMP-16, MMP-17, MT-MMP-1 and -6; and a heterogeneous group MMP-7, MMP-12, MMP-18. MMP activity is closely regulated by a variety of mechanisms; transcriptional control at the RNA level; proteolytic activation by plasmin, trypsin or other MMP's; inhibition by an endogenous family of tissue inhibitors of metalloproteinases - TIMP-1, 2 and 3 (Woessner, 1991).

The MMP's are made up of a variety of domain structures, which demonstrate a specific function or sequence homologue within the MMP family. Whilst no enzyme contains all the domains, three domains are present within all MMP's - a signal peptide, a propeptide and a catalytic domain. The other common domains are hemopexin-like, linker, fibronectin type-II, vitronectin-like, furin-recognition sequence and transmembrane.

The classification of MMP's according to substrate specificity is rather arbitrary, because many of the MMP's share substrates between groups and little is known about their *in vivo* activities. For example the characteristic feature of a collagenase is

the ability to cleave native fibrillar collagens (types I, II and III) at precise Gly-Ile or Gly-Leu bonds yielding ¾ and ¼ length fragments (Gross et al., 1974). However, the gelatinases MMP-2 and MMP-9 degrade non-fibrillar collagens, elastin, gelatin as well as partially degraded fibrillar collagen (Aimes et al., 1995). The stromelysins have broad substrate specificity, including proteoglycans and laminin (Okada et al., 1986). To confound things completely, the membrane type MMP's (MT-MMP's) are grouped together by virtue of their transmembrane domain structure rather than function. Comprehensive reviews by Birkedal-Hansen (Birkedal-Hansen, 1993) and Nagase and Woessner (Nagase and Woessner, 1999) describe the MMP family in detail. The principal substrate specificities of each MMP are shown in Table 3.1.

MMP's are normally expressed at a background level in healthy tissue and are involved in processes such as wound healing (Philipp et al., 2005; Mulholland et al., 2005; Toy, 2005), pregnancy and parturition (Becher et al., 2004; Merchant et al., 2004) and bone resorption (Andersen et al., 2004; Shorey et al., 2004). However, this family of enzymes are implicated in a range of disease processes in which destruction of the extracellular matrix is a key feature, such as invasion and metastasis of cancer (Handsley et al., 2005; Lee et al., 2005), rheumatoid arthritis (Garcia-Vicuna et al., 2004), periodontal disease (Pozo et al., 2005; Ramamurthy et al., 2005), liver and renal fibrosis (Lichtinghagen et al., 2003; Gong et al., 2005), atherogenesis (Molloy et al., 2004; Fabunmi et al., 1998) and aortic aneurysm formation (Thompson et al., 2002).

Enzyme	MMP	Matrix substrate and function	Location
Collagenases			
Interstitial Collagenase	MMP-1	Collagens (I, II, III, VII, VIII, X), Gelatins, Entactin, Aggrecan, link protein, pro-MMP-2 & MMP-9	11q22-q23
Neutrophil collagenase	MMP-8	Collagens (I, II, III, V, VII, VIII, X), Aggrecan, α_2-antiplasmin, link protein	11q21-q22
Collagenase 3	MMP-13	Collagens (I, II, III, IV, IX, X, XIV), Gelatin, Plasminogen activator inhibitor, pro-MMP-9	11q22.3
Collagenase 4	MMP-18	Collagen I	Not determined
Gelatinases			
Gelatinase A	MMP-2	Gelatins, Collagens, Fibronectin, Laminin, Aggrecan, Elastin, large tenascin-C, Vitronectin, pro-MMP-1,9,13	16q13
Gelatinase B	MMP-9	Gelatins, Collagens IV, V, XIV, Aggrecan, Elastin, Entactin, Vitronectin, IL-1β, Plasminogen	20q11.2-q13.1
Stromolysins			
Stromolysin 1	MMP-3	Gelatins, Collagens, Fibronectin, Laminin, Aggrecan, large tenascin-C, Vitronectin, plasminogen, pro-MMP-1 "superactivation", MMP-2/TIMP 2 complex, pro-MMP-7,8,9,13.	11q23
Stromolysin 2	MMP-10	Aggrecan, Fibronectin, Collagens (III, IV,V), MMP-1,8	11q22.3-q23
Stromolysin 3	MMP-11	Weak activity on fibronectin, collagen IV, aggrecan, gelatins	22q11.2
Metalloelastase	MMP-12	Collagen IV, gelatin, elastin, casein, fibronectin, vitronectin, laminin, entactin, fibrinogen, fibrin, plasminogen	11q22.2-q22.3
Membrane-type MMP's			
MT1-MMP	MMP-14	Collagens (I,II,III), Fibronectin, Laminin-1, Vitronectin, Activates pro-MMP-2 & pro-MMP-13	14q11-q12
MT2-MMP	MMP-15	Fibronectin, large tenascin-C, entactin, laminin, aggrecan, perlecan, pro-MMP-2	16q12.2-q21
MT3-MMP	MMP-16	Collagen III, gelatin, casein, fibronectin, Activates proMMP-2	8q21
MT4-MMP	MMP-17	Not Known	Not determined
Others			
Matrilysin	MMP-7	Collagens (IV, X), Aggrecan, Fibronectin, Laminin, Gelatins, Collagen IV, Elastin, Entactin, small & large tenascin-C, Vitronectin, plasminogen, pro-MMP-1,2,9, MMP9/TIMP 1 complex	11q21-q22
(Unnamed)	MMP-19	Gelatin	12q14
Enamelysin	MMP-20	Amelogenin	Not determined
Tissue Inhibitors			
TIMP 1,3,4		Inhibits MMP-1, MMP-3, MMP-9	
TIMP 2		Inhibits MMP-2	

Table 3.1: Principal substrate specificities of each MMP

Table 3.2 illustrates the normal physiological and pathological role in which the MMP's are implicated.

Normal Mechanisms	Pathological Mechanisms
Development	**Tissue Destruction**
Blastocyst implantation	Rheumatoid arthritis
Embryonic development	Osteoarthritis
Nerve growth	Cancer invasion
Growth plate cartilage removal	Cancer metastasis
Skeletal, bone growth	Gastric ulceration
Nerve outgrowth	Corneal ulceration
Primary tooth resorption	Periodontal disease
Reproduction	**Fibrotic Diseases**
Endometrial cycle	Liver cirrhosis
Graafian follicle rupture	Fibrotic lung disease
Luteolysis	Otosclerosis
Cervical dilation	Atherosclerosis
Postpartum uterine involution	Multiple sclerosis
Mammary gland morphogenesis	
Mammary gland involution	
Rupture of fetal membrane	
Maintenance	**Weakening of Matrix**
Remodelling of bone	Dilated cardiomyopathy
Hair follicle cycle	Epidermolysis
Wound healing	*Aortic aneurysm*
Angiogenesis	
Apoptosis	
Nerve regeneration	
Macrophage function	
Neutrophil function	

Table 3.2. Normal and Pathological Mechanisms in which MMP's are involved.

The pathological changes seen in the extracellular matrix of abdominal aortic aneurysms result from an imbalance in MMP activity and many studies now report increased elastase and collagenase activity within the wall of aortic aneurysms. The cellular sources of the MMP's implicated in abdominal aortic aneurysm pathogenesis are described in Table 3.3.

Enzyme	Cellular Source	Author	Year
Collagenases			
MMP-1	Mesenchymal Cell	Newman et al	1994
	Vascular Endothelial Cell	Newman et al	1994
MMP-13	Smooth Muscle Cell	Mao et al	1999
Elastases			
MMP-2	Macrophage	McMillan et al	1995
		Curci et al	1998
	Smooth Muscle Cell	McMillan et al	1995
		Curci et al	1998
		Davis et al	1998
		Crowther et al	2000
	Fibroblasts	Curci et al	1998
MMP-9	Macrophage	Newman et al	1994
		McMillan et al	1995
		Thompson et al	1995
		Curci et al	1998
		Davis et al	1998
		Herron et al	1991
		Sakalihalsan et al	1993
		Palambo et al	1999
	Smooth Muscle Cell	Newman et al	1994
	Neutrophil	Nagashima et al	2002
MMP-12	Macrophage	Curci et al	1998
Others			
MMP-3	Macrophage	Newman et al	1994
MMP7	Cell type not specified	Curci et al	1998
Tissue Inhibitors			
TIMP-1	Smooth Muscle Cell	McMillan et al	1995
TIMP-2	Macrophage	McMillan et al	1995
	Smooth Muscle Cell	McMillan et al	1995
		Crowther et al	2000

Table 3.3: Cellular sources of the MMP's and TIMPs identified by immunohistochemistry or in situ hybridisation in aneurysmal abdominal aorta.

The Elastases

MMP-2 (gelatinase A)

MMP-2 is a 72kiloDalton gelatinase that cleaves elastin and nonfibrillar collagens types IV, V, VII and X (Okada et al., 1990; Senior et al., 1991). The successful cleaving by MMP-2 of type I collagen is reported at non-physiological conditions (Aimes et al., 1995), however, at 37°C MMP-2 can only lyse denatured and fragmented fibrillar collagens (Willhelm et al., 1989; Collier et al., 1988). Zymography and immunoblotting studies describe higher active levels of MMP-2 in smaller compared to larger abdominal aortic aneurysms, representing an inverse correlation between MMP-2 activity and aneurysm diameter (Freestone et al., 1995). A significantly higher proportion of MMP-2 is present as the active isoform in aneurysmal tissue, with the ratio of the active fraction correlating to the level of inflammation (Freestone et al., 1995). McMillan et al., demonstrate a five-fold elevation in the mRNA levels of MMP-2 in vascular smooth muscle monoculture from aneurysmal over normal aorta (McMillan et al 1995), however, an elevation in MMP-2 transcript was not determined in other studies (Tamarina et al., 1997; Elmore et al., 1998).

Immunohistochemical studies have localised MMP-2 to mesenchymal cells, probably smooth muscle cells, within the aortic media and subadventitia (Davis et al., 1998; Crowther et al., 2000). This may lead to elastin and non-fibrillar collagen breakdown in the aortic media, but not interfere with matrix turnover within the adventitia. Less

commonly MMP-2 has been shown to localise to macrophages (McMillan et al., 1995).

MMP-9 (gelatinase B)

MMP-9 is the most prominent elastolytic MMP expressed in AAA tissue (Vine and Powell, 1991; Elmore et al., 1998; Tamarina et al., 1997). Being similar to MMP-2, MMP-9 can also rapidly fragment partially degraded fibrillar collage (Wilhelm et al., 1989; Collier et al., 1988). Highly purified MMP-9, in the absence of TIMP and synthetically activated with para-aminophenylmercuric acetate, is also reported to degrade types III, IV and V collagens (Watanabe et al., 1993). Gelatin-zymography indicates significantly raised MMP-9 activity in aneurysmal compared with normal aorta (Sakalihasan et al., 1996). MMP-9 levels have been shown to correlate positively with increasing aneurysm size (Freestone et al., 1995), though a negative correlation between MMP-9 activity and aneurysm diameter is also reported (Petersen et al., 2000; Sakalihasan et al., 1996). At transcription level, MMP-9 shows a 18-fold elevation in aneurysmal over normal aorta (McMillan et al., 1995). Expression of MMP-9 mRNA is highest in medium sized aneurysms of 5 to 6.9cm but significantly lower in aneurysms of 7cm or greater (McMillan et al., 1997). A critical role for MMP-9 is supported by targeted disruption of the MMP-9 gene in mice, which suppresses experimental aneurysm formation. Pro-aneurysmal phenotype is restored following transplantation of wild type bone marrow cells (Pyo et al., 2000). Levels of MMP-9 are also elevated in plasma from patients with aneurysms compared with

patients with aortoiliac occlusive disease (McMillan et al., 1999) and have been shown to correlate positively with aneurysm expansion (Lindholt et al., 2000).

The majority of cell culture studies support the macrophage as the primary source of MMP-9 within aneurysmal tissue of the abdominal aorta (Thompson et al., 1995; Lopez et al., 1997; Davis et al., 1998). Immunohistochemistry preferentially localises MMP-9 to macrophages of the adventitia (Palombo et al., 1999). Fewer studies proportion MMP-9 production to aortic mesenchymal cells (Evans et al., 1991; Ishii and Asuwa et al., 2000; Patel et al., 1996).

MMP12 (metalloelastase)

In 1998 Curci et al., determined the expression of MMP-12 in abdominal aortic aneurysms, athero-occlusive aorta, and normal aorta, however, no age or biopsy site details were provided in the manuscript (Curci et al., 1998). The group demonstrated MMP-12 mRNA in all aneurysmal and athero-occlusive aortic tissues. Other studies have failed to demonstrate MMP-12 mRNA (Elmore et al., 1998). Protein levels of MMP-12, recorded from aortic tissue, show a seven-fold increase in aneurysmal compared to normal aorta. Production of MMP-12 is demonstrated in abdominal aortic aneurysm tissue by *in situ* hybridisation and immunohistochemistry, and is localised to macrophages within the media (Curci et al., 1998). Immunohistochemical studies on longitudinal sections crossing the transition between dilated aneurysmal aorta and non-dilated aorta proximally, reveal an absence of MMP-12 in the non-dilated portion but high expression at the transition zone (Curci et al., 1998).

Pyo et al., found that isolated genetic deficiency of MMP-12 does not have a significant influence on elastin fiber degradation or aneurysm development, indicating that MMP-12 is not essential for elastase-induced abdominal aortic aneurysm formation and that its expression cannot compensate for the absence of MMP-9. This author also demonstrated a slight enhancement of the aneurysm-resistant phenotype in double-deficient mice compared with those with MMP-9 deficiency alone indicating that MMP-12 might still be acting to assist elastin fiber degradation (Pyo et al 2000).

The Collagenases

MMP-1 (collagenase I)

Collagenase I (MMP-1) was the first metalloproteinase to be described. MMP-1 cleaves type III collagen up to15 times faster than type I collagen (Hasty et al., 1987) and has poor activity against type II collagen and other extra-cellular proteins (Welgus et al., 1985). Immunoblotting has shown an elevation of MMP-1 at protein level in aneurysmal abdominal aorta compared to normal aorta (Irizzary et al., 1993; Newman et al., 1994; Annabi et al., 2002), however, expression in aneurysms may not be significantly different from athero-occlusive disease (Mao et al., 1999). Tamarina et al., confirmed a twelve-fold transcriptional elevation in MMP-1 mRNA expression in aneurysmal tissue compared to non-age-matched normal aorta (Tamarina et al., 1997). Immunohistochemistry localises MMP-1 to adventitial smooth muscle cells

and vascular endothelial cells in aneurysms (Newman et al., 1994; Sasaguri et al., 1994).

MMP-13 (collagenase III)

MMP-13 preferentially cleaves fibrillar type II collagen, the predominant collagen in cartilage, but is 5 or 6 time less efficient at cleaving types I and III collagen (Knauper et al., 1993). In addition, MMP-13 degrades type IV collagen and can activate latent pro-MMP's. MMP-13 protein and mRNA are uniformly expressed in aneurysmal abdominal aortic biopsies, but not in normal aorta (Mao et al., 1999). The expression of MMP-13 is considered to be more prominent in aneurysmal than atherosclerotic aorta. Immunoreactivity to MMP-13 is detected in the outer aortic wall of aortic aneurysmal biopsies, where it is localized, in part to smooth muscle cells. Smooth muscle cells from normal aorta express MMP-13 only in the presence of growth factor stimulants (Mao et al., 1999).

MMP-8 (collagenase II)

The collagenase, MMP-8 like other collagenases, cleaves fibrillar collagens at the triple helical domain. MMP-8 cleaves type I collagen the predominant fibrillar collagen in blood vessels (Hasty et al., 1987; Murphy et al., 1991). MMP-8, was first reported in human neutrophils in 1968 (Lazarus et al., 1968) hence its alias "Neutrophils Collagenase." Initially MMP-8 transcription was considered to occur

only in maturing bone marrow associated neutrophils, the enzyme to be stored in specialized granules, and transcription to cease once the neutrophil was mature (Mainardi et al., 1991). This assumption has been overturned since the finding of MMP-8 transcripts in circulating neutrophils, articular chondrocytes, synovial fibroblasts and human umbilical vein endothelial cells (Cole et al., 1995).

Interest was renewed following elaboration *in vitro* and *in situ* of MMP-8 from vascular atheroma associated endothelial cells, macrophages and smooth muscle cells (Herman et al., 2001). Expression of MMP-8 from atheroma associated cell types required prolonged exposure to proinflammatory cytokines suggesting that MMP-8 was not stored in granules by these cells but released soon after synthesis (Herman et al., 2001).

The collagenolytic activity of this enzyme has never been described in aortic tissue – healthy or otherwise. However, PRC/Southern blotting of MMP-8 by Moa et al., characterised the "at best" inconsistent expression of MMP-8 mRNA in aneurysmal and athero-occlusive abdominal aorta, and total absence in normal aorta (Mao et al., 1999). In other chronic inflammatory conditions MMP-8 is regarded as playing a central role in of matrix degradation; MMP-8 activity is shown in chronic bronchiectasis (Sepper et al., 1995), cystic fibrosis, in which the level of activity correlated to the disease state (Power et al., 1994), and rheumatoid arthritis (Matsuki et al., 1996).

Other Proteolytic Effectors

MMP-3 (stromelysin I)

MMP-3 digests collagen type III and IV, and fibronectin (Collier et al., 1988). MMP-3 also plays a central role in the pericellular MMP activation cascade by cleaving other MMP proenzymes to their active forms, including interstitial collagenase, matrilysin and gelatinase B. Carrell et al., reported the transcription level of MMP-3 to be 40-fold higher in the aneurysmal aortic tissue compared with occlusive aortic samples (Carrell et al., 2002). Immunohistochemical studies localize the expression of MMP-3 to macrophages within the AAA wall (Newman et al., 1994).

MMP-7 (matrilysin)

MMP-7 has substrate activity against elastin and collagen type IV but is poorly described in aortic tissue. Elmore et al., failed to detect MMP-7 mRNA transcripts in any of the 15 abdominal aortic aneurysms examined (Elmore et al., 1998). Carrell et al., detected MMP-7 in 8 abdominal aortic aneurysms and 8 athero-occlusive aortic biopsies, though they failed to demonstrate any difference in levels between the two groups (Carrell et al., 2002). Curci et al., attempted immunohistochemical detection of MMP-7 in AAA tissue but reported it to be rare and did not specify a cell type of origin (Curci et al., 1998).

Tissue Inhibitors of MMP's

Inhibition of MMP activity is accomplished by the Tissue Inhibitors of MMP's (TIMP's), which currently include TIMP-1, TIMP-2, TIMP-3 and TIMP–4 (Borden et al., 1997; Fridman et al., 1993). MMP's form tight non-covalently bonded complexes with TIMP's in a 1:1 stiochiometric fashion (Gomis-Ruth et al., 1997). TIMP-1 and TIMP-2 are principal TIMP's described in the aorta, though their characterisation is incomplete.

McMillan et al., reported no difference in transcription levels of TIMP-1 and TIMP-2 between age and site-matched aneurysmal, athero-sclerotic and normal aortic tissue using Northern blot technology (McMillan et al., 1995). Constitutive expression of TIMP-1 and TIMP-2 mRNA was detected in cultured vascular smooth muscle cells from normal and aneurysmal aorta (McMillan et al., 1995). *In situ* hybridisation revealed TIMP-1 and TIMP-2 co-localised to adventitial smooth muscle cells with TIMP-2 also localising to macrophages within the wall of the abdominal aortic aneurysm. Neither TIMP-1 nor TIMP-2 could be demonstrated by *in situ* hybridisation in normal aorta (McMillan et al., 1995). Elmore et al., utilising Northern blot analysis confirmed a lack of difference in TIMP-1 transcript levels but found a small yet significant increase in TIMP-2 mRNA levels in aneurysmal tissue relative to a much younger site-matched control cohort (Elmore et al., 1998). Tamarina et al., described a two- and four-fold elevation in TIMP-1 and TIMP–2 mRNA in aneurysmal aorta compared with normal abdominal aorta (Tamarina et al., 1997). Carrel et al., found a two-hundred-fold increase of TIMP-3 mRNA levels in aneurysmal aorta over athero-occlusive aorta (Carrell et al., 2002).

At protein level gelatin zymography results show a six-fold higher TIMP-1 content in the culture medium of aneurysm wall relative to normal aorta (Thompson et al., 1995). Western blotting shows a two- and four-fold increase in TIMP-1 and TIMP-3, with 60% decrease in TIMP–2 protein content of abdominal aneurysm tissue compared to normal thoracic aorta (Annabi et al., 2002). A polyclonal antibody to recombinant human TIMP-1 was produced in rabbit and used to establish an immunoassay. Seeding of TIMP-1 transfected cell lines onto xenograft guinea pig aorta in rats results in local over expression of TIMP-1 protein, suppression of MMP-9 protein and the prevention of chronic inflammation and aneurysmal formation normally in this aortic model (Allaire et al., 1998).

The role of TIMP's in modulating extracellular matrix turnover extends beyond the simple competitive inhibition of MMP activity. Indeed the processing of pro-MMP-2 to its active form by membrane-bound MT1-MMP is a TIMP-2 dependant process. TIMP-2 is known to bind selectively to pro-MMP-2, and by doing so presents pro-MMP-2 for activation by MT1-MMP (Howard et al., 1991). In addition, TIMP-2 appears to activate MT1-MMP and its absence is associated with MT1-MMP autocatalysis (Hernandez-Barrantes et al., 2000). TIMP-2 also stimulates fibroblast proliferation (Corcoran et al., 1995), and TIMP-1 has an erythroid potentiating activity distinct from its antiproteolytic function (Chesler et al., 1995).

3.4 Other Proteolytic Activators in Abdominal Aortic Aneurysms

Cysteine Proteases

Cysteine-dependent elastolytic enzymes or the Cathepsins are a group of proteases with optimal activity within an acidic pH. Since optimal conditions are found within intracellular acidic lysosomes, an extracellular role for cathepsins in extracellular matrix degradation was initially overlooked. Cathepsin S and K are unique among cysteine proteases because they posses potent elastolytic activity at a neutral pH so can degrade proteins within the extracellular domain. Increased cathepsin S expression has been documented in atherosclerotic plaques and aortic aneurysms by immunohistochemistry and Western blot analysis. Furthermore, atheroma associated vascular smooth muscle cells have been established as a source of cathepsins S and K transcripts in vivo and in vitro (Sukhova et al., 1998). Vascular smooth muscle cells co-cultured in interferon-ɣ causes the secretion of a cysteine protease. A reciprocal decrease in the endogenous inhibitors of cathepsin, cystatin C, is observed in atherosclerotic plaques and aneurysmal tissue compared with normal aorta. Cathepsin S, therefore, shows a shift of enzyme/inhibitor balance in atherosclerosis and aneurysmogensis (Shi et al., 1999).

Plasminogen Activators

The plasminogen activators of the fibrinolytic system are known to mediate proteolysis within the extracellular matrix. Elevated fibrinolytic activity is described in abdominal aortic aneurysms compared to atherosclerotic or normal abdominal aorta. Plasmin is generated from its proenzyme, plasminogen, by the urokinase-type and tissue-type plasminogen activators, all of which are actively expressed and secreted at protein and mRNA levels in abdominal aortic aneurysms in comparison to both normal and atherosclerotic occlusive aorta (Reilly et al., 1994; Jean-Claude et al., 1994). Both tissue- and urokinase-plasminogen activator have been localized to macrophages by immunohistochemistry and *in situ* hybridisation (Reilly et al., 1994; Schneiderman et al., 1995). Tissue-plasminogen activator demonstrates immunostaining in the intima only of normal aorta, while staining the aneurysmal aorta is more diffuse staining intima and media (Reilly et al., 1994).

3.5 Matrix Metalloproteinase in Abdominal Aortic Aneurysm Rupture

Dobrin et al., described the perfusion of canine carotid and human iliac artery with elastase and collagenase enzymes. Degradation of arterial elastin produced vessel dilatation, decreased vessel distensibility, and vessel elongation, while degradation of collagen produced greater dilatation, increased distensibility, and vessel rupture. These reports suggested that the critical element in both the gross enlargement and rupture of aneurysms was collagen loss (Dobrin et al., 1984).

The first report of collagenase activity in ruptured aneurysmal tissue, in 1987 is attributed to Menashi et al., Charring Cross Hospital, London. Significant concentrations of active and latent collagenase were detectable in ruptured aneurysms but only following the resolution of the collagenase from its endogenous tissue inhibitors. However, satisfactory demonstration of collagenase activity in asymptomatic and inflammatory aneurysms was not achieved (Menashi et al., 1987).

The Charring Cross group furthered this work by describing the behaviour of smooth muscle cells in culture from ruptured and non-ruptured aneurysmal derivation (Powell and Greenhalgh, 1989). Collagenase activity in non-ruptured aortic explants was increased six-fold following the addition of macrophage-conditioned culture medium. This increase fell someway short of the collagen activity in unstimulated ruptured aortas. Interestingly, a comparatively small increase in collagenase activity was observed with ruptured aortic explants stimulated by the same macrophage products. The group went on to report an increase in inflammatory cells in ruptured aneurysms, however, no histological plates were presented. This study was the first to support a

possible endogenous mechanism of aneurysm rupture whereby an inflammatory infiltrate up-regulates smooth muscle cell expression of a collagenase so risking vessel rupture. The study did offer an admonition, as the difference in latent collagenase expression between ruptured and non-ruptured aneurysms was less substantial following correction for the cellular content rather than the wet weight of the respective aneurysms.

Since these important initial experiments, the principal group of enzymes with elastase and collagenase capability, investigated in abdominal aortic aneurysms have been the matrix metalloproteinases (MMP's). The changes at pre and post transcription levels for MMP's are extensively delineated within control and asymptomatic aneurysmal aorta. However, only a limited number of studies to date approach the MMP changes associated with aneurysm expansion and rupture.

Freestone et al., described more prominent immunostaining for MMP-9 in aneurysms greater than 5.5cm in diameter than those below 5.5cm, and the reciprocal was observed for MMP-2 immunostaining. They also concluded that adventitial inflammation (predominantly macrophages and B lymphocytes) was the only histological feature associated with increasing aneurysm diameter ($p=0.022$) (Freestone et al., 1995). Sakalihasan et al., showed that activated MMP-9 was present on 5 aneurysms with a mean diameter of 7.5cm and absent in 5 smaller aneurysms with mean diameter 5.8cm (Sakalihasan et al., 1996).

McMillan et al., illustrate the 18-fold elevation of mRNA levels of MMP-9 in aneurysmal aorta compared to control aorta (McMillan et al., 1995). Subsequently

they demonstrated significantly higher expression of MMP-9 mRNA in medium sized aneurysms (n=10, diameter 5 to 6.9cm) when compared to smaller (n=3, p=0.03) and larger aneurysms (n=6, p=0.01). All aneurysms in the study did have MMP-9 mRNA levels significantly greater than the control group (McMillan et al., 1997).

Peterson et al., compared active and total levels of MMP-9 and MMP-2 proteins in non-ruptured and ruptured abdominal aortic aneurysms (Petersen et al., 2000; Petersen et al., 2002). Their studies failed to definitively illustrate a clear difference in either MMP-9 or MMP-2 between non-ruptured and ruptured abdominal aortic aneurysms. However, subgroup analysis did show elevation of MMP-9 (p=0.012) in ruptured aneurysms compared to large non-ruptured aneurysms - >7cm (Petersen et al., 2000) but not medium sized aneurysms - 5-7cm (Petersen et al., 2000). An inverse correlation between MMP-9 levels and the diameter of non-ruptured aneurysms was observed, however, the coefficient of correlation was low (p=0.04, r=-0.31) (Petersen et al., 2000; Petersen et al., 2002). Overall MMP-2 protein levels did not correlate with aneurysm size or rupture (Petersen et al., 2000). Subgroup analysis showed a positive correlation for MMP-2 concentrations and non-ruptured aneurysm diameter but only in aneurysms > 7cm (Petersen et al., 2002). Indeed higher levels of MMP-2 were noted in large non-ruptured aneurysms than medium sized ruptured aneurysms (p=0.007) (Petersen et al., 2002).

The case for a possible decrease in MMP-9 mRNA and protein in larger aneurysms is not statistically robust given that significance was achieved only with subgroup analysis in a small patient cohort (McMillan et al., 1997; Petersen et al., 2000; Petersen et al., 2002). Despite this several hypothesis are reported to support these

correlations. Firstly, given that elastin loss is considered an early event in aneurysm formation, a decrease in elastin substrate may result in the failure of positive feedback required to maintain MMP-9 levels (McMillan et al., 1997). It has also been suggested that the negative correlation between MMP-9 levels and aneurysm diameter is caused by aneurysms with lower MMP-9 levels remaining un-ruptured and simply expanding but aneurysms with higher MMP-9 levels rupturing at a slightly smaller diameter (Petersen et al., 2000). This theory does not concur with post mortem studies showing that approximately 20% of aneurysms of diameter less than 7cm and under were ruptured whilst up to 60% of aneurysms over 7cm and 95% of aneurysms over 10cm were found to be ruptured at post-mortem (Darling et al., 1970).

It possible that the respective associations of MMP-2 and MMP-9 with diameter are coincidental and clearly other protease must potentially influence the risk of rupture. MMP-2 and -9 may facilitate the continued expansion of aneurysms but their substrate specificity, as well as the current evidence relating to expression with respect to aneurysm diameter, suggests a peripheral role in aneurysm rupture. Certainly Pyo et al., confirmed the central role of MMP-9 in aneurysm generation (Pyo et al., 2000) but to conclude that MMP-9 is critical to the rupture process is controversial at this stage.

A clear association between levels of the tissue inhibitors of MMP's (TIMP's) and aneurysm development has been described in animal models. Normally a xenograft aorta becomes aneurysmal within a few weeks and is associated with a dense inflammatory cell infiltrate (Allaire et al., 1994). The cell based gene transfer of TIMP-1 to the decellularised luminal surface of xenograft aorta, results in local

TIMP-1 up-regulation, suppression of MMP-2 and –9 and possibly suppression of MMP-3 and –12. These changes prevented elastin depletion, aneurysm formation and rupture (Allaire et al., 1998). This clearly represents an association between an imbalance in inhibitor activity and the pathogenesis of aortic aneurysms. This data is conditional since TIMP's are not specific to MMP-2 and –9. Therefore, the possibility of other protease(s) being inhibited by TIMP-1 in this model may have to be considered.

3.6 Rational for Thesis

A lack of consideration has been given to the genuine fibrillar collagenases. The reason for this may be found in the initial experiments conducted by Busuttil et al., and Menashi et al., where they concluded that collagenase activity was difficult to demonstrate and required total annulment of the collagenase/inhibitor/substrate interaction (Busuttil et al., 1980; Menashi et al., 1987). According to Dobrin et al., the presence of a collagenase capable of breaking down fibrillar arterial collagens (types I and III) is required to induce aneurysm rupture (Dobrin et al., 1984; Dobrin et al., 1991). None of the aforementioned enzymes are capable of degrading native fibrillar collagens in vivo. Under physiological conditions it is acknowledged that MMP-2 and –9 have collagenolytic activity towards nonfibrillar collagens, however, their actions against fibrillar collagens relate to the degradation of partially hydrolysed components, not intact fibrils (Okada et al., 1990; Wilhelm et al., 1989; Collier et al., 1988).

Genuine fibrillar collagenases MMP-1, MMP-8 and MMP-13 have substrate specificities favouring the breakdown of collagen types III, I and II respectively (Hasty et al., 1987; Horwitz et al., 1977; Knauper et al., 1993). The detection of MMP-1 and –13, at protein and RNA level, has been reported in asymptomatic but not ruptured aneurysms (Irizarry et al., 1993; Mao et al., 1999). Moa et al's., in vitro characterisation of the "at best" inconsistent expression of MMP-8 mRNA from smooth muscle cells of aneurysmal and athero-occlusive abdominal aorta remains the only report pertaining to this collagenase in the aortic tissue (Mao et al., 1999).

In conclusion, it would appear that we are far from determining the origins of aneurysm rupture. The levels of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13 are well described in non-ruptured AAA. Given that degradation of collagen is an important step in AAA formation and rupture (Dobrin et al., 1984), a good starting point would be the consideration of the fibrillar collagenase, MMP-8, in normal aorta and non-ruptured abdominal aortic aneurysm tissue. The expression of all MMP's, including the novel collagenase MMP-8, at the actual site of AAA rupture has not been reported. Thus, the analysis of MMP's, both globally within ruptured AAA and locally at the site of rupture, will be of great benefit. The observation of an elevated MMP within ruptured AAA represents a therapeutic target. Statins are known to lower MMP levels in carotid plaques (Molloy et al., 2004). The influence of statins in AAA should be observed between a group of patients, pre-operatively optimised on a statin, and a group not taking a statin. Furthermore, a clear picture of the cellular changes that occur within ruptured AAA have not been described. The site of expression of MMP's and the histological changes within non-ruptured and ruptured abdominal aortic aneurysms requires quantification.

CHAPTER 4

METHODOLOGY

4.1 Patient Recruitment

AAA Biopsies
Normal Aorta

4.2 Laboratory Experiments

Protein Assay

MMP Extraction
Enzyme Linked Immunosorbent Assay (ELISA)
Conventional Sandwich ELISA
Activity ELISA

Immunohistochemistry

Antigen Retrieval
Pilot study for antigen detection
Immunohistochemistry Controls
Further Histological Stains

In Situ Hybridisation

In Situ Hybridisation Process
In Situ Hybridisation Controls

Objective Assessment of Histology

Histological Analysis
Objective Quantitation of Histology

4.3 Statistical Methods

4.1 Patient Recruitment

AAA Biopsies

Sixty-three patients with non-ruptured AAA and 21 patients with ruptured AAA were recruited from the University Hospitals of Leicester, in accordance with ethical guidelines (Reference LREC 6242). Pre-operatively all patients gave full consent for the removal of aortic tissue biopsies and the use of these biopsies in medical research. Figures 4.1 and 4.2 show the biopsy process. Biopsies were taken from the anterior aneurysm sac, 5cm distal to the left renal vein, in non-ruptured and ruptured AAA. A further biopsy, from the edge of the rupture site, was attempted in ruptured AAA.

With the first 8 non-ruptured AAA, biopsies were washed in saline, dissected of luminal thrombus and adipose tissue, and frozen in liquid nitrogen. For the subsequent 55 patients, biopsies were divided in two, with half frozen and half fixed in 4% paraformaldehyde then embedded in paraffin wax. This situation arose because the decision to conduct histology was taken after the initial study inception.

Twenty-one patients with ruptured AAA were recruited to the study. Biopsies from the anterior aneurysm sac were taken in all 21 cases. When identified, an additional biopsy from the ruptured AAA was taken from the edge of the rupture site. This was possible in 12 ruptured AAA patients. Ten of the 12 identified rupture sites were posterior with 2 being anterior. No rupture site coincided with the site of anterior aneurysm wall biopsy. Biopsies of the rupture site were not possible in 9 patients due

to the technical challenges of the rupture surgery. All rupture biopsies, irrespective of site were divided in two, with half frozen in liquid nitrogen and half fixed in paraformaldehyde.

Thus homogenates of anterior aneurysm sac biopsies were available for 63 non-ruptured AAA and 21 ruptured AAA. Homogenates from the edge of the rupture site, paired with anterior aneurysm sac biopsies, were available for 12 of the ruptured AAA. Histological biopsy blocks of the anterior aneurysm sac were available from 55 non-ruptured AAA and 21 ruptured AAA. Histological biopsy blocks, taken from the rupture site, paired to anterior aneurysm biopsy blocks, were available in 12 of the ruptured AAA.

Patient demographic information including age, gender, smoking history (current or ex-smoker of less than 10 years versus non-smoker or ex-smoker of greater than 10 years), presence of a cardiovascular event (documented myocardial infarction, cerebrovascular or peripheral vascular disease, angina requiring medication), hypertension (requiring medication), and diabetes (requiring medication or dietary modification) were recorded. Cardiovascular medications were also recorded (statin, beta-blocker, calcium channel-blocker, acetylcholine-esterase inhibitor and non-steroidal anti-inflammatory). The maximum external diameter of each non-ruptured AAA was determined from a pre-operative computed tomogram. The diameter of each ruptured AAA was measured intra-operatively.

Normal Aortic Biopsies

Ethical permission was obtained for the use of normal aorta provided by the United Kingdom Human Tissue Bank (UK-HTB), De Montfort University, Leicester (Birmingham MREC Reference MREC/03/7/19). Patients undergoing organ donation were recruited for this research. Renal grafts unsuitable for transplant were transported to the UKHTB laboratory. Aortic patches from the renal graft were fixed in 4% paraformaldehyde and embedded in paraffin wax or frozen in liquid nitrogen.

Over the course of sample collection, 22 normal aortas from organ donors were collected by the UK Human Tissue Bank. Organ donors were younger than non-ruptured AAA patients (mean age 51 +/- 4 yrs versus 72 +/- 7 yrs). To ensure close age-matching only normal subjects older than 60 yrs were used. This resulted in a subgroup of the 10 oldest normal aortic samples (mean age 66 +/- 2 yrs). The remaining 12 younger patients were excluded and not analysed (mean age 40 +/- 4 yrs). Only the 40 youngest non-ruptured AAA (age < 75 yrs), were compared to the subgroup of older normal aorta. This resulted in a comparison of MMP-8 and TIMP levels between similarly aged cohorts: mean age AAA = 68 +/- 6 yrs, mean age normal aorta = 66 +/- 2 yrs, independent t-test - $p = 0.2$.

Patient demographic information including age, gender, smoking history, presence of a cardiovascular event, hypertension, and diabetes were recorded. Cardiovascular medication (statin, beta-blocker, calcium channel-blocker, acetylcholine-esterase inhibitor and non-steroidal anti-inflammatory) and cause of death were also recorded.

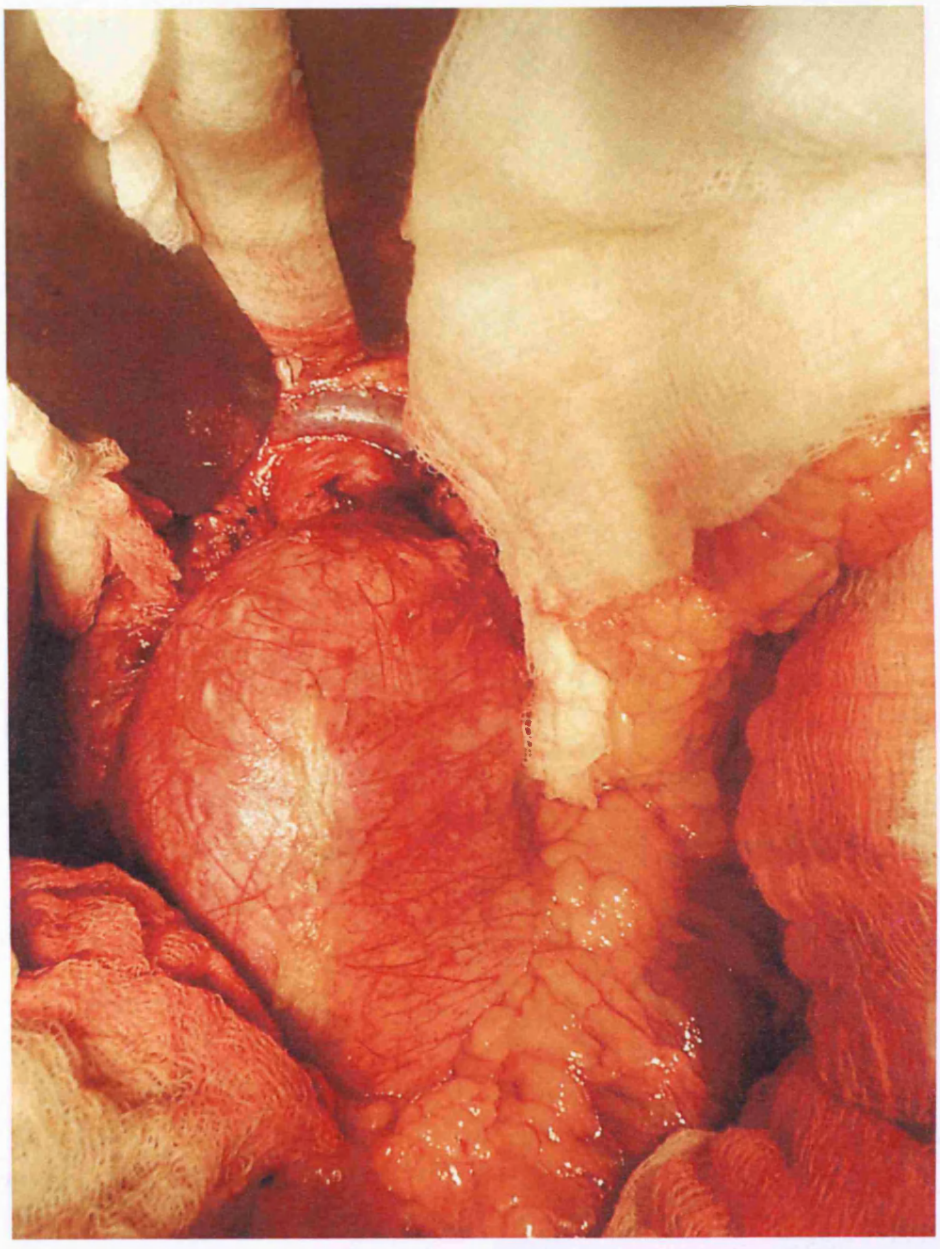


Figure 4.1
Abdominal aortic aneurysm displayed prior to arteriotomy

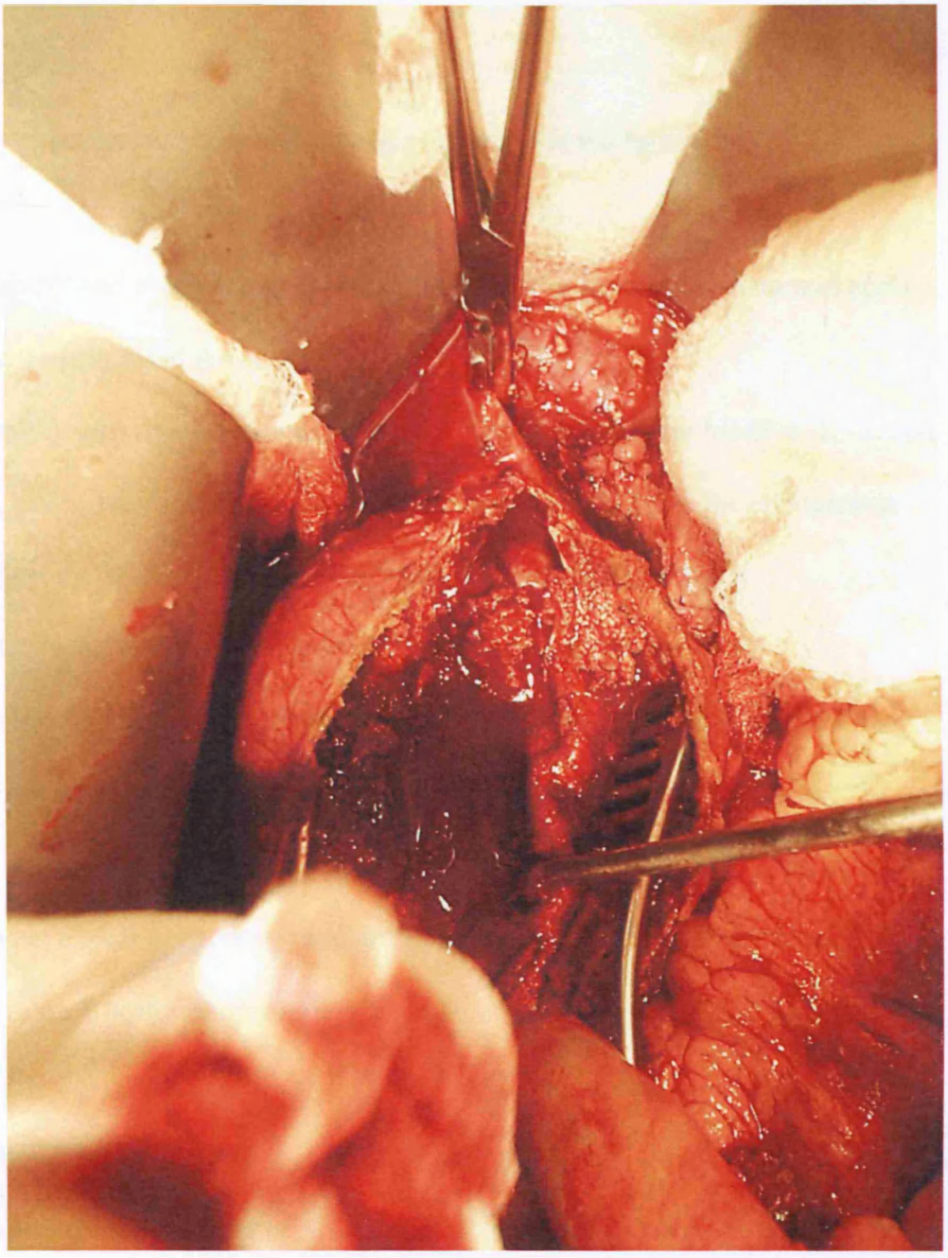


Figure 4.2
Abdominal aortic aneurysm displayed with arteriotomy.
Biopsies were taken from the arteriotomy edge.

4.2 Laboratory Experiments

Laboratory experiments were undertaken to investigate the following:

i) quantify and characterise expression of MMP-8 expression in normal aorta and non-ruptured AAA

ii) quantify and characterise expression of the collagenolytic MMP's -1, -8, and -13, elastolytic MMP's –2 and -9, stromelytic MMP-3 and the endogenous inhibitors TIMP-1 and –2, in non-ruptured and ruptured AAA

iii) observe the cellular changes associated with AAA rupture

iv) observe the effect of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) on MMP expression in non-ruptured AAA.

The following experiments were conducted to determine the aforementioned objectives in each aortic biopsy -

i) protein assay (ELISA)

ii) immunohistochemistry

iii) *in situ* hybridisation

iv) histological quantification of cellular content

Initial ELISA were undertaken and results determined the extent of immunohistochemistry and *in situ* hybridisation. All work was conducted in the laboratories of the University of Leicester.

Protein Assay

The components of the protein assay are MMP extraction and Enzyme Linked Immunosorbent Assay (ELISA).

MMP extraction

The tissue was snap frozen in liquid nitrogen and stored at –70°C until extraction. Tissue samples were thawed on ice, weighed, diced into small pieces with a scalpel blade and placed in Du Pont tubes (Du Pont, USA). To each tube was added 1ml of cold homogenising buffer (2M urea, 50mM Tris-Hydrochloric acid, 1g/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1 % Brij35 at pH 7.6) for every 100mg of wet weight tissue. The tissue was mechanically homogenised using a using a polytron homogeniser (Kinematica, Switzerland) for 5 minutes at 24000rpm. Cellular debris was then removed by centrifugation of these homogenates at 10000g for 1 hour at 4°C. The resulting supernatants were then aspirated and transferred to small sections of pre-boiled visking tubing with a 12-14 kilodalton molecular weight cut-off (Fisher, Loughborough, UK) and both ends sealed with plastic clips. Dialysis occurred against dialysis buffer (Calcium Chloride, 0.1% Brij-35, PMSF 0.lmM 25mM Tris HCl pH 8.5) for 18 hours at 4°C. Each sample was allowed 150ml of dialysis buffer. Proteins of molecular weight greater than 12-14 kilodalton's were retained by dialysis.

The protein concentration in each sample was determined by the Bio-Rad protein concentration assay (Biorad Laboratories, Hemel Hempstead, UK). This method

assesses the colour change when Coomassie blue R-250 binds primarily with arginine and aromatic amino acid residues. The dye binds proportionally to the concentration of total protein and can be read spectrophotometrically at 595nm (UV1601, Shimadzu Corporation, Japan). Known concentrations of bovine serum albumin were used to construct a standard curve from which protein concentrations of homogenates could be read and standardised to 1 mg/ml. The remaining homogenate was than snap frozen in liquid nitrogen. Homogenates were completely de-thawed to room temperature and vortex mixed prior to performing the ELISA.

Enzyme Linked Immunosorbent Assay (ELISA)

Target MMP and TIMP levels were quantified by ELISA validated for use with human tissue homogenates (Fujimoto et al., 1993). Casein and gelatin zymography are only semiquantitative. Conventional ELISA provides a specific and precise quantitative determination of total enzyme levels and are based on a 2-site ELISA sandwich format. They employ antibodies directed against the epitopes of the enzyme. Activity ELISA's were attempted where significant differences in total MMP levels were detected.

Conventional Sandwich ELISA

The principal of the conventional sandwich ELISA is as follows. Target standard and sample homogenates are applied to the microtitre wells of the ELISA plate (Figure 4.3), which are pre-coated with anti-target antibody. The ELISA plate is incubated for 1hr to room temperature. Any target present is bound to these antibodies and other components of the sample are removed by washing and aspiration. A further anti-target antibody conjugated to horseradish peroxidase, is added to the wells to detect the bound target. Further washes and aspiration removed any excess horseradish peroxidase conjugated anti-target antibody. The amount of peroxidase present in each well, which is directly proportional to the amount of target, is determined by the addition of 3,2',5,5'-tetramethylbenzidine substrate. The reaction is stopped by addition of sulphuric acid. The resulting colour is read using a microtitre plate spectrophotometer measured at 405nm. The concentration of enzyme is determined by interpolation from a standard curve from known target standards. The principal of the conventional sandwich ELISA is demonstrated pictorially in Figure 4.4

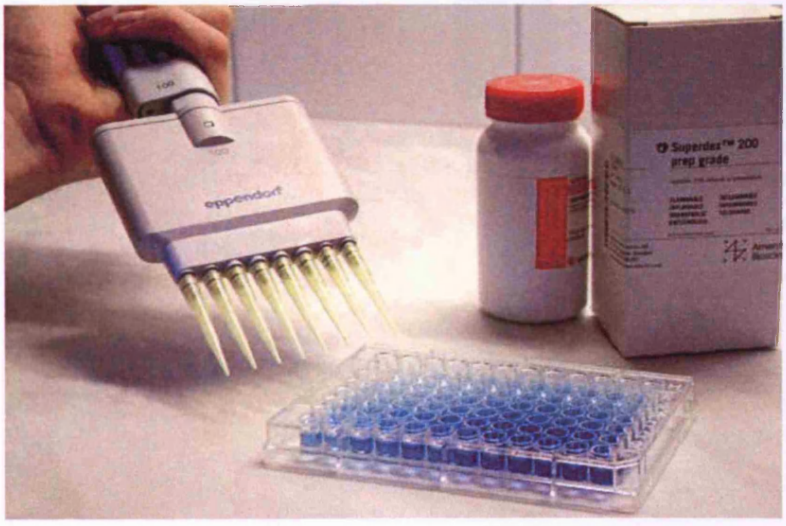


Figure 4.3 ELISA Plate

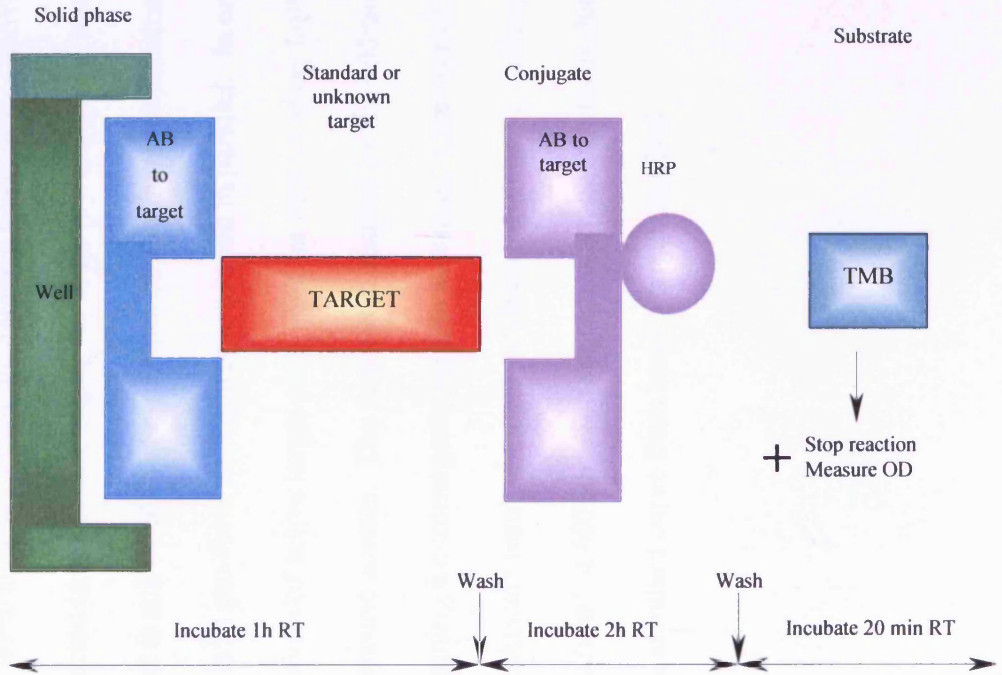


Figure 4.4: Conventional MMP/TIMP Sandwich ELISA assay design. AB = antibody; HRP = horse raddish peroxidase; OD = optical density; RT = room temperature.

Activity ELISA

The principal of the activity ELISA is as follows (Figure 4.5). 100 µl of anti-target antibody is applied to a microplate, pre-coated with anti-target antibody. Target standards and sample homogenates are incubated in microtitre wells and any target is bound to the immobilised antibody. Washing and blotting on tissue paper removes the other components of the sample. Either endogenous levels of free active-target or total-target can be detected in the same sample. In order to measure the total levels of target, bound pro-target is activated by the addition of p-aminophenylmercuric acetate. The standard is in pro-form so requires activation in parallel. In order to measure endogenous levels of active target only, samples receive assay buffer rather than p-aminophenylmercuric acetate. The target is quantified by the addition of a detection reagent containing a chromogenic peptide substrate. Cleavage of the chromogenic peptide substrate results in a colour change read using a microtitre plate spectrophotometer measured at 405nm. The concentration of enzyme is determined by interpolation from a standard curve from known target standard.

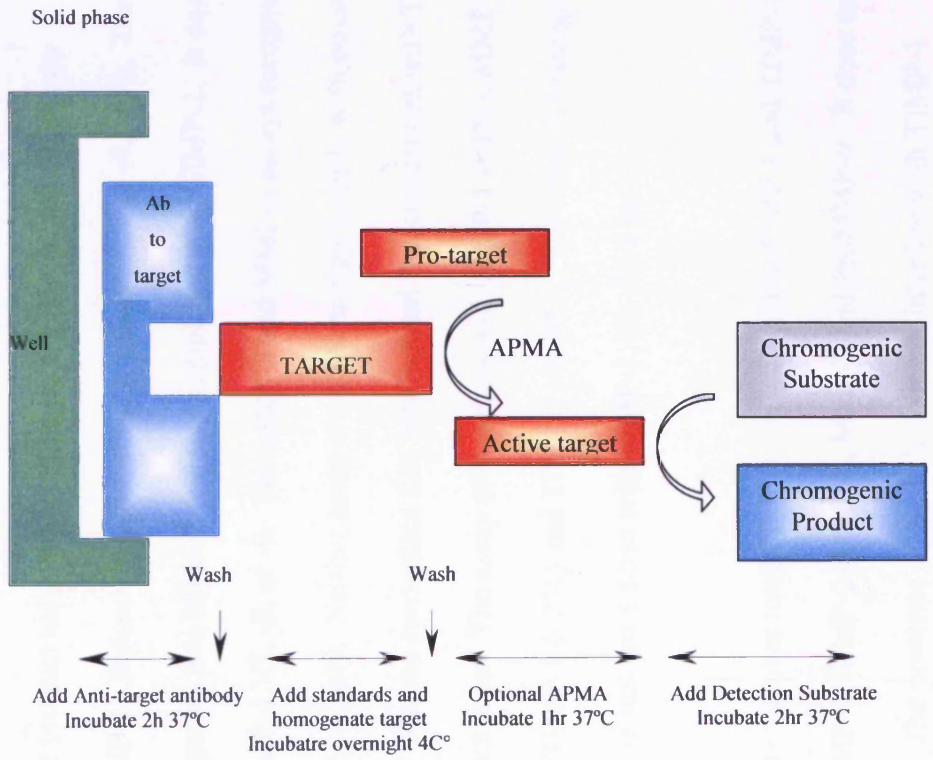


Figure 4.5: Activity MMP/TIMP ELISA design. Ab = antibody; AMPE = aminophenylmercuric acetate ; RT = room temperature.

Standardised samples of homogenate were measured according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK) in duplicate for the presence of total levels of MMP-1 (RPN2610), MMP-2 (RPN2617) MMP-3 (RPN2613), MMP-8 (RPN 2619), MMP-9 (RPN2614), MMP-13 (RPN2621), TIMP-1 (RPN2611) and TIMP-2 (RPN2618) using conventional sandwich ELISA.

Data taken from the product information sheet shows that the MMP-1 assay recognises free MMP-1. It does not cross-react with MMP-2, -3, -8, or -9. The MMP-2 assay recognises free MMP-2 and that complexed with TIMP-2. It does not cross-react with MMP-1, -3, -8, or -9. The MMP-3 and MMP-8 assays recognise free MMP-3 and –8 respectively. Neither assay cross reacts significantly with other MMP's. The MMP-9 assay recognises free MMP-9 and that complexed with TIMP-1 but not TIMP-2 complexes. The assay cross-reacts with MMP-1 (<1%), MMP-2 (<3), MMP-3 (<2), TIMP-1 (<2%) and TIMP-2 (<1.4%). The MMP-13 recognises free MMP-13. It does not cross-react with MMP-1, -2, -3, -8 or –9.

The TIMP-1 assay recognises total TIMP-1, including free TIMP-1 and TIMP-1 complexed with MMP-1, MMP-2, MMP-3, MMP-9 and ProMMP-9. It does cross react with TIMP-2. The presence of MMP's does not interfere with TIMP-1 quantification. The TIMP-2 assay recognises free TIMP-2 and that complexed to the active form of MMP's, but not TIMP-2 complexed with the proMMP-2.

Based on the ELISA results of total MMP and TIMP levels, the active levels of MMP-8 and MMP-9 were determined by ELISA (MMP-8 RPN2635; MMP-9 RPN2634; Amersham Pharmacia Biotech, Buckinghamshire, UK). The MMP-8

activity assay recognises all active forms of MMP-8. Other MMP's have some cross reactivity (MMP-1 = 7.9%, MMP-2 = 1.2%, MMP-3 = 1.5, MMP-9 = 1.3%, MMP-13 = 2.2%, TIMP-1 = 0.5%). The MMP-9 activity assay recognises the active forms of MMP-9. It does not cross-react with other MMP's or TIMP's. The assay only partly recognises MMP-9 bound to TIMP-1and TIMP-2 complexes (active MMP-9/TIMP-1 complex = 21.6%; active MMP-9/TIMP-2 complex = 7.0%) in the assay.

Prior to the final ELISA's being conducted, test runs were undertaken to ensure optimal overlap of the test samples with the standard curves. The purpose of this was to minimise extrapolation error. From the test runs, dilutions were determined for each MMP and TIMP (Table 4.1).

Target	Homogenate Dilution
MMP-1(t)	1:1
MMP-2(t)	1:10
MMP-3(t)	1:4
MMP-8(t)	1:10
MMP-8(a)	1:4
MMP-9(t)	1:10
MMP-9(a)	1:2
MMP-13(t)	1:1
TIMP-1(t)	1:10
TIMP-2(t)	1:1

Table 4.1: Dilution of tissue homogenates each for ELISA (total – (t), active – (a)).

Immunohistochemistry

Paraformaldehyde-fixed paraffin embedded tissue has the advantage of maintaining greater architectural integrity than frozen sections. Therefore, a piece of each aortic biopsy was fixed in fresh, cold 4% paraformaldehyde for 24 hours. The samples were dehydrated in 95% IMS for 2 hours followed by 99% IMS for 7 hours, and finally xylene for a further 3 hours. The dehydrated samples were embedded in paraffin.

Sectioning of paraffin blocks was performed in the General Diagnostic Laboratory (Figure 4.6). Three to four micron sections were cut from the paraffin blocks, floated onto silane-coated slides on the day prior to staining and air dried at 37°C overnight. The following morning sections were dewaxed in xylene and rehydrated through graded alcohols to water. Antigen retrieval was then conducted.

Antigen Retrieval

A combination of the following retrieval processes were used:

i) Microwave antigen retrieval

Microwaving was carried out in a Matsui 85OW oven. After dewaxing and washing well in running water, the slides were placed in a coplin jar containing citrate buffer at pH 6.0. The coplin jar was placed inside the oven along with several other coplin jars empty of slides but filled with water. The door was closed and time set for 15 or 25

Figure 4.6
Section of Paraffin Blocks

minutes. Every 3 minutes, distilled water was added to the citrate buffer to ensure the sections did not dry out. At the end of the cycle, the jars were allowed to cool to room temperature then washed in running water.

ii) Proteolytic enzyme digestion

0.1% trypsin (ICN Flow) solution in 0.1% calcium chloride (Sigma) was prepared at 37 °C. This was buffered to a pH of 7.8 prior to use. The sections were dewaxed, washed in running water, allowed to come to 37 °C and incubated in trypsin for 8 to 12 minutes. The reaction was halted in running water.

iii) Acid Denaturation

The sections were dewaxed then treated in a 2 molar Hydrochloric acid (Sigma) in a preheated water bath at 37^{o}C for 12 minutes. The slides were then washed in running water.

Following antigen retrieval the sections were then immersed in phoshpate-buffered saline (PBS) for 5mins, then dried around and placed in an incubation tray.
Any intrinsic peroxidase activity was then blocked by submersion in 6% hydrogen peroxide for 10 minutes, followed by 5 minutes wash in PBS. To reduce non-specific antibody binding, normal goat serum diluted 1 in 20 (Dako, Ely, UK) sufficient to cover the whole section, was added to the slides. The slides were left in a humid chamber for 10 minutes. After this the goat serum was carefully removed using a

tissue. Primary antibody was diluted as necessary in 5% goat serum and 100 µl was added to each section. The sections were then incubated for 1 hour at room temperature in a humidification chamber. Any unbound antibody was removed by two 10 minute washes in PBS. After drying round the sections, 100 µl of secondary antibody solution was applied to each slide and incubated for 1 hour at room temperature. Unbound secondary antibody was removed after 1 hour by two 10 minute washes in PBS, then excess PBS was removal with tissue paper. Streptavidin-Biotin Complex (Dako) was added and the sections incubated for 30 minutes at room temperature. Following a PBS rinse, diaminobenzidine (DAB, DAKO) at a concentration of 0.05% was added to each slide and left at room temperature for 10 minutes. Protective gloves were worn when using DAB. The sections were then washed in distilled water, lightly counterstained with hematoxylin for 15 seconds and washed in tap water. Finally, the sections were dehydrated in absolute alcohol and mounted, from clean xylene, in DPX mounting medium. Microscopically, areas of immuno-reactivity were visualised as dark brown staining.

Pilot study for antigen detection

A pilot study was conducted to determine the optimal antigenicity retrieval and antibody concentration for each target on paraformaldehyde fixed tissue. Antibody concentrations varied around the quoted concentration on the manufacturers literature in factors, being either the quoted literature figure, half or twice the quoted figure. Denaturing pre-treatments were performed using six different regimens. The regimens used were as follows:

1. Microwaving for 15 minutes.
2. Microwaving for 25 minutes.
3. Trypsinisation 37 °C for 12 minutes
4. Microwaving for 15 minutes followed by trypsinisation 37 °C for 8 minutes.
5. Microwaving for 25 minutes followed by trypsinisation 37 °C for 8 minutes.
6. 2 molar hydrochloric acid at 37 °C for 12 minutes then trypsinisation at 37 °C for 8 minutes.

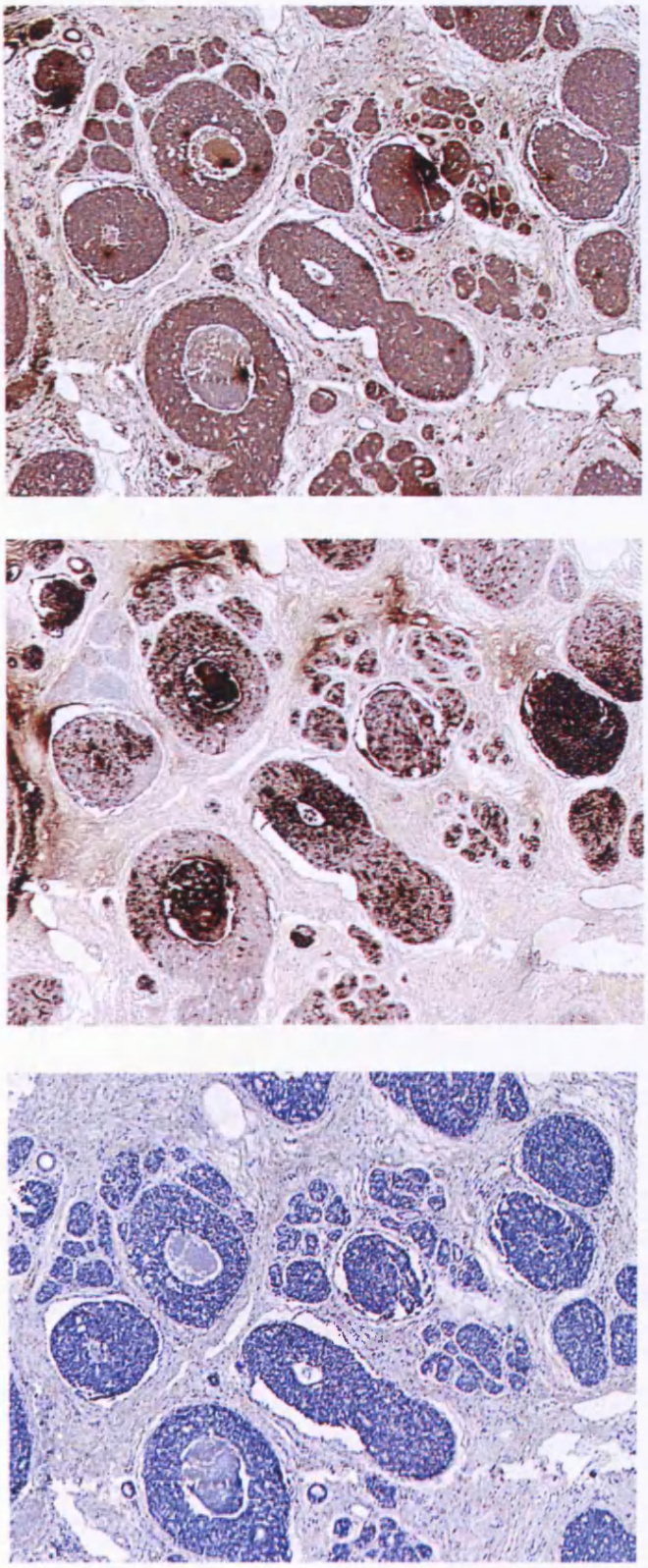
Optimal pre-treatment for retrieval of antigenicity was determined as the regime that resulted in the maximal positive immunostaining, minimal background immunostaining, with best preservation of tissue morphology. Optimal pre-treatment for retrieval of antigenicity was varied against antibody concentrations. The results for optimal retrieval and antibody concentration are shown in Table 4.2.

Immunohistochemistry Controls

A positive control was conducted to ensure that the immunohistochemistry steps worked. Positive control involved immunohistochemistry conducted on a breast adenocarcinoma, known to express MMP-8 and –9 (Figures 4.7(a), 4.7(b)). Negative control was included to ensure there was no non-specific nuclear staining. This involved the method detailed above being conducted on aortic and breast tissue with omission of the primary antibody step (Figures 4.7(c), 4.8, 4.9).

Target	Antibody	Manufacturer	Dilution	Retrieval
MMP-8	Anti-human MMP-8®	R&D Systems, Minneapolis, Minnesota	1:50	T 20 mins
MMP-9	Anti-human MMP-9 ™	Novocastra Laboratories, Newcastle upon Tyne	1:100	T 20 mins
Mesenchyme	Anti-human Vimentin ™	Dako, Glostrup, Denmark	1:100	μ 15 mins
Smooth Muscle Cells	Anti-human Smooth Muscle α-actin ™	Dako, Glostrup, Denmark	1:1000	Nil
Leucocytes	Anti-human CD-45, Leucocyte Common Antigen ™	Dako, Glostrup, Denmark	1:50	T 20 mins
Macrophages	Anti-Human CD-68 ™	Dako, Glostrup, Denmark	1:150	T 20 mins
Neutrophils	Anti-human CD-15®	Becton Dickinson, San Jose, California	1:50	μ 15 mins

Table 4.2: Results of the pilot study for immunohistochemical antigen detection. (T – trypsinisation, μ – microwaving)

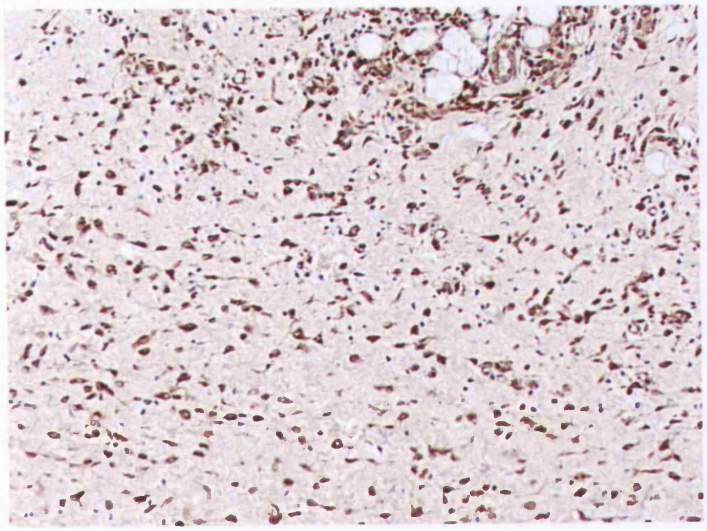


(a)

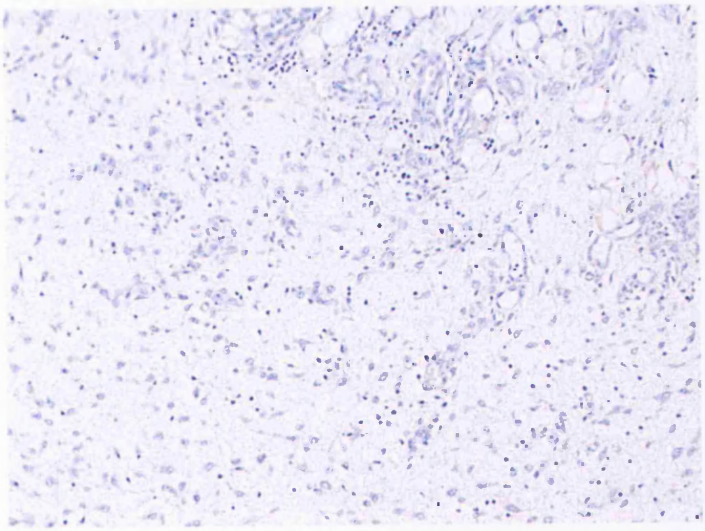
(b)

(c)

Figure 4.7
Immunohistochemistry Positive Controls on breast adenocarcinoma for (a) MMP-8 (b) MMP-9 (c) Negative control.



(a)



(b)

Figure 4.8
Immunohistochemistry for (a) MMP-8 and (b) negative control

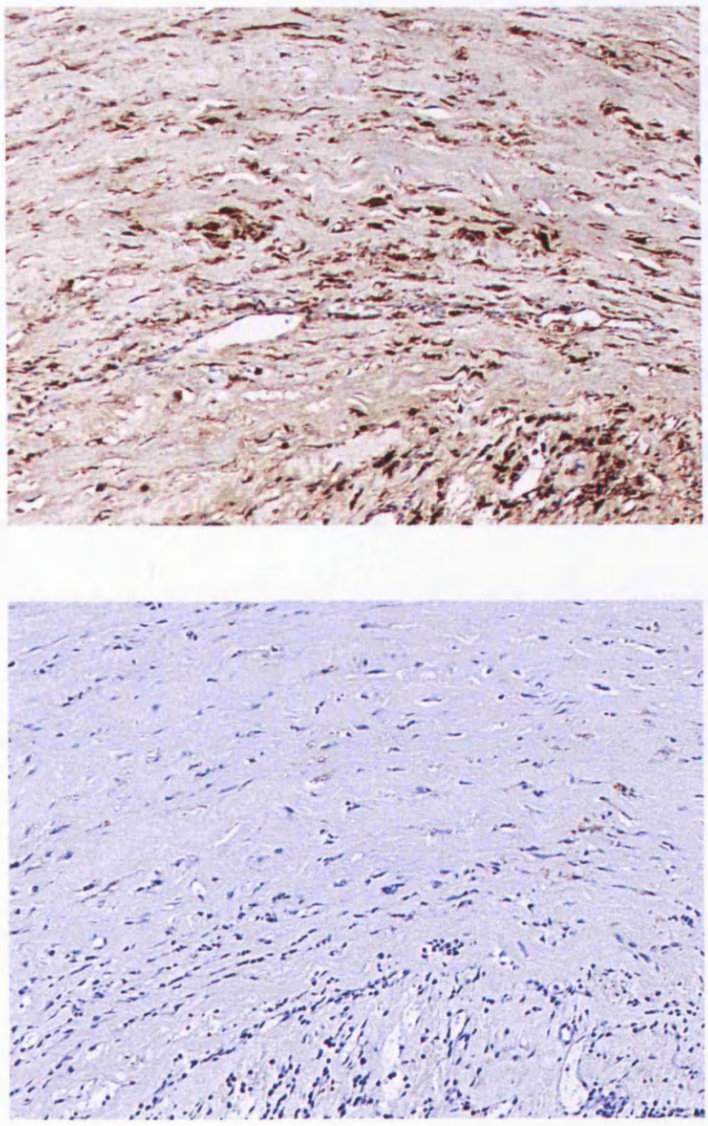


Figure 4.9
Immunohistochemistry for (a) MMP-9 and (b) negative control.

Further Histological Stains

Hematoxylin and Eosin Staining

Arterial structure was examined using hematoxylin and eosin (H&E), which stained the nuclei dark. Wax embedded slide mounted tissue sections were hydrated by sequentially passing them through solutions of xylene, 99% ethanol, 95% ethanol and finally water. They were then submerged in hematoxylin for 4 minutes and washed in running water until clear. Next they were submerged in 0.5% eosin for 5 seconds followed by a further wash in running water until clear. The stained sections were then dehydrated by sequentially passing them through water, 95% ethanol, 99% ethanol and finally finishing in xylene prior to mounting with cover slips using DPX mountant.

Elastin Van Geison Staining

Elastin Van Geison (EVG) staining is a useful technique for visualising elastin fibers in arterial tissue. Elastin fibers are stained black, collagen fibers are pink and smooth muscle cells are yellow. EVG staining involved rehydrated tissue sections submerged in 0.25% potassium permanganate for 5 minutes, 0.1 % oxalic acid for 5 minutes, then washed in running water until clear. After submersion in 95% ethanol, sections were placed in Millers elastin stain for 45 minutes. Next the sections were returned to 95% ethanol then left in running water until clear. The slides were placed in Van Geison counterstain for 2 minutes. Finally, the stained sections were rehydrated to xylene and mounted with cover slips using DPX.

In Situ Hybridization

In situ hybridisation relies on the re-annealing of complementary sequences of nucleic acid. The technique allows the target nucleic acid to be identified at its original cellular site. The cellular expression of nucleic acid is obtained using complementary hybridisation probes to that of the target sequence. MMP-8 and MMP-9 *in situ* hybridisation was conducted using a well-established technique. A cocktail of oligonucleotides, 20 to 30 bases in length and end-labelled with Digoxigenin, were used. Anti-digoxigenin secondary antibody was purchased from Sigma.

MMP-8 Probe Sequences (Herman et al., 2001)

5'-tcgacagtctccgactccatctttctcgat-3';

5'-cggaacgacagagggttgatacgaaagtcc-3';

5'-ttgtatgaagaaacatttactggttaagac-3';

5'-tcttgatctaaaaccaatcttcattcctaa-3'.

MMP-9 Probe Sequences (Loftus et al., 2000)

5'-actggcagggtttcccatcagcattgccgt-3'

5'-tccggcactgaggaatgatctaagcccagc-3'

5'-gttgcaggcatcgtccaccggactcaaagg-3'

5'-gctccccctgccctcagagaatcgccagta-3'

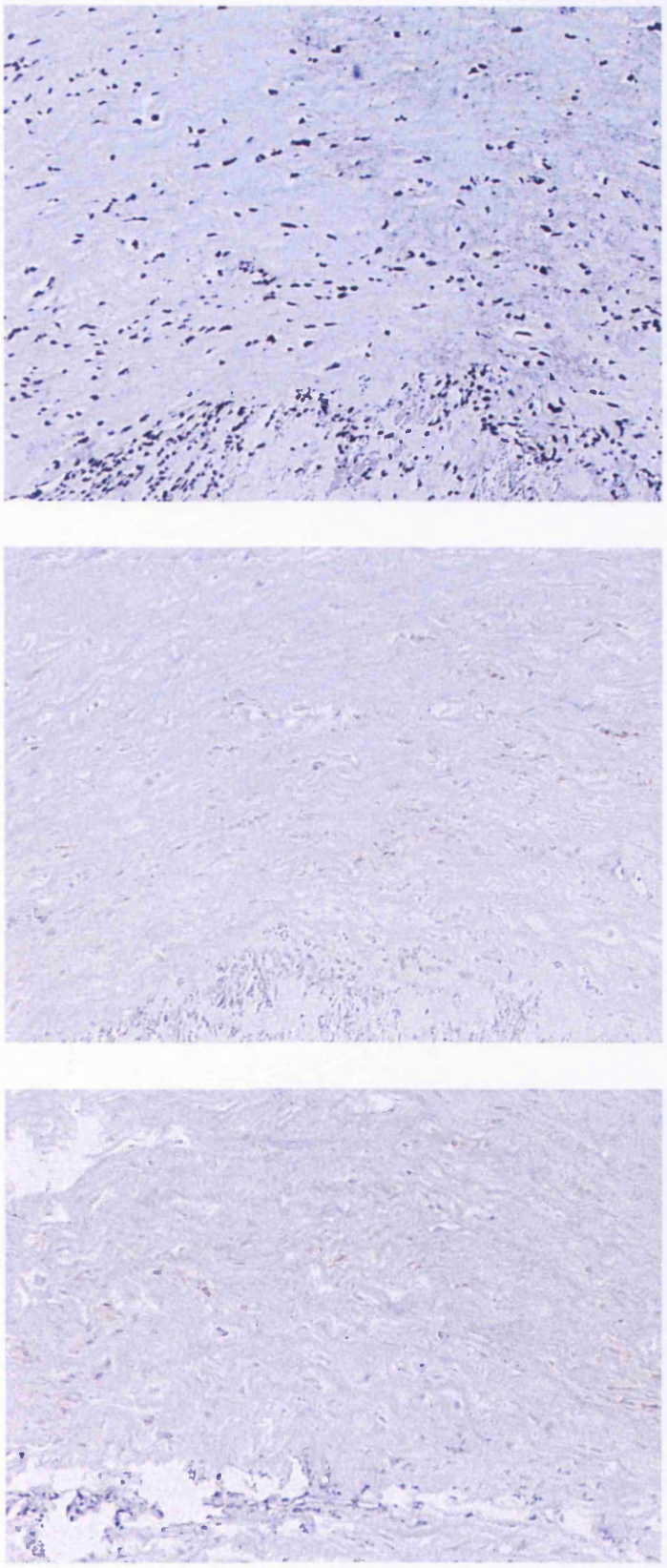
In Situ Hybridisation Process

The procedure is conducted with RNAase Treated Glassware. Three to four micron sections were cut, floated onto silane-coated slides and air dried at 36°C overnight, prior to ISH. The following morning the sections were de-wax in xylene and rehydrated using 100% alcohol followed by ultra pure water and diethylpyrocarbonate (DEPC, Sigma) treated water. The slides were placed in an incubation tray and 4 µg/ml Proteinase K was added (37°C for 30 minutes), and then rinsed off with DEPC treated water. The sections were dehydrated in 95% alcohol (3 minutes), 100% alcohol (2 X 3 minutes), and air dried for a further 2 minutes. One hundred microliters of pre-hybridisation solution was added and incubated at 60 °C for 15 minutes. After this the pre-hybridisation solution was tipped off. One hundred microliters of hybridisation solution containing the probes were then added to each section and RNAase free cover slips applied. Hybridisation was continued at 37 °C for 2 hours. The coverslips were carefully removed by momentarily dipping the slides into PBS, 0.1% Triton X100 in order to release them. One hundred microliters of 2X Standard Saline Citrate / 30% formamide (30ml Formamide, 7ml 20X Standard Saline Citrate and 63ml DEPC water) was made up and use to wash the slides on a rocker for 10 minutes. This was followed by a wash in distilled water. The slides were returned to the incubation tray and freshly made and filtered blocking solution was added for 5 minutes. Next anti-digoxigenin-alkaline phosphatase 1:600 was added to the unused blocking solution and this was add to the sections for 60 minutes. A final wash in TBS for 10 minutes was followed by a dry round the sections and addition of freshly made substrate buffer for 5 minutes. Next 5-bromo-4-chloro-3 - indylphosphate and nitroblue tetrazolium solution (1 tablet in 10ml of DEPC water)

was added to the substrate buffer on the sections. The sections were incubated overnight in the dark at room temperature. The following morning the sections were checked with a microscope to ensure a signal had developed with no background staining. The sections were washed in water (to stop the reaction) and mounted in Glycergel (Dako).

In Situ Hybridisation Controls

The protocol was conducted with an RNAse step prior to proteinase K for one section in each experiment. The protocol was also conducted with negative controls, using a random sequence probe (Figures 4.10 and 4.11).



(a)

(b)

(c)

Figure 4.10
In situ hybridisation on AAA tissue for (a) MMP-8, (b) negative controls using random sequences and (c) RNAase prior to the addition of MMP-8 anti-sense probes.

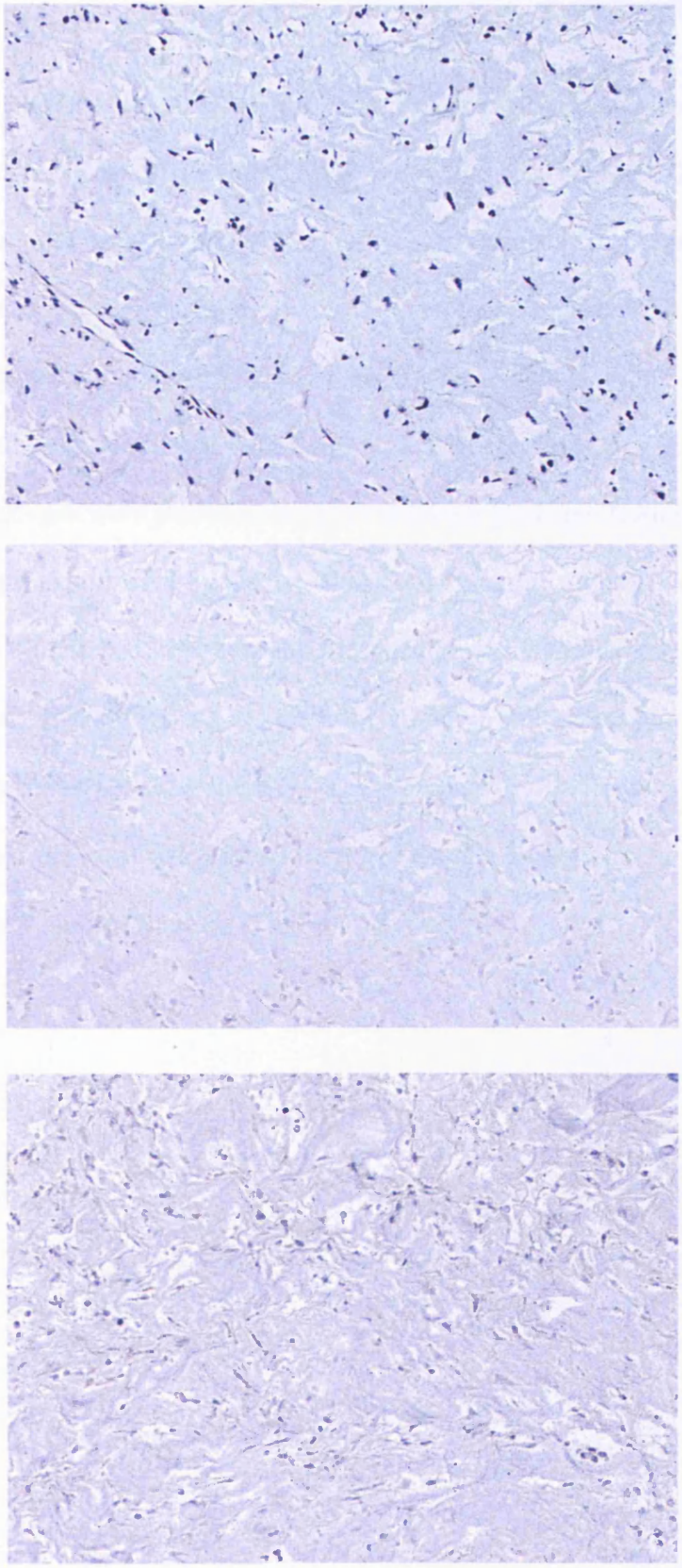


Figure 4.11
In situ hybridisation on AAA tissue for (a) MMP-9, (b) negative controls using random sequences and (c) RNAase prior to the addition of MMP-9 anti-sense probes.

Objective Quantitation of Histology

Further immunohistochemistry for mesenchymal cells (Vimemtin), smooth muscle cells (αSMA), lymphocytes (CD-45), macrophage (CD-68) and neutrophils (CD-15) was conducted on sections of non-ruptured and ruptured AAA biopsies from the anterior aneurysm sac and the edge of the rupture site. Quantitation of the IHC staining was undertaken using a Nikon E800 microscope with an attached JVC KYF50 3 chip color video camera linked to an Apple G3 computer through a Scion CG-7 frame grabber. Images were analysed using the freeware package 'National Institute of Health Image', automated by the use of in-house macros. In each case the aortic adventitia was distinguished. Background illumination was digitally subtracted from each image before thresholding at a pre-set level, which distinguished consistently between stained and non-stained areas. A percentage area fraction of immunostaining per field area was calculated for each aneurysm biopsy from a mean of 10 images taken over 5 stained sections (Figure 4.12).

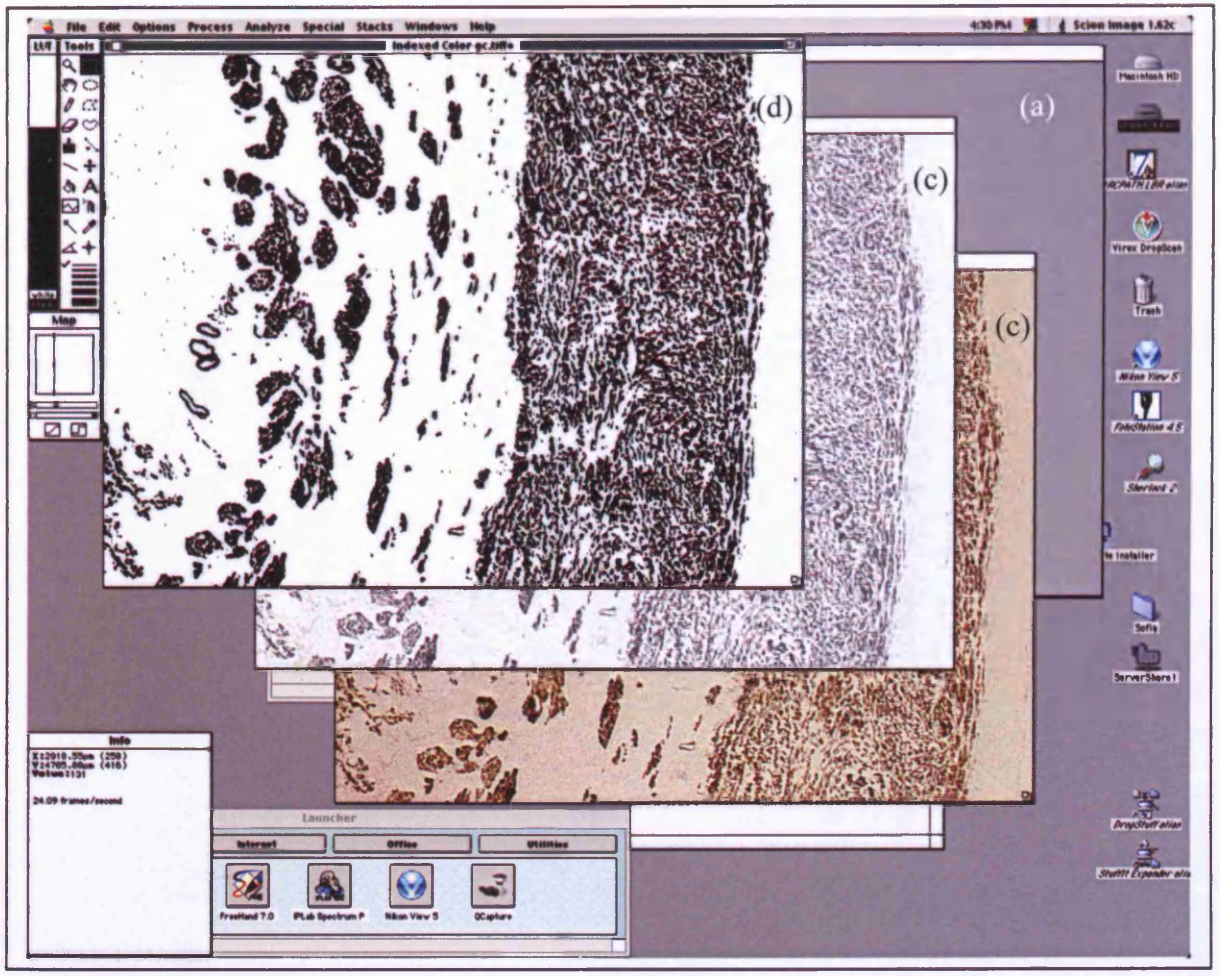


Figure 4.12
Process of (a) background capture, (b) image capture, (c) digital subtraction, (d) thresholding and quantitation

4.3 Statistical Methods

Statistical analysis used GraphPad Prism 5. Discrete variables were presented as numbers and percentages and compared using Fisher's exact test. The continuous variable age was normally distributed, presented as a mean (and standard deviation) and compared using the independent t-test. Other continuous variables were non-normally distributed, reported as a median and interquartile range (AAA diameter, MMP levels, TIMP levels and cellular quantitation data) and compared using the Mann-Whitney U-test or Wilcoxon Paired test. Spearman's Correlation was used to test for correlations. Statistical significance was assumed at the $p<0.01$ level.

CHAPTER 5

RESULTS

CHARACTERISATION OF MMP-8 EXPRESSION IN NORMAL AORTA AND ABDOMINAL AORTIC ANEURYSMS

5.1 Protein Quantification

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Levels Of MMP-8 In Normal Aorta And AAA
Results - Levels Of TIMP's In Normal Aorta And AAA

5.2 Aortic Wall Histology

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Hematoxylin and Eosin, Elastin Van Geison Staining
Results - MMP-8 Immunohistochemistry and Localisation Studies
Results - MMP-8 In Situ Hybridisation

5.3 Summary of Findings

5.1 Protein Quantification

Introduction

The structural integrity of the abdominal aorta is dependent upon the extracellular matrix proteins and mesenchymal cells within the aortic media and adventitia. Two groups of extracellular matrix proteins predominate – elastins and collagens (Dobrin., et al 1994). Elastins are an amorphous group of proteins that provide extensile properties. The fibrillar collagens, types I and III, impart tensile strength to the aorta (Dobrin et al., 1984). In the normal abdominal aorta there is 2 to 3 times more type I than type III collagen, and this ratio is maintained in the aneurysmal state (Menashi et al., 1987; Rizzo et al., 1987).

Aneurysm formation is associated with a chronic inflammatory response (Brophy et al., 1991; Annabi et al., 2002), depletion of the smooth muscle cell population (Lopez-Candales et al., 1997) and excessive MMP production (Freestone et al., 1995; Annabi et al., 1995), causing abnormal degradation of elastins and collagens. *In vitro* models suggest that loss of elastin is responsible for early aortic expansion and loss of recoil, whilst late expansion and possibly rupture are modulated by collagen breakdown (Dobrin et al., 1994). The elastolytic MMP's, MMP–2 and -9 appear to play a significant role in initiating aneurysm formation (Longo et al., 2002). The traditional aortic collagenases, MMP-1 and MMP–13, are present in relatively low concentrations in the aortic wall (Freestone et al., 1995; Mao et al., 1999; Tamarina et al., 1997), and have substrate specificities

favouring breakdown of collagen types II and III (Hasty et al., 1987; Knauper et al., 1996).

Neutrophil collagenase or MMP-8 is a potent type I collagenase implicated in the atherosclerotic process (Herman et al., 2001). Given that type I collagen is the most abundant collagen in the AAA wall, we hypothesised that a specific type I collagenase must be involved in the aneurysmal process. The aim of the present study was to determine the scale and site of MMP-8 expression in normal and AAA wall.

Aims

To determine the level of MMP-8 expression in normal aorta and AAA. To determine the level of the MMP inhibitors, TIMP-1 and TIMP-2, expressed in age-matched normal aorta and AAA.

Patient Selection

Twenty-two normal aortas from organ donors were collected by the UK Human Tissue Bank. Organ donors were younger than non-ruptured AAA patients (mean age 51+/- 4 yrs versus 72 +/- 7 yrs). To ensure close age-matching of the study cohorts, only normal aorta from the 10 oldest patients (age > 60 yrs) were used (mean age 66 +/- 2 yrs). The remaining 12 patients (age ≤ 60 yrs) were excluded and not analysed (mean age 40 +/- 4 yrs). Over the course of the study 63 non-ruptured aneurysm patients were recruited (mean age 72 +/-7 yrs). Only the youngest 40 non-ruptured aneurysm patients (age < 75 yrs) were included in the comparison within this chapter (mean age 68 +/- 6 yrs). The remaining 23 non-ruptured aneurysm patients, representing the oldest third of non-ruptured aneurysm patients, were excluded (mean age 79 +/- 0.6 yrs). Therefore, two age-matched subgroups were studied, the 40 youngest non-ruptured AAA (age < 75 yrs) and the 10 oldest normal aortas (age > 60 yrs). This resulted in a comparison of MMP-8 and TIMP levels between similarly aged cohorts: mean age AAA = 68 +/- 6 yrs, mean age normal aorta = 66 +/- 2 yrs, independent t-test - p = 0.2.

Table 5.1 summarises the characteristics of the age-matched subgroups. There were no statistical differences in age, gender, cardiovascular events (documented myocardial infarction, cerebrovascular or peripheral vascular disease, angina requiring medication), hypertension, smoking habit or diabetes between the age-matched groups of normal aorta and non-ruptured AAA.

Characteristics	Normal Aorta (n=10, age>60yrs)	AAA (n=40, age<75yrs)	p-value
Mean (Std) Age (years)	66 (+/- 2)	68 (+/-6)	0.177†
Gender	7 males (70%)	37 males (93%)	0.086
Cardiovascular Event	3 (30%)	9 (23%)	0.686
Hypertension	3 (3%)	23 (58%)	0.164
Current or ex-smoker	5 (5%)	31 (78%)	0.118
Diabetes	2 (2%)	4 (10%)	0.586

Table 5.1: Characteristics of age-matched normal aorta and AAA subjects (Fisher's Exact Test, † Independent t-test with group mean and standard deviation, $p<0.01$).

Table 5.2 summarises the characteristics of the normal and non-ruptured AAA patients excluded, on the basis of age, from the analysis. It is interesting to note, as well as age, other factors associated with cardiovascular risk were significantly less frequent in the excluded group of normal aorta compared to the excluded group of non-ruptured AAA. Thus an exclusion criteria based on age resulted in close matching of other risk factors in the studied groups (Table 5.1). Indeed, even following the exclusion of half the normal aortas and one-third of the aneurysmal aortas, this study represents one of the largest cohorts of age-matched normal versus aneurysmal aorta in vascular literature.

Characteristics	Normal Aorta (n=12, age≤60)	AAA (n=23, age≥75)	p-value
Mean (Std) Age (years)	40 (+/- 4)	79 (+/-0.6)	<0.001†
Gender	7 males (58%)	21 males (91%)	0.033
Cardiovascular Event	2 (17%)	12 (52%)	0.070
Hypertension	1 (8%)	16 (70%)	<0.001
Current or ex-smoker	1 (8%)	20 (87%)	<0.001
Diabetes	1 (8%)	2 (9%)	1.0

Table 5.2: Characteristics of excluded normal aorta and AAA subjects (Fisher's Exact Test, † Independent t-test with group mean and standard deviation, p<0.01).

Laboratory Methods

Biopsies were processed according to the protocols described in the methods section 4.2. In brief, tissue from normal aorta and AAA were homogenised, centrifuged and dialysed in buffer overnight. The resultant homogenates underwent ELISA analysis for the quantification of MMP-8, TIMP-1 and TIMP-2.

Levels Of MMP-8 In Normal Aorta And AAA

The levels of total and active MMP-8 were compared in normal and aneurysmal abdominal aorta (Figure 5.1 and 5.2). Both forms of the enzyme were present in normal and aneurysmal aorta, however, MMP-8 was greatly elevated in aneurysmal aorta. There was no correlation between MMP-8 concentration and aorta size (MMP-8(a) p=0.537, r=0.089; MMP-8(t) p=0.494, r=-0.098).

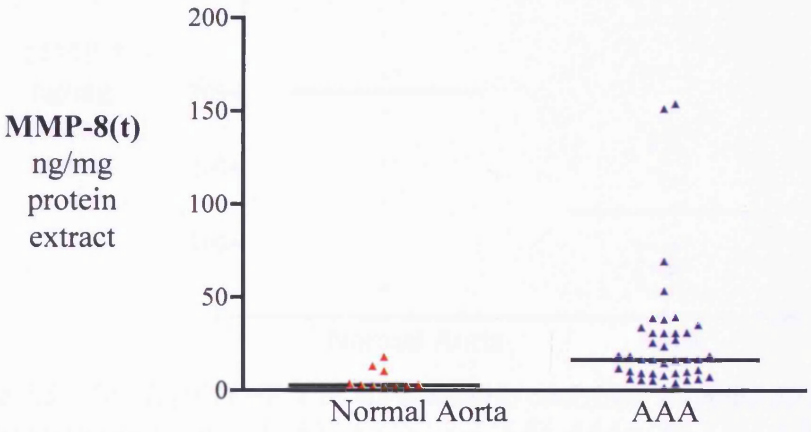


Figure 5.1: Levels of total MMP-8 in normal aorta and AAA, detected by ELISA (median (interquartile) NA =2.8 (1.8-11.6), AAA =16.6 (9.3-30.9), p<0.001).

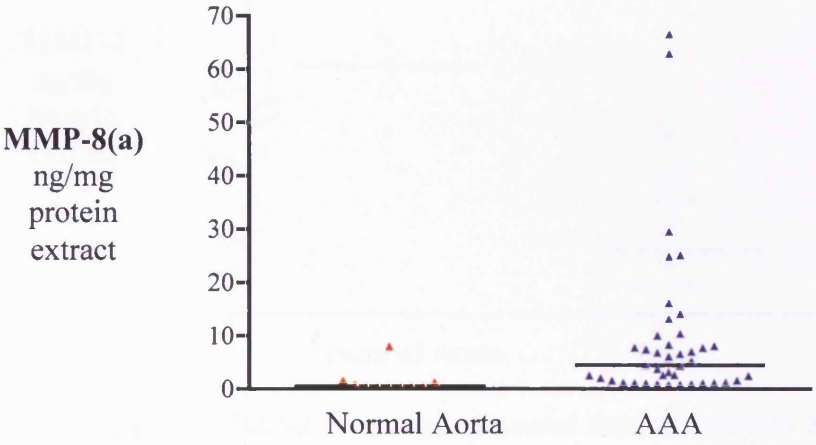


Figure 5.2: Levels of active MMP-8 in normal aorta and AAA, detected by ELISA (median (interquartile) NA=0.5 (0.4-1.4), AAA=4.5 (1.2-10.3), p<0.001).

The levels of TIMP-2 were markedly lower in AAA tissue compared to normal aorta ($p<0.001$). The decrease in TIMP-1 in AAA was less pronounced ($p=0.05$). The ratio of TIMP-1 and –2 to MMP-8 in each of the normal and AAA tissues were not calculated because of the potential cross-reactivity of the assay kits.

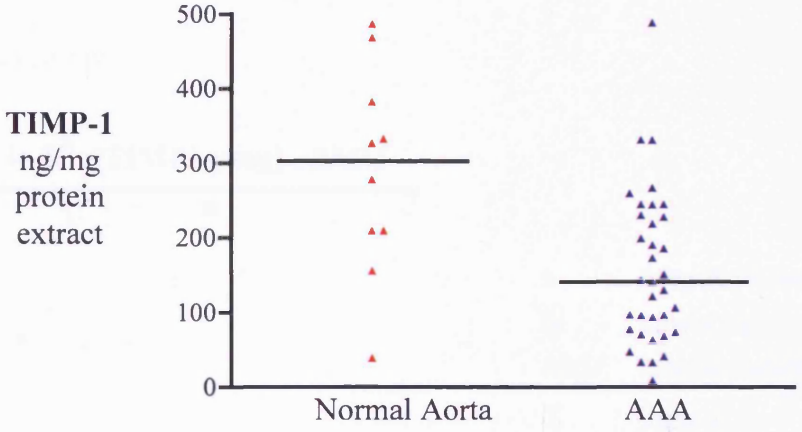


Figure 5.3: Levels of TIMP-1 in normal aorta and AAA, detected by ELISA (median (interquartile) NA=302.8 (182.8-425.8), AAA=142.2 (75.6-235.4), p=0.05)

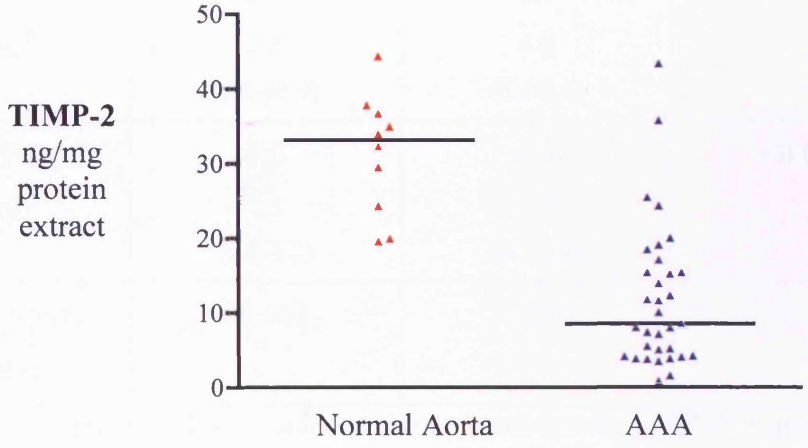


Figure 5.4: Levels of TIMP-2 in normal aorta and AAA, detected by ELISA (median (interquartile) NA=33.1 (26.1-37.2), AAA=9.2 (4.2-16.2), p<0.001).

The median levels of TIMP-1 were much higher than TIMP-2, however, a direct comparison could not be made based on the mass of each, since the two TIMPs are of differing relative molecular mass. Expressing the molar concentrations of each TIMP per volume protein extract allowed for proper comparison. Intra- and inter-group comparison of median values is limited. Comparison of geometric means, calculated by exponentiation of the logarithmic transformed mean is preferable. The formula for the geometric mean for the molar concentration of the TIMPs is as follows:

$$e^{\frac{\ln \Sigma^{x} [\text{TIMPng/mg}] \bullet RMM}{n}}$$

n	Avogadro's Number
ln	*natural log*
RMM	Relative Molecular Mass
Σ	Sum of

	Normal Aorta mmols/litre	Aneurysmal Aorta mmols/litre	Independent t-test P value
TIMP-1 $X10^6$	8.9 3.0 (5.8-10.9)	4.9 4.0 (3.8-6.4)	0.046
TIMP-2 $X10^6$	1.0 2.3 (0.9-1.2)	0.3 3.7 (0.2-0.4)	<0.001
Paired t-test P value	<0.001	<0.001	

Table 5.3: Comparison between the molar concentrations of TIMP-1 and TIMP-2 in aneurysmal and normal abdominal aortic tissue. Data presented as geometric means, standard deviation and confidence limits of the means at the 95% interval.

The data in Table 5.3 confirmed that the molar concentration of TIMP-1 was significantly greater than that of TIMP-2, in both normal and aneurysmal abdominal aorta. It may well be that much of the TIMP-2 was bound to pro-MMP-2 and the TIMP-2 ELISA assay failed to detect this complex. TIMP-1 assays, on the other hand, recognised all total TIMP-1 including that complexed to MMP's. This may account for the relatively higher molar levels of TIMP-1 compared to TIMP-2. More importantly, the decrease in TIMP-2 between normal and aneurysmal aorta was highly significant, the decrease in the molar level of TIMP-1 was of borderline significance.

There was a significant elevation in the quantity of protein extracted from aneurysmal aorta, in comparison to normal aorta, as determined by photometric analysis. MMP and TIMP levels determined by ELISA (expressed as ng of target protein per ml of total protein extract) were corrected for the quantity of protein extracted (expressed as mg of extracted protein per ml of homogenising buffer). Hence the final value of each MMP was expressed as ng of target protein per mg of protein extract and thus circumventing the difference in protein extraction.

An alternative to protein correction is the analysis of dry weight aorta. The removal of water alters the concentrations of proteins. Thus comparisons are based on a protein environment far removed from the *in vivo* arrangement. Further, the extract may be corrected to the DNA content of the biopsy sample. This was not considered due to the small size of the biopsies.

5.2 Aorta Wall Histology

Introduction

Good evidence exists for the active expression of various elastin- and collagen-degrading MMP's by SMC's, macrophages, and microvascular endothelium. How these different cell types and their proteolytic products might interact during various stages of the evolution of an AAA, however, remains to be delineated. It appears likely that no single MMP or cell type is solely responsible for the entire spectrum of proteolytic events underlying aneurysmal degeneration. This process probably represents the net result of multiple proteases and their mechanisms of regulation *in vivo*.

Aims

To determine the cell types synthesising MMP-8 at protein and mRNA levels, in representative samples of AAA and normal aorta.

Patient Selection

Representative histology sections of AAA and normal aorta were studied.

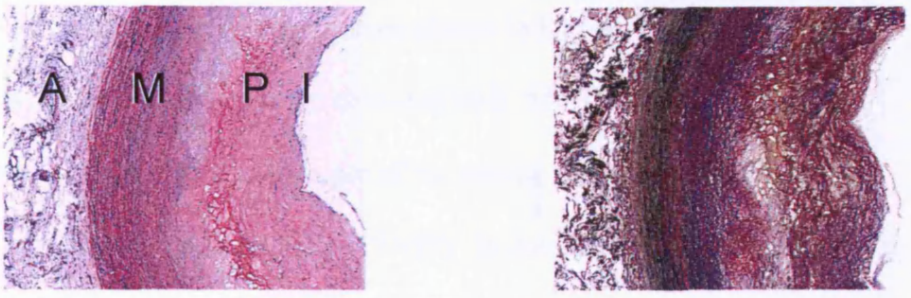
Laboratory Methods

Biopsies were processed for histology according to the protocols described in the methods section 4.2. In brief, normal aorta and AAA were paraformaldehyde-fixed and paraffin embedded. Sections were cut from the paraffin blocks for immunohistochemistry. Immunohistochemistry was conducted for MMP-8 and cell localisation studies including macrophages, lymphocytes, neutrophils, smooth muscle cells and native mesenchymal cells. *In situ* hybridisation was conducted for MMP-8 mRNA. Staining with hematoxalin /eosin and elastin Van Geison was also conducted.

Hematoxalin and Eosin, Elastin Van Geison Staining

The histological appearance of the normal aortic wall is illustrated in Figure 5.5 (hematoxylin and eosin, and elastin Van Geison). An area of atheromatous plaque (P) is noted within the intima of the normal aorta. The media contains confluent elastin layers (EVG). Figure 5.5 also shows a representative section of aneurysmal abdominal aorta with disruption of the intima and deposition of intramural thrombus (H+E). Within the aneurysmal media elastin fibers are qualitatively decreased (EVG). The adventitia is thickened (H+E).

Normal Aorta



Non-ruptured Abdominal Aortic Aneurysm

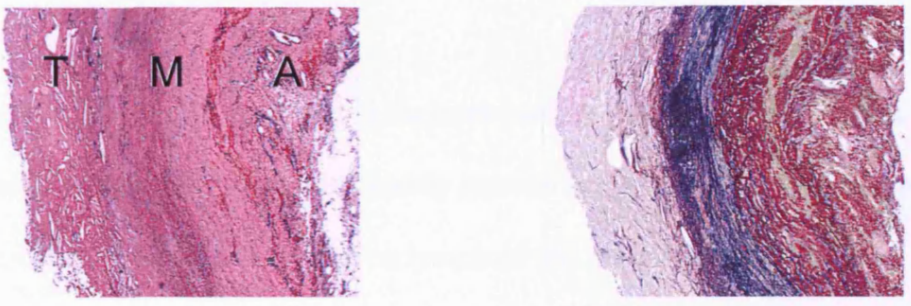


Figure 5.5
Hematoxylin and eosin (H+E), elastin van Geison (EVG) at paired anterior wall of normal and non-ruptured AAA.
(magnification x40, I – intima, M – media, A – Adventita, T - thrombus)

MMP-8 Immunohistochemistry and Localisation Studies

Representative MMP-8 IHC sections are shown in Figures 5.6. Two distinct patterns of MMP-8 expression are demonstrated. MMP-8 immunostaining is noted within the atheromatous plaque of the normal aorta (Figure 5.6), which localises to macrophage-foam cells (CD-68). In contrast, MMP-8 expression in aneurysmal aorta is strongly positive throughout the media and adventitia (Figure 5.7). This localises to medial smooth muscle cells and vimentin positive cells in the adventitia on serial section (α-SMA and Vimentin). Extracellular staining is evident in both tissue types reflecting the site of action of this enzyme.

CD15 positive neutrophils are noted in the intra-luminal thrombus of aneurysms, but neither CD15 nor CD45 are significantly represented in the aneurysm (Figure 5.7). The vimentin antigen is present on lymphoid and mesenchymal cells (smooth muscle cells and fibroblasts). Since CD15, CD45 and α-SMA fail to localise to MMP-8 expression in the adventitia, this suggests that vimentin positive fibroblasts or de-differentiated smooth muscle cells lacking α-SMA expression produce adventitial MMP-8. The quantification of MMP-8 IHC, by image analysis, was not undertaken. MMP's are extracellular proteins with a high degree of matrix binding. This results in unacceptable levels of matrix binding and the inability of image analysis to differentiate the cellular cytoplasm from the matrix staining.

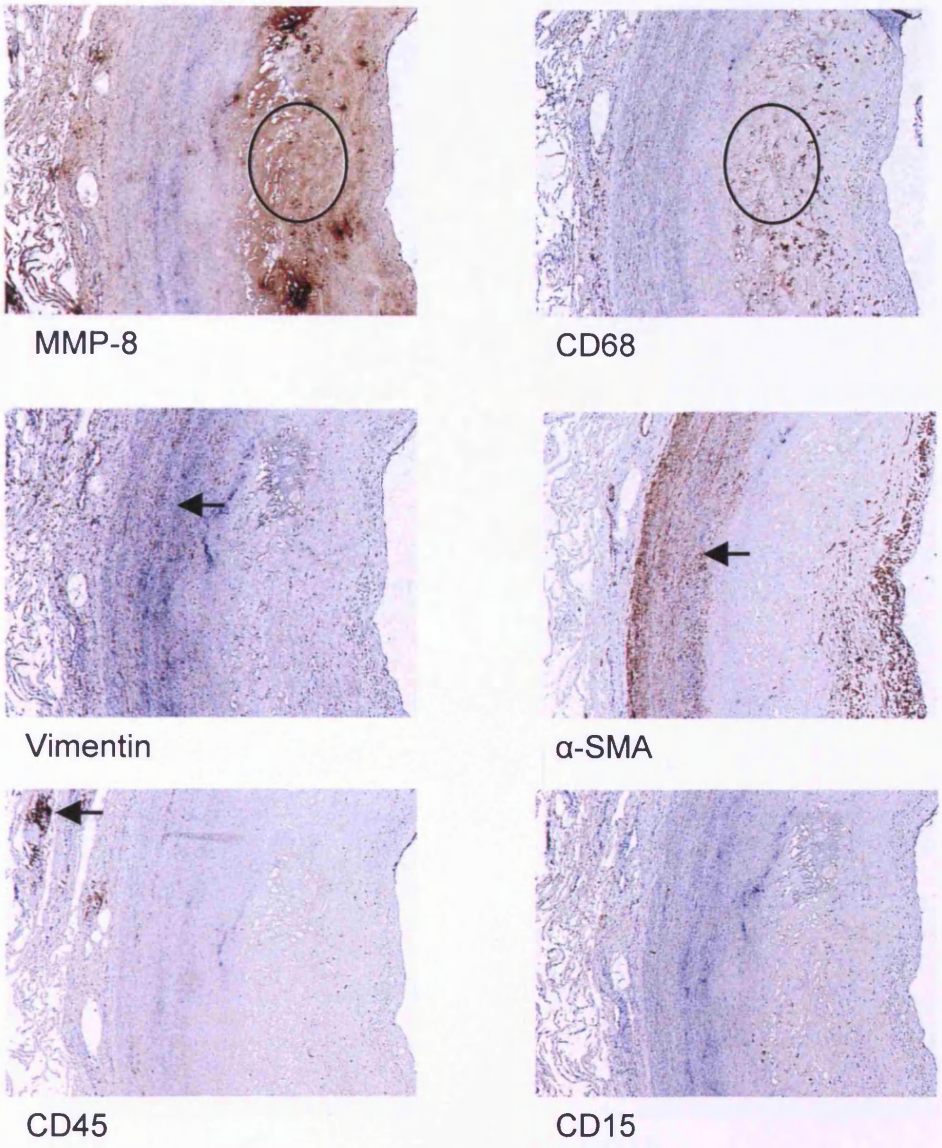


Figure 5.6
MMP-8 co-localisation study from normal aorta (magnification x40, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of CD-68 with MMP-8).

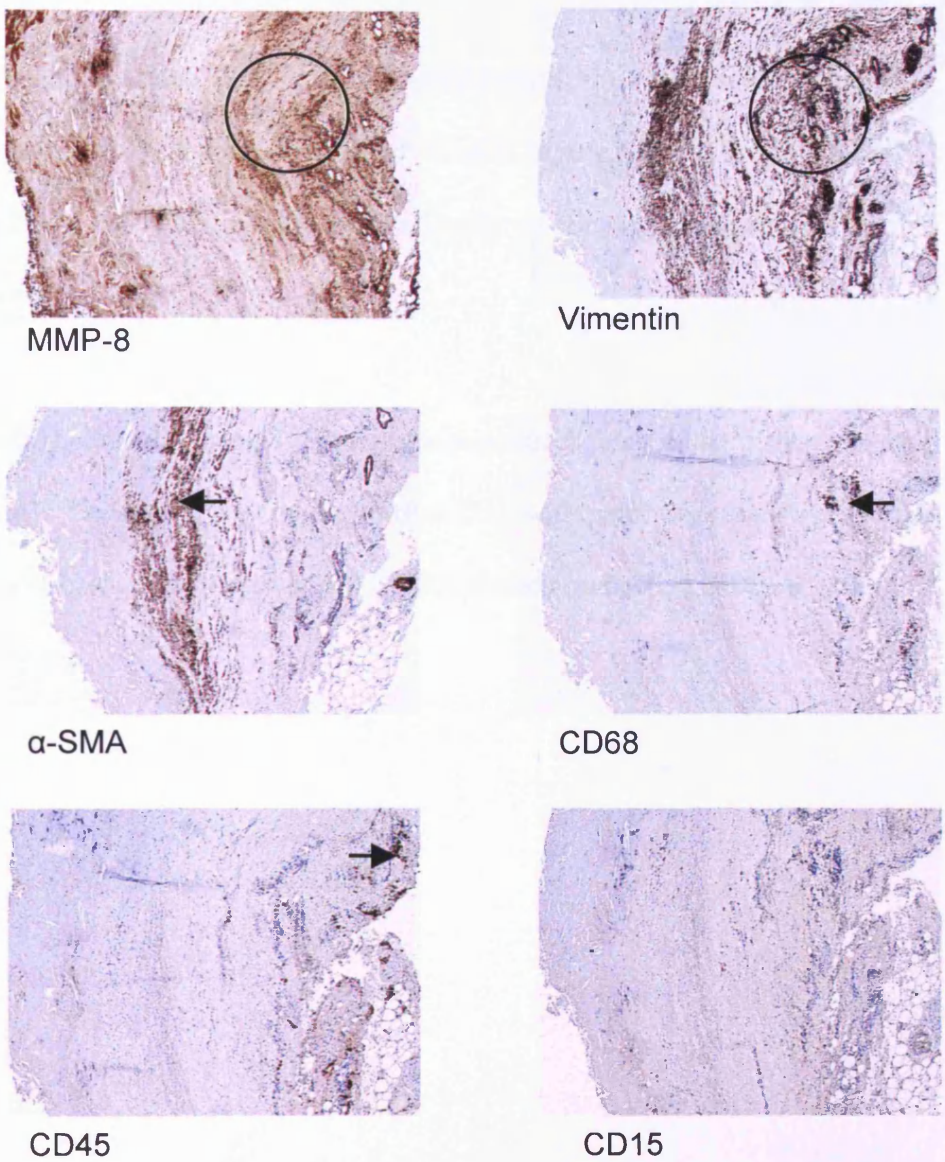


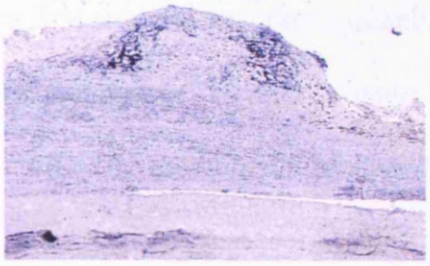
Figure 5.7
MMP-8 co-localisation study from the anterior wall of a non-ruptured AAA (magnification x40, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of Vimentin with MMP-8).

MMP-8 In Situ Hybridisation

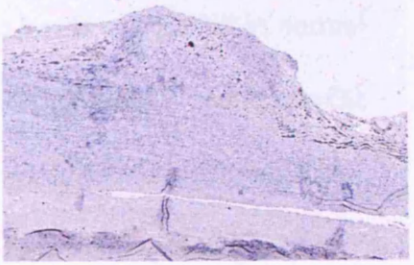
Figure 5.8 demonstrates positive ISH for MMP-8 mRNA within spindle shaped mesenchymal cells of the aneurysmal adventitia. Figure 5.8 also shows MMP-8 positive ISH in macrophage cells at the shoulder of atheromatous plaque in normal aorta.

Negative ISH controls, using 4 sense probe sequences returned no positivity (Figure 5.8). The quantification of MMP-8 ISH, with point counting or grey-scale image analysis, was not undertaken. ELISA already quantified MMP-8 protein objectively.

Normal Aorta

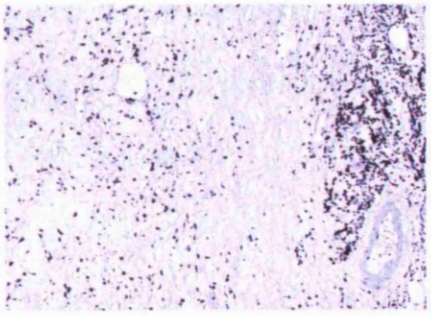


ISH MMP8

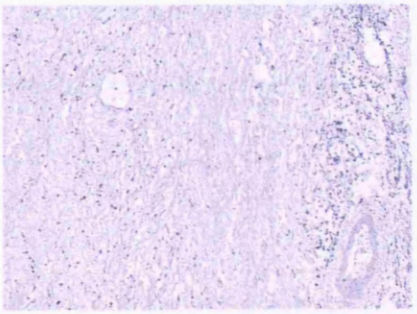


ISH Random probe

Non-ruptured Abdominal Aortic Aneurysm



ISH MMP8



ISH Random probe

Figure 5.8
MMP-8 In situ hybridisation at the anterior wall of normal and non-rupture AAA (magnification x100).

5.3 Summary of Findings

This is the first study to accurately quantify protein levels of MMP-8 in normal and aneurysmal abdominal aortic tissue. Furthermore, this study represents the largest cohort of age-matched normal and aneurysm aorta ever analysed. The results of this study suggest that MMP-8 (a potent type I collagenase) may be implicated in aneurysm formation. The levels of active and total MMP-8 were significantly elevated in aneurysmal tissue in comparison to normal control aorta. The level of its principal inhibitor, TIMP-1, was decreased in AAA. This would predispose an aneurysm to increased type I collagen breakdown. However, the absolute diameter of an aneurysm did not appear to be dependant on the MMP-8 concentration. MMP-8 may represent a target for pharmacotherapy aimed at aneurysm stabilisation.

Immunohistochemistry co-localised MMP-8 principally to vimentin-positive mesenchymal cells (either smooth muscle cells or fibroblasts) in the adventitia of the abdominal aortic aneurysm. The failure of α-SMA immuno-reactivity in the adventitia of the abdominal aortic aneurysm biopsies does not conclusively prove these adventitial cells to be fibroblasts. Significant loss of actin expression is reported in aneurysms (Annabi et al., 2002). This change is associated with smooth muscle cell de-differentiation following physical or chemical insult (Casscells, 1992). Hence, the exact nature of the mesenchymal cells within the adventitia of aortic aneurysms, whether smooth muscle or fibroblastic, is indeterminate. In the normal aorta, MMP-8 co-localised strongly to CD-68 positive macrophage-foam cells within atheromatous plaque, but only weakly to

mesenchymal smooth muscle cells and fibroblasts. The lack of CD-15 and CD-45 immuno-reactivity excludes the leukocyte and lymphocyte as a confounding source of MMP-8 in both tissue types. Thus the subtleties of MMP-8 expression were only apparent with immunohistochemistry. The process of atherosclerosis is different to aneurysm formation and this study serves to highlight important cellular subtypes and their topographical orientation within each process.

There were a number of confounding factors within the study and attempts were made to overcome these. The effect of the organ donor patient's terminal disease on the results of this study could not be controlled for and a future study comparing grossly occlusive disease to abdominal aortic aneurysms may be of value. The comparison of a single factor between two patient cohorts requires all other factor to be identical. Rigorous efforts were made ensure age-matching of the study cohorts. The youngest normal aortas (age $\leq$ 60 yrs) and the oldest AAA (age $\geq$ 75yrs) were excluded from the analysis. It could be argued that the exclusion of large numbers of subjects, half the normal aorta and one-third of the AAA, biased the results. However, the analysis of excluded patients demonstrated grossly different age and risk factor profiles that would have caused even greater uncertainty.

In conclusion, despite the subgroup analysis, this study represents the largest cohort of age-matched normal and aneurysm aorta ever analysed in vascular literature. Furthermore, given the predominance of type I collagen in the aorta, its proteolysis by a specific collagenase must be a prerequisite to continued aneurysm expansion and rupture. This study demonstrated up-regulation of MMP-8 (a

potent type I collagenase) and a fall in its endogenous inhibitors, TIMP-1 and TIMP-2, within the adventitia of the aneurysm wall. This finding is very important since the majority of arterial type I collagen is located there (Davidson et al., 1985). These findings indicate an *in vivo* pathway for adventitial type I collagen breakdown analogous to the animal models described by Dobrin et al. (Dobrin et al., 1984).

CHAPTER 6

RESULTS

CHARACTERISATION OF MMP EXPRESSION IN NON-RUPTURED AND RUPTURED ABDOMINAL AORTIC ANEURYSM

6.1 Anterior Sac - Non-ruptured versus Ruptured AAA

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Correlation between Non-Ruptured AAA diameter and MMP Levels
Results - MMP and TIMP in Anterior Sac of Non-Ruptured and Ruptured AAA

6.2 Rupture Anterior Sac versus Rupture Edge

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Aims
Patient Selection
Laboratory Methods
Results - MMP and TIMP in paired Rupture Anterior Sac and Rupture Edge

6.3 Aortic Wall Histology

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Hematoxylin and Eosin, Elastin Van Geison Staining
Results - MMP-8 and MMP-9 Immunohistochemistry and Localisation Studies
Results - MMP-8 and MMP-9 In Situ Hybridisation

6.4 Summary of Findings

6.1 Anterior Sac - Non-ruptured versus Ruptured AAA

Introduction

The formation and expansion of an abdominal aortic aneurysm (AAA) is characterized by extracellular matrix degradation, increased proteolytic activity, and an inflammatory cell infiltrate (Annabi et al., 2000). Fragmentation of elastin fibers and a reduction in elastin concentration appears to be mediated by increased concentrations of matrix metalloproteinases (MMP's) secreted by various cell types within the aortic wall (Sakalihasan et al., 1993). With progressive loss of elastin, established AAA's are largely composed of collagens types I and III (Rizzo et al., 1989).

It seems reasonable to suggest that the final common pathway, leading to aortic rupture, might involve proteolytic degradation of the collagen matrix. This concept was first investigated by Dobrin et al., (Dobrin et al., 1984), who investigated the proteolytic effects of purified collagenase and elastase on isolated arterial tissue perfused at supraphysiological pressures. Treatment with elastase caused the vessels to dilate markedly and become less compliant, but was not related to rupture. In contrast, treatment with collagenase caused the blood vessels to dilate, become more compliant and rupture. These findings supported the hypothesis that elastin degradation was a key step in aneurysmal dilatation but that collagen degradation was ultimately required for AAA rupture.

The role of MMP's in initiating AAA formation has been extensively delineated (Longo et al., 2002; Freestone et al., 1995; McMillan et al., 1997; Sakalihasan et al., 1996), in comparison only a limited number of studies examine MMP changes associated with AAA rupture (Petersen et al., 2002).

Aims

To compare the levels of the elastases MMP-2 and -9, the collagenases MMP-1, -8, -13, the stromelysin MMP-3, and MMP inhibitors TIMP-1 and -2 in biopsy samples taken from the anterior sac of non-ruptured and ruptured AAA. To determine if the expression of MMP's correlates with aneurysm diameter. To determine if MMP expression is up-regulated in ruptured AAA, compared to non-ruptured AAA, in site-matched anterior aneurysm sac biopsies.

Patient Selection

Over the study period 63 non-ruptured and 21 ruptured AAA were recruited. All of the recruited patients underwent intra-operative biopsy of their AAA. Biopsies were frozen in liquid nitrogen, then homogenised at per protocol for the extraction of MMP's. The MMP content of each homogenate was quantified using ELISA. The clinical features of the 63 non-ruptured and the 21 ruptured AAA subjects are described in Table 6.1. Median AAA diameter was greater in ruptured than non-ruptured AAA. There were no other differences in the characteristics of the study cohorts.

Patient Characteristics	Non-Ruptured AAA (n=63)	Ruptured AAA (n=21)	p-value
Age	72 (+/-7.2) yrs	70 (+/- 5.3) yrs	0.389*
AAA size	6.3 (5.8-7.1)	8.5 (6.3 - 10)	0.005†
Gender Distribution	58 males (92%)	18 males (86%)	0.406
Cardiovascular Event	21 (33%)	3 (14%)	0.161
Hypertension	39 (62%)	11 (52%)	0.454
Smoking History	51 (81%)	14 (67%)	0.229
Diabetes	6 (9%)	0 (0%)	0.329

Table 6.1: Characteristics of patients with non-ruptured and ruptured abdominal aortic aneurysms (AAA). (Comparison used independent t-test (*) with group mean and standard deviation in years, Mann-Whitney U-test (†) with group median and interquartile range in cm, and Fisher's Exact Test, $p<0.01$).

Laboratory Methods

Biopsies were processed for ELISA according to the protocols described in the methods section 4.2. In brief, anterior aneurysm sac biopsies from non-ruptured and ruptured AAA were homogenised, centrifuged and dialysed in buffer overnight. The resultant protein extract underwent ELISA analysis for the quantification of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, TIMP-1 and TIMP-2.

Correlation between Non-Ruptured AAA diameter and MMP Levels

Table 6.2 demonstrates the correlations between aneurysm diameter in non-ruptured AAA and MMP concentrations. MMP concentration did not increase with AAA diameter. The correlation between MMP-9 and AAA diameter was negative and approached significance (p=0.028). A similar correlation within ruptured AAA was not tested for since the diameter of ruptured AAA could not be accurately obtained.

Enzyme Group	Co-efficient of correlation	p-value
MMP-1(t)	0.110	0.424
MMP-2(t)	0.035	0.779
MMP-3(t)	0.110	0.425
MMP-8(t)	-0.202	0.139
MMP-9(t)	-0.296	0.028
MMP-13(t)	-0.159	0.286
TIMP-1(t)	-0.090	0.512
TIMP-2(t)	0.130	0.353

Table 6.2: Correlation between non-ruptured abdominal aortic aneurysm (n=63) diameter and matrix metalloproteinase concentration. (Comparison used Spearman Correlation, p<0.01).

The level of protein extraction did not differ significantly between anterior aneurysm sac biopsies of non-ruptured and ruptured AAA (non-ruptured AAA = 3.79 +/- 1.49 mg/ml, ruptured AAA = 4.26 +/- 1.60, p=0.45).

Elastase Levels

The total levels of the elastase MMP-2 and MMP-9 (ng/mg) were compared in anterior sac biopsies from non-ruptured and ruptured AAA. There were no significant differences between the non-ruptured and rupture cohorts.

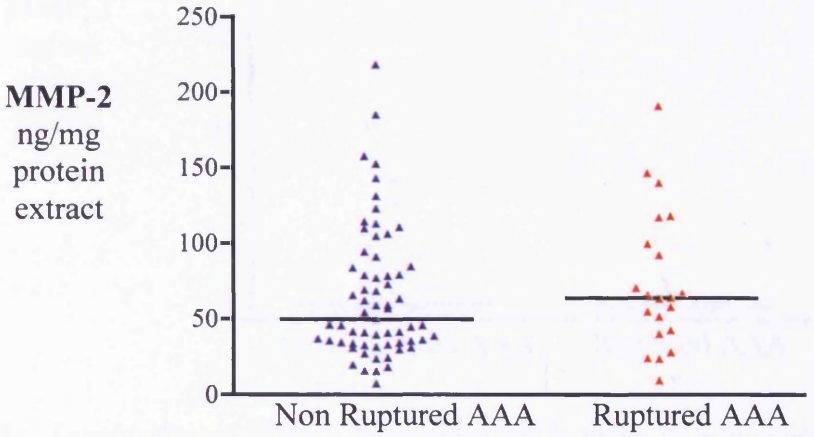


Figure 6.1: Levels of total MMP-2 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=49.5 (34.1-84.5), RAAA=63.8 (41.0-108.0), p=0.337, Mann-Whitney U-test).

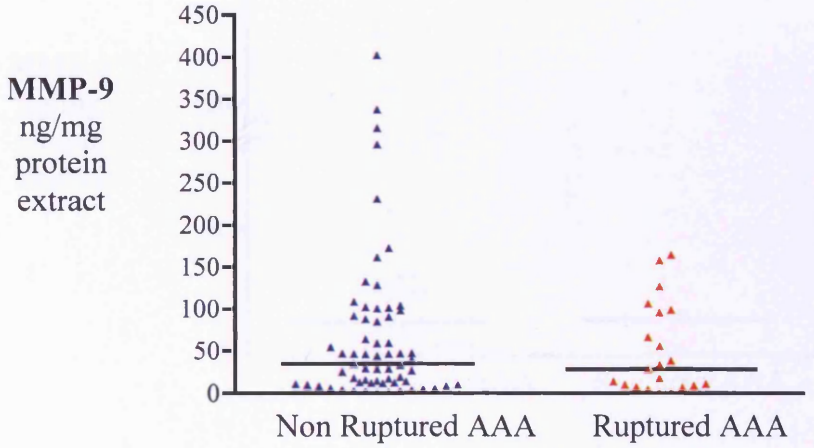


Figure 6.2: Levels of total MMP-9 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=34.5 (12.1-91.6), RAAA=28.5 (8.0-97.0), p=0.529, Mann-Whitney U-test).

Collagenase Levels

The levels of the collagenases MMP-1, MMP-8 and -13 (ng/mg) were compared in anterior sac biopsies from non-ruptured and ruptured aneurysmal abdominal aorta. There were no significant differences in the levels of collagenase between the non-ruptured and rupture cohorts.

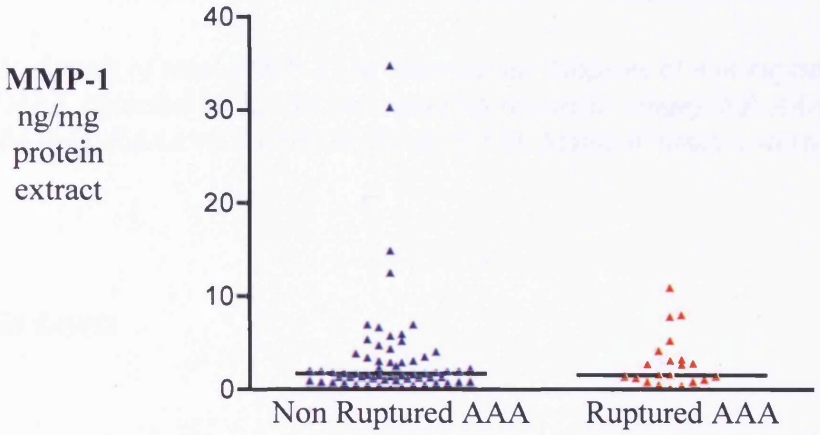


Figure 6.3: Levels of total MMP-1 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=1.7 (1.0-3.5), RAAA= 1.6(0.9-3.7), p=0.869, Mann-Whitney U-test).

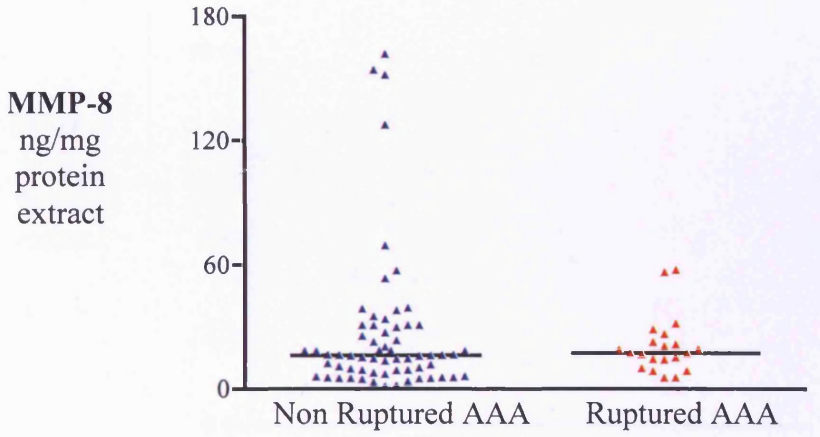


Figure 6.4: Levels of total MMP-8 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=16.3 (7.9-30.7), RAAA=17.3 (12.1-24.6), p=0.542, Mann-Whitney U-test).

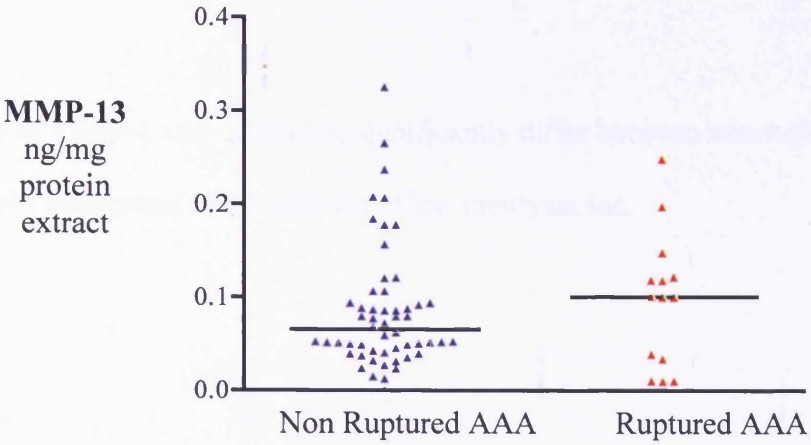


Figure 6.5: Levels of total MMP-13 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=0.07 (0.04-0.1), RAAA=0.1 (0.02-0.13), p=0.519, Mann-Whitney U-test).

Stromelysin Levels

Total levels of the stromelysin MMP-3 (ng/mg) were compared in anterior sac biopsies from non-ruptured and ruptured aneurysmal abdominal aorta; there was no difference.

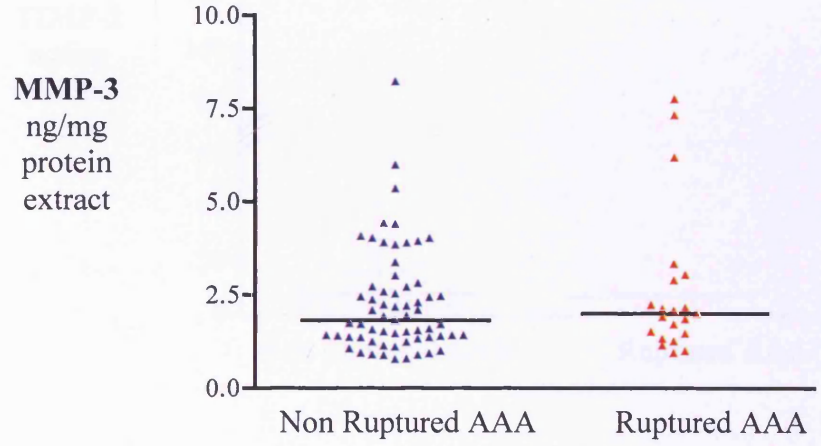


Figure 6.6: Levels of total MMP-3 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=1.8 (1.4-2.7), RAAA=2.0 (1.4-3.0), p=0.657, Mann-Whitney U-test).

TIMP Levels

The levels of TIMP-1 and –2 did not significantly differ between non-ruptured and ruptured aneurysms at the anterior of the aneurysm sac.

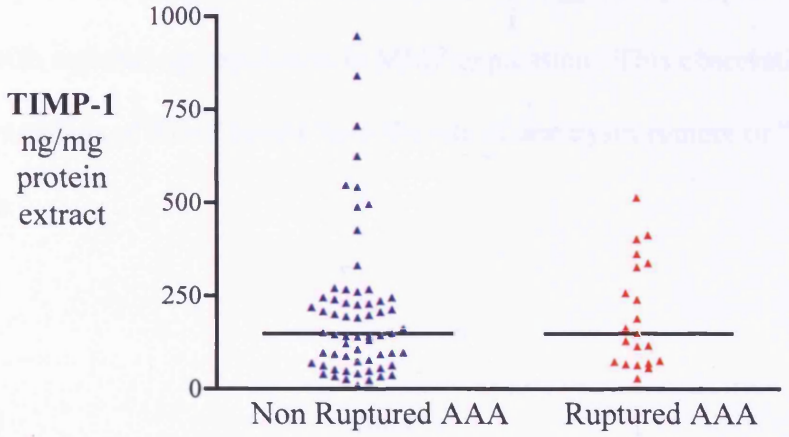


Figure 6.7: Levels of total TIMP-1 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=148.0 (73.2-245.0), RAAA=148.0 (70.8-333.0), p=0.884, Mann-Whitney U-test).

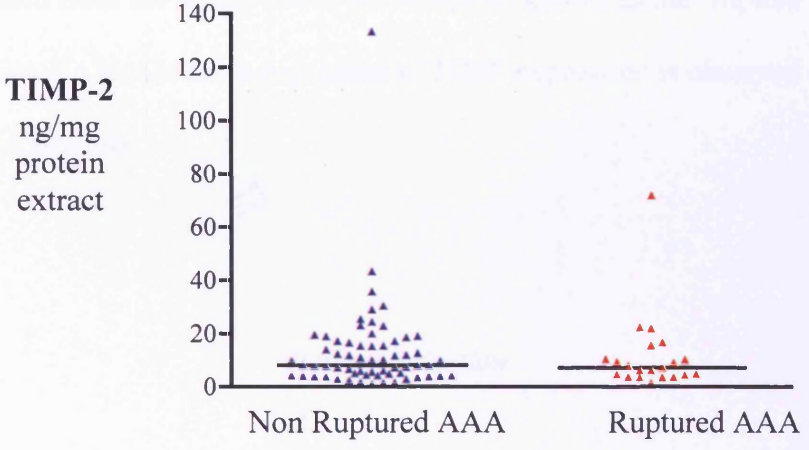


Figure 6.8: Levels of total TIMP-2 in anterior sac biopsies of non-ruptured and ruptured AAA, detected by ELISA (median (interquartile range), NR-AAA=8.1 (4.2-17.1), RAAA=7.2 (4.1-12.9), p=0.432, Mann-Whitney U-test).

6.2 Rupture Anterior Sac versus Rupture Edge

Introduction

The failure to demonstrate significant differences in MMP levels, in the anterior sac of non-ruptured and ruptured AAA (section 6.1), suggests that rupture is not associated with a global up-regulation in MMP expression. This observation thus justifies the analysis of MMP levels from the site of aneurysm rupture or "the rupture edge."

Aims

To compare the levels of the elastases, collagenases, stromelysins, and TIMP's in biopsy samples taken from the anterior sac of ruptured AAA with MMP levels in biopsies taken from the actual site of AAA rupture known as the "rupture edge". To determine if a localised up-regulation of MMP expression is observed at the site of AAA rupture.

Patient Selection

In total, 21 ruptured AAA were recruited over the course of the study. Due to the challenging anatomy of the ruptured aneurysm the "rupture edge" was identified in only 12 patients. The 12 ruptured aneurysms had biopsies taken from the

anterior of the aneurysm sac and from the edge of the rupture site. Ten of the 12 identified rupture sites were posterior with 2 being anterior. No rupture site coincided with the site of anterior aneurysm wall biopsy. In the remaining 9 ruptured AAA, biopsies were only taken from the anterior aneurysm sac. Paired rupture edge biopsies were not possible in these 9 patients due to the technical challenges of the rupture surgery. Thus, the paired comparison of MMP levels of anterior sac with rupture edge biopsies was conducted in 12 patients.

Laboratory Methods

Biopsies were processed for ELISA according to the protocols described in the methods section 4.2. In brief, paired ruptured AAA biopsies from anterior aneurysm sac and rupture edge were homogenised, centrifuged and dialysed in buffer overnight. The resultant protein extract underwent ELISA analysis for the quantification of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, TIMP-1 and TIMP-2.

MMP and TIMP in paired Rupture Anterior Sac and Rupture Edge

Elastase Levels

The total level of MMP-2 did not differ between the anterior sac and rupture edge of ruptured aneurysms. In contrast, the levels of total MMP-9 were significantly higher at the rupture edge in comparison to the anterior sac of the rupture.

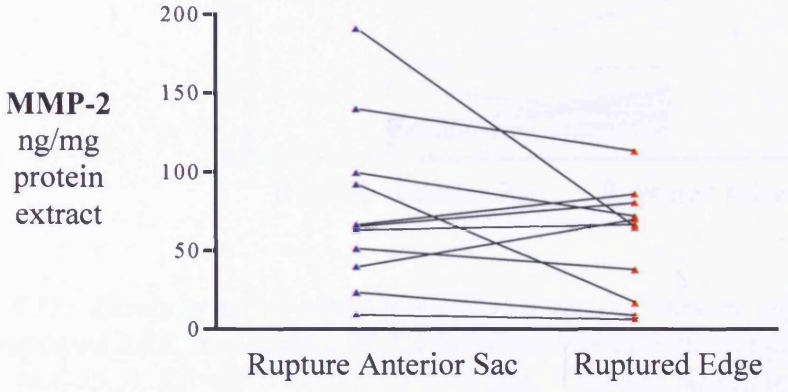


Figure 6.9: Levels of total MMP-2 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=66.1 (45.6-95.9), RE=65.9 (12.8-83.4), p=0.160, Wilcoxon paired test).

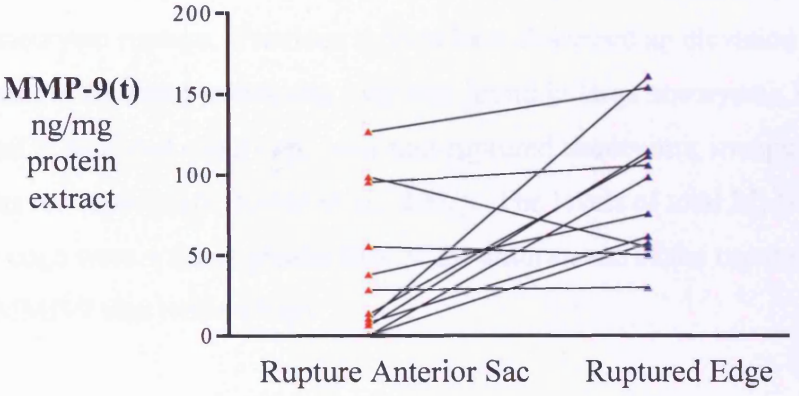


Figure 6.10: Levels of total MMP-9 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=21.2 (7.79-75.5), RE=87.1 (56.3-113), p=0.010,Wilcoxon paired test).

Further to the analysis of total MMP-9, an analysis of the active fraction of MMP-9 was conducted. Active MMP-9 was also found to be significantly elevated at the rupture edge over the anterior aneurysm sac levels.

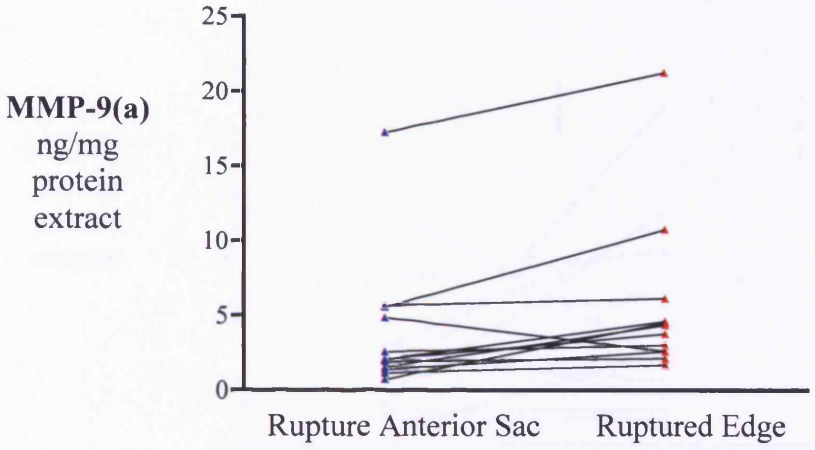


Figure 6.11: Levels of active MMP-9 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=6.2 (4.4-15.5), RE=12.2 (7.7-16.2), p=0.008, Wilcoxon paired test).

This is the first study to accurately quantify MMP-9 levels at the actual site of aortic aneurysm rupture. Previous reports have described an elevation of MMP-9 in the wall of ruptured aneurysms over that found in large aneurysms, however, an elevation in ruptured aneurysms over non-ruptured aneurysms, irrespective of size, was not reported (Petersen et al., 2002). The levels of total MMP-9 at the rupture edge were 4 times greater than at the anterior sac of the rupture, while active MMP-9 was twice a high.

Collagenase Levels

The levels of both MMP-1 and MMP-13 collagenases, detected by ELISA, were very low. There was no difference in MMP-1 or –13 levels in paired anterior wall and rupture edge biopsies.

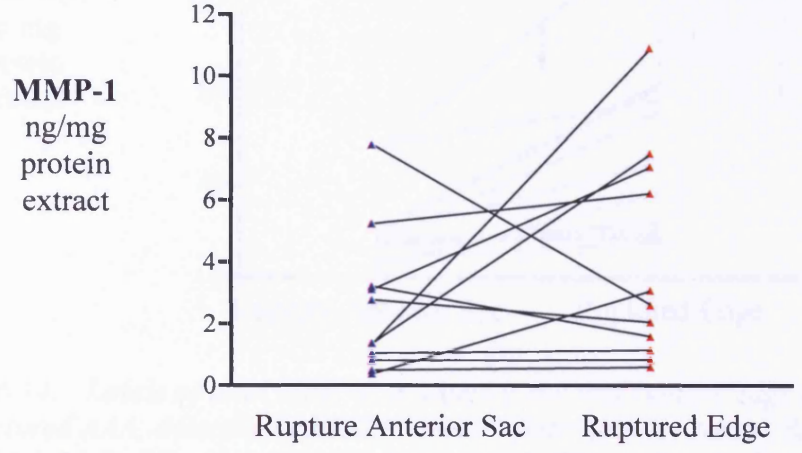


Figure 6.12: Levels of total MMP-1 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=1.38 (0.81–3.20), RE=2.59 (1.14 –7.08), p=0.240, Wilcoxon paired test).

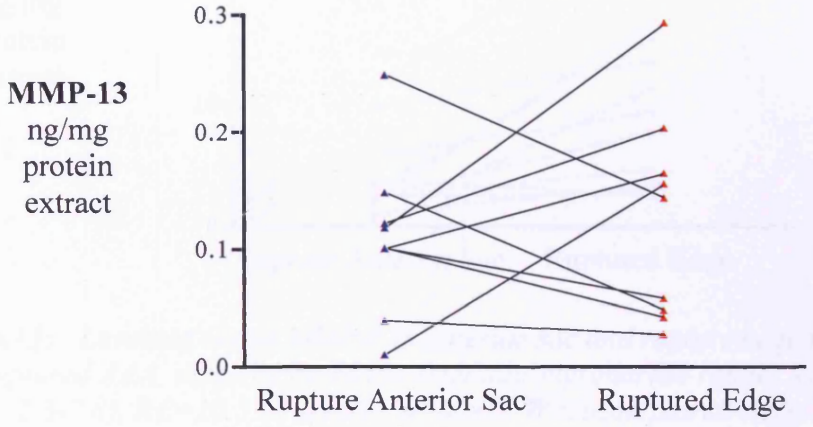


Figure 6.13: Levels of total MMP-13 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=0.11 (0.07–0.14), RE=0.14 (0.04 –0.18), p= 0.734, Wilcoxon paired test).

In contrast to the other collagenases, both the active and total levels of the potent type I collagenase MMP-8, were elevated at the ruptured edge in comparison to the anterior sac.

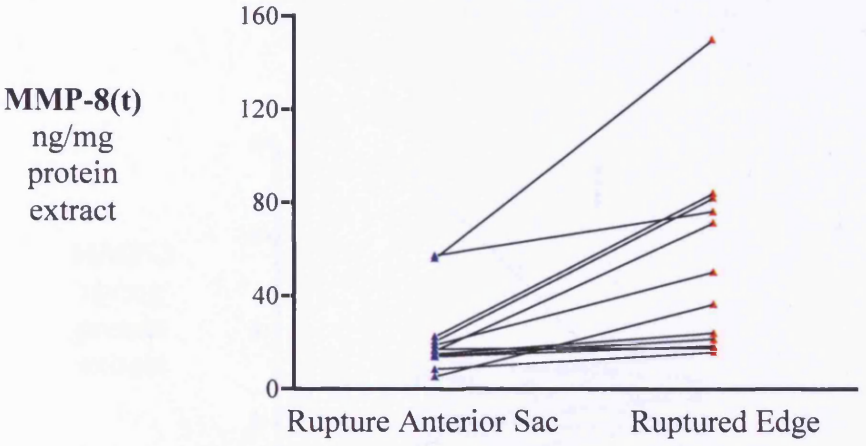


Figure 6.14: Levels of total MMP-8 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA: (median(interquartile range) RAS=17.0 (14.1-21.7), RE=43.6 (20.1–79.3), p<0.001, Wilcoxon paired test).

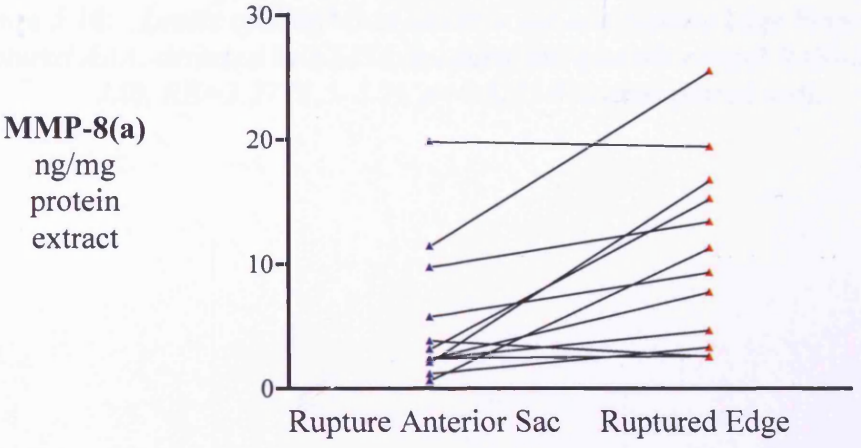


Figure 6.15: Levels of active MMP-8 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=2.8 (2.3-7.8), RE=10.3 (3.4-16.0), p=0.005, Wilcoxon paired test).

Stromelysin Levels

Stromelysin levels were unchanged between the anterior sac and the rupture edge.

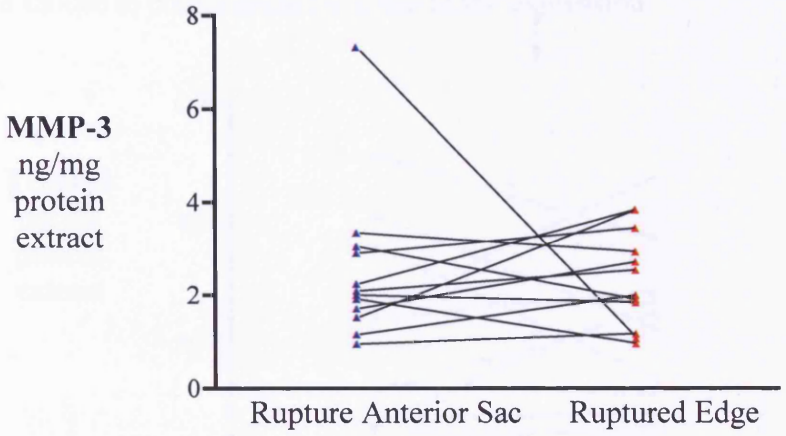


Figure 5.16: Levels of MMP-3 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median(interquartile range) RAS=2.1 (1.6-3.0), RE=2.27 (1.5–3.2), p=0.622, Wilcoxon paired test).

TIMP Levels

There was no significant difference in the levels of TIMP-1 in the rupture edge biopsies when compared to the rupture anterior sac biopsies. Also TIMP-2 levels failed to show any compensatory increase in expression at the rupture edge. This suggested a failure to compensate for a rise MMP expression.

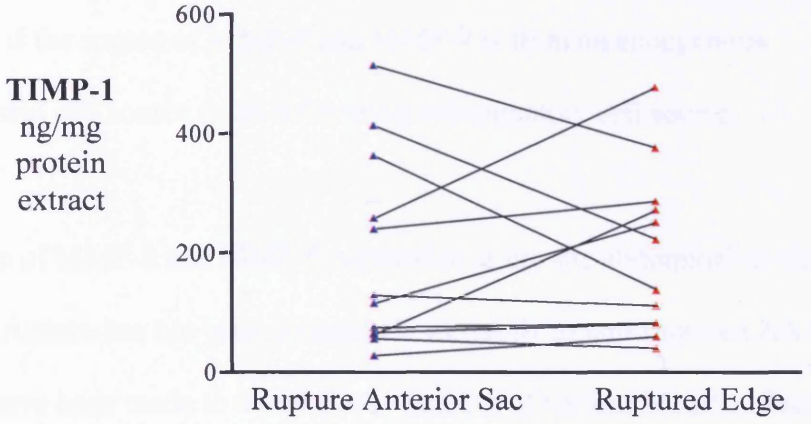


Figure 6.17: Levels of active TIMP-1 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median (interquartile range), RAS=123.0 (65.3–331.0), RE=180.0 (82.3-280.0), p=0.850, Wilcoxon paired test).

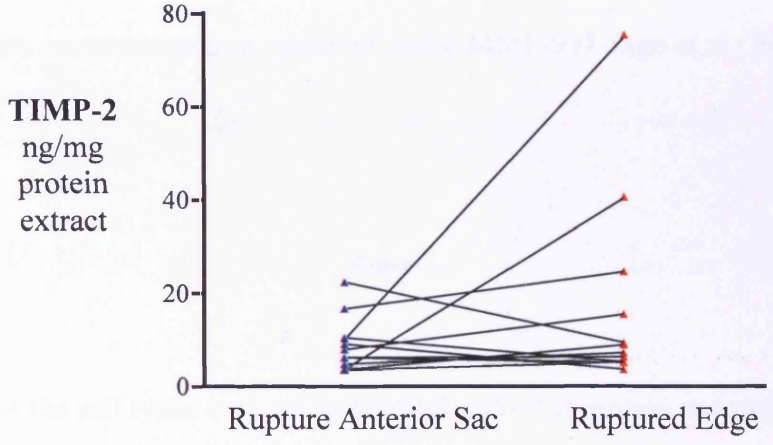


Figure 6.18: Levels of total TIMP-2 in anterior sac and rupture edge biopsies from ruptured AAA, detected by ELISA (median (interquartile range) RAS=7.0 (4.3-10.3), RE=8.0 (5.3-20.0), p=0.226, Wilcoxon paired test).

6.3 Aorta Wall Histology

Introduction

Section 6.2 clearly demonstrates the up-regulation of total and active fractions of MMP-8 and MMP-9 at the site of AAA rupture over levels found in anterior aneurysm sac biopsies. Given this increase in expression, it is important to determine if the source of MMP-8 and MMP-9 is from an endogenous mesenchymal cell source or an infiltrating inflammatory cell source.

The source of MMP-8 and MMP-9 expression at the site abdominal aortic aneurysm rupture has not been delineated. Certainly in non-ruptured AAA attempts have been made to delineate the sources of various MMP's. Previous studies support the mesenchymal cell as a source of MMP's-1, -2 and –13 (Mao et al., 1999; Davis et al., 1998; Sasaguri et al., 1994). Immunohistochemical studies localise MMP-9 to macrophages and native mesenchymal cells (Irizarry et al., 1993; Patel et al., 1996) and animal models present compelling evidence supporting the macrophage as a source of aortic MMP-9 (Longo et al., 2002).

Aims

To determine the cell types expressing MMP-8 and –9 at protein and mRNA level, by immunohistochemistry and *in situ* hybridisation, in representative samples of anterior sac and rupture edge biopsies from ruptured AAA.

Patients Selection

Representative samples of anterior aneurysm sac and rupture edge biopsies were studied.

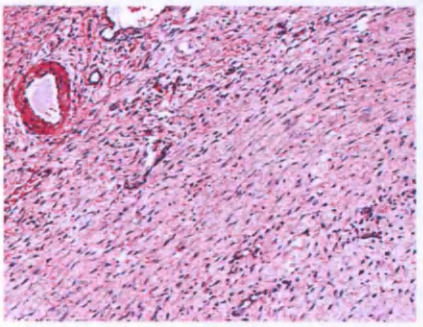
Laboratory Methods

Biopsies were processed for histology according to the protocols described in the methods section 4.2. In brief, paired biopsies of ruptured AAA from the anterior aneurysm sac and the rupture site, were paraformaldehyde-fixed and paraffin embedded. Sections were cut from the paraffin blocks for immunohistochemistry. Immunohistochemistry was conducted for MMP-8 and MMP-9 and cell localisation studies including macrophages, lymphocytes, neutrophils, smooth muscle cells and native mesenchymal cells. *In situ* hybridisation was conducted for MMP-8 and MMP-9 mRNA. Staining with hematoxalin /eosin and elastin Van Geison was also conducted.

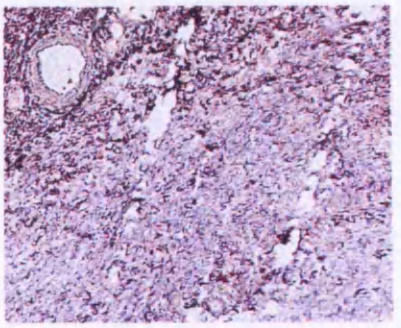
Hematoxylin and Eosin, Elastin Van Geison Staining

Representative pairs of anterior sac and rupture edge biopsies from ruptured abdominal aortic aneurysms are shown in Figures 6.19. The micrographs demonstrate disruption of the intima. Medial elastin fibers are unrecognisable with elastin Van Geison staining. Lack of medial elastin causes difficulty in determining the medial-adventitial boundary.

Ruptured AAA-1

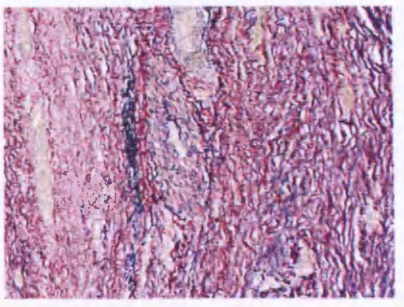


H+E Anterior Sac



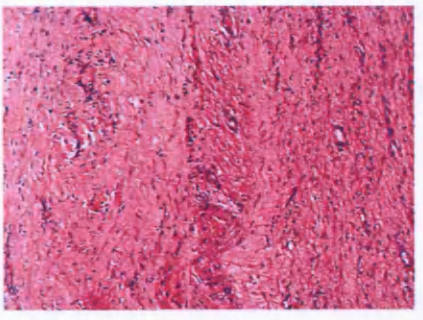
EVG Anterior Sac

H+E Rupture Edge

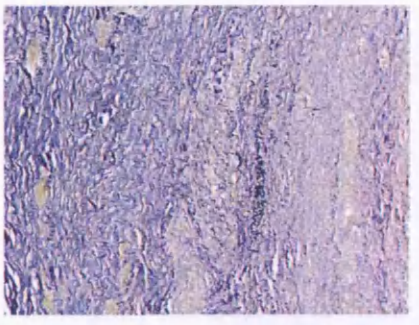


EVG Rupture Edge

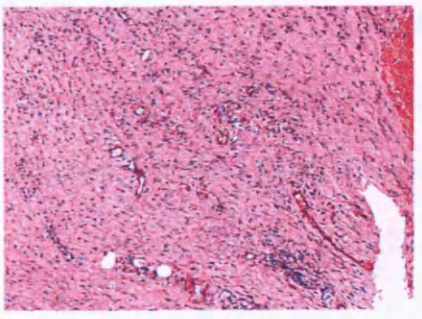
Ruptured AAA-2



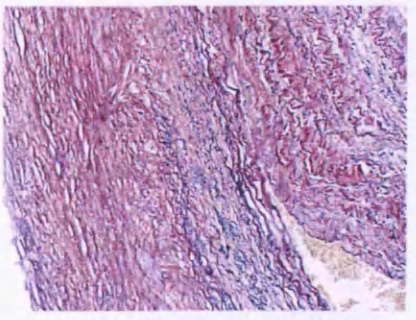
H+E Anterior Sac



EVG Anterior Sac



H+E Rupture Edge



EVG Rupture Edge

Figure 6.19
Hematoxylin and eosin (H+E), elastin van Geison (EVG) at paired anterior sac and rupture edge of two ruptured AAA.
(magnification x100)

MMP-8 and MMP-9 Immunohistochemistry and Localisation Studies

Immunohistochemistry for MMP's -8 and -9 were performed on paired biopsies from ruptured AAA. MMP-8 and MMP-9 were present at both the anterior aneurysm wall and rupture site biopsies with prominent adventitial staining for both MMP's (Figures 6.20, 6.21, 6.22, and 6.23). Smooth muscle α-actin and vimentin-antigen immunostaining was noted throughout the anterior aneurysm wall and rupture edge biopsies. A significant co-localisation for α-SMA and vimentin with MMP-8 and MMP-9 was observed (circled areas, Figures 20, 21, 22, 23). The expression of CD-45 (lymphocytes) and CD-68 (macrophages) was observed throughout some sections but only around the vasa vasorum in others (Figures 6.20, 6.21, 6.22, and 6.23). Though present, immunohistochemical expression of CD-15 (neutrophils) was low in most histological sections (Figures 6.20, 6.21, and 6.22), with some sections demonstrating no neutrophils (Figure 6.23).

The co-localisation of α-SMA to MMP-8 and –9 was slightly less consistent than that of vimentin with occasional areas of vimentin-positive but α-SMA-negative MMP staining. Smooth muscle α-actin denotes smooth muscle cells (SMC's), however, myofibroblasts and endothelial cells may also express α-SMA. The vimentin antigen is present on mesenchymal cells including fibroblasts and SMC's. The close localisation of MMP-8 and MMP-9 staining with α-SMA and vimentin, and the failure of CD-68, CD-45, and CD-15 to co-localise, indicates the cellular source of these MMP's in the human aorta to be the native mesenchymal cells, either fibroblasts, SMC's or a myofibroblast intermediate.

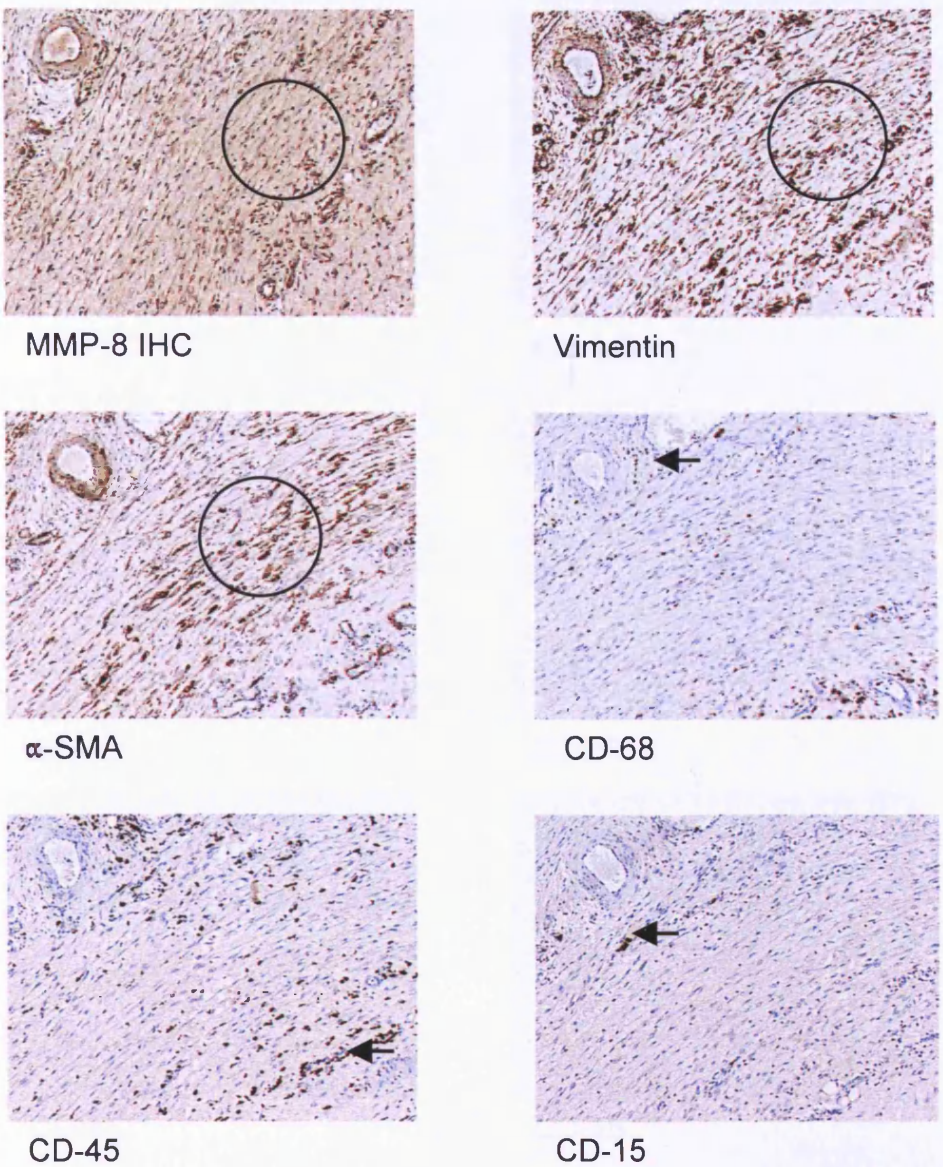


Figure 6.20
MMP-8 co-localisation study from the anterior aneurysm wall of a ruptured AAA (magnification x100, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of α–SMA and Vimentin with MMP-8).

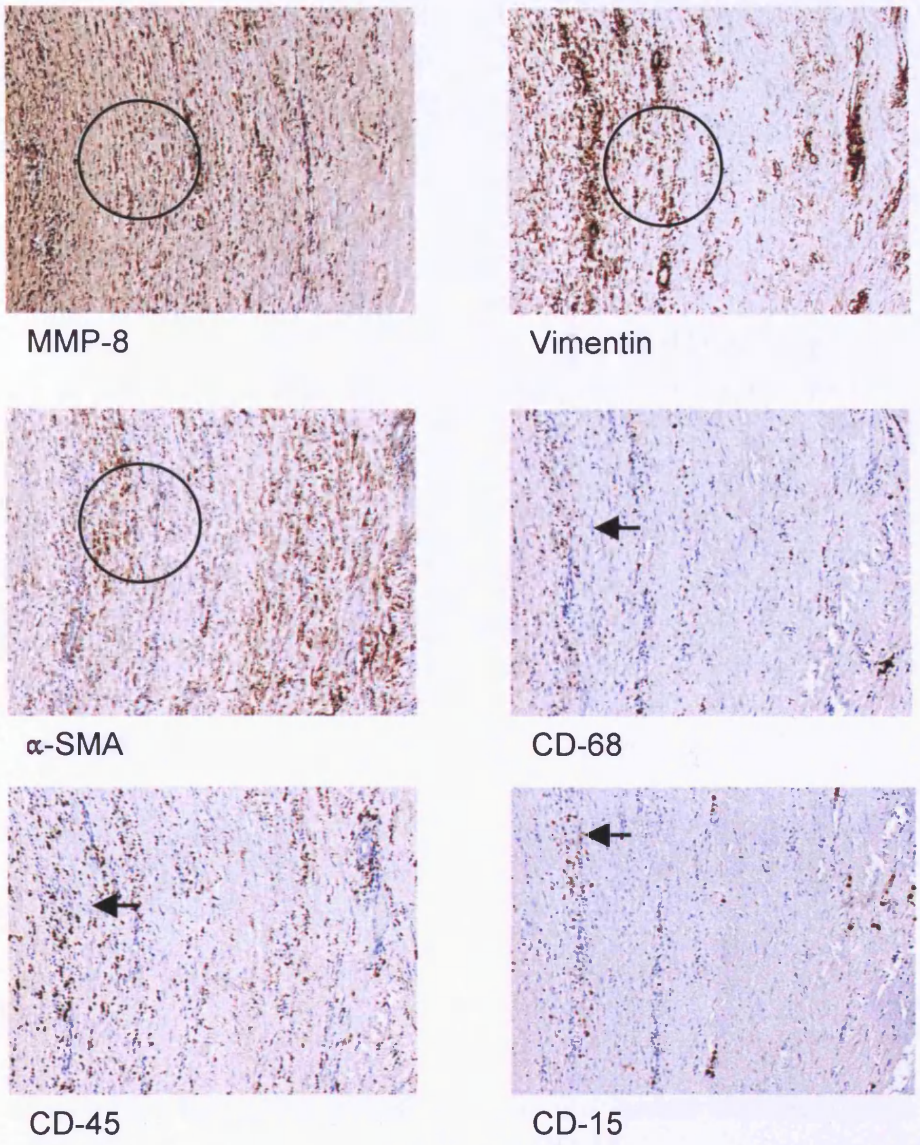


Figure 6.21
MMP-8 co-localisation study from the site of rupture of a ruptured AAA (magnification x100, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of α–SMA and Vimentin with MMP-8).

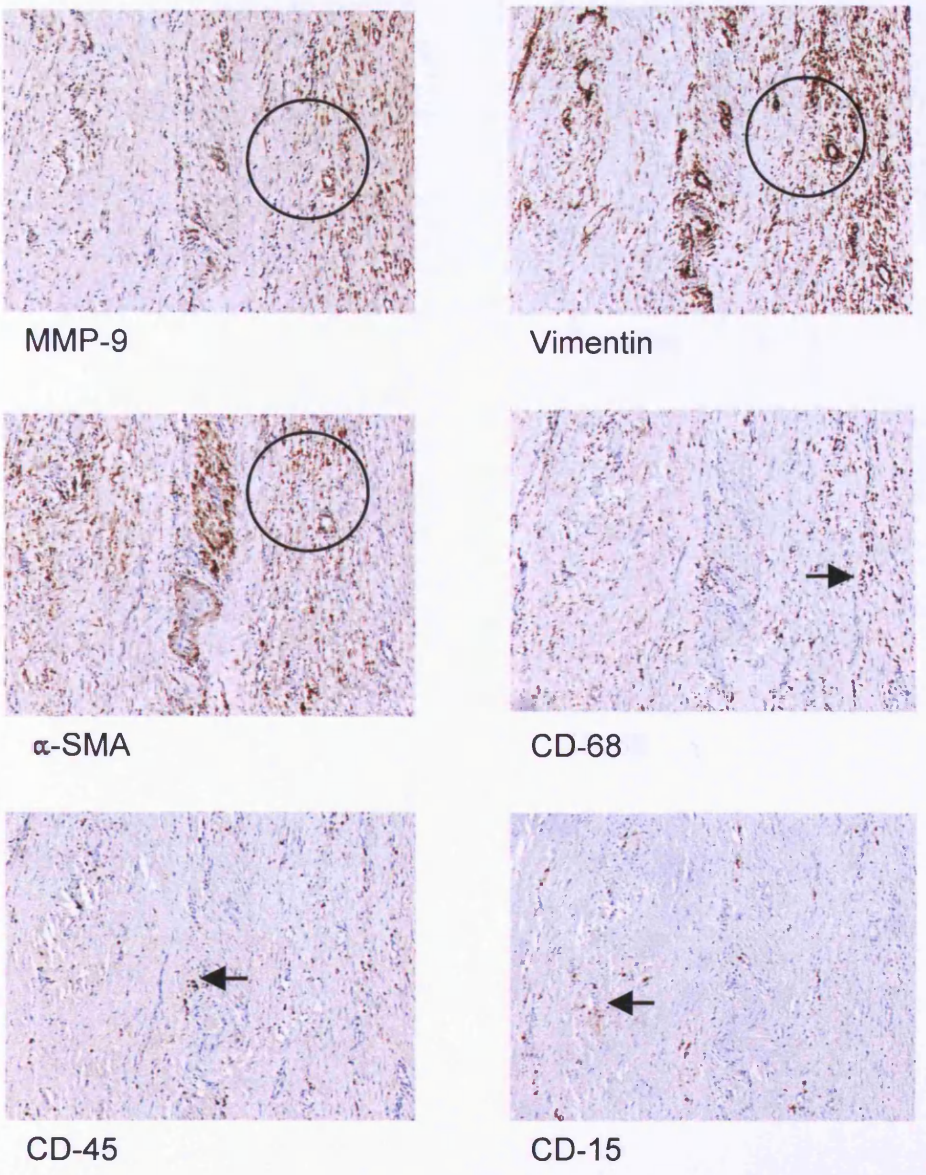


Figure 6.22
MMP-9 co-localisation study from the anterior aneurysm wall of a ruptured AAA (magnification x100, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of α–SMA and Vimentin with MMP-9).

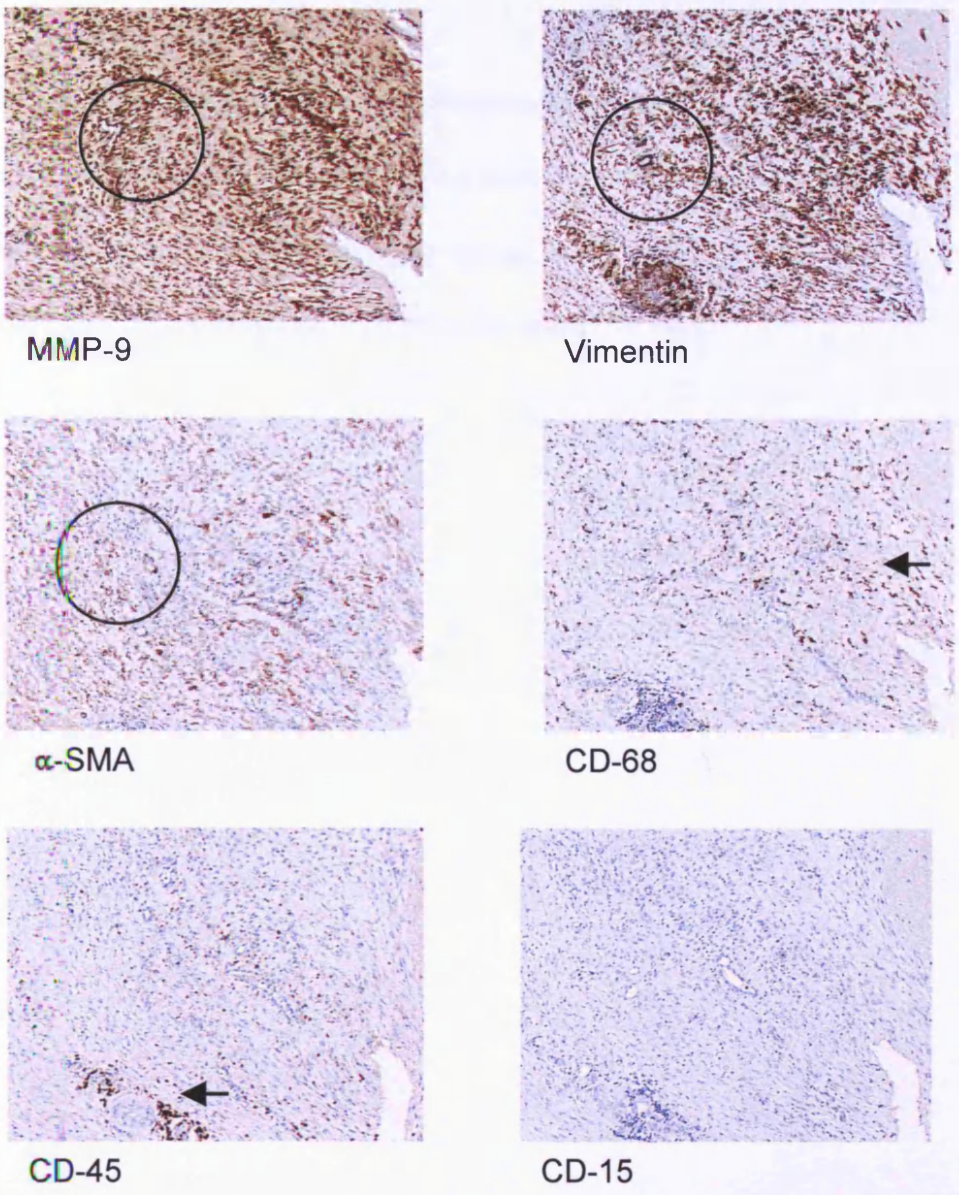


Figure 6.23
MMP-9 co-localisation study from the site of rupture of a ruptured AAA (magnification x100, counter stain – hematoxylin, ← example of immunostaining, ◯ area of strong co-localisation of α–SMA and Vimentin with MMP-9).

MMP-8 and MMP-9 In-situ hybridisation

In-situ hybridisation performed on paired rupture biopsies demonstrated MMP-8 and MMP-9 mRNA within the AAA wall at both the anterior aneurysm wall and the rupture site (Figures 6.24) supporting the native mesenchymal cell as the primary source of these two MMP's within the aneurysm wall.

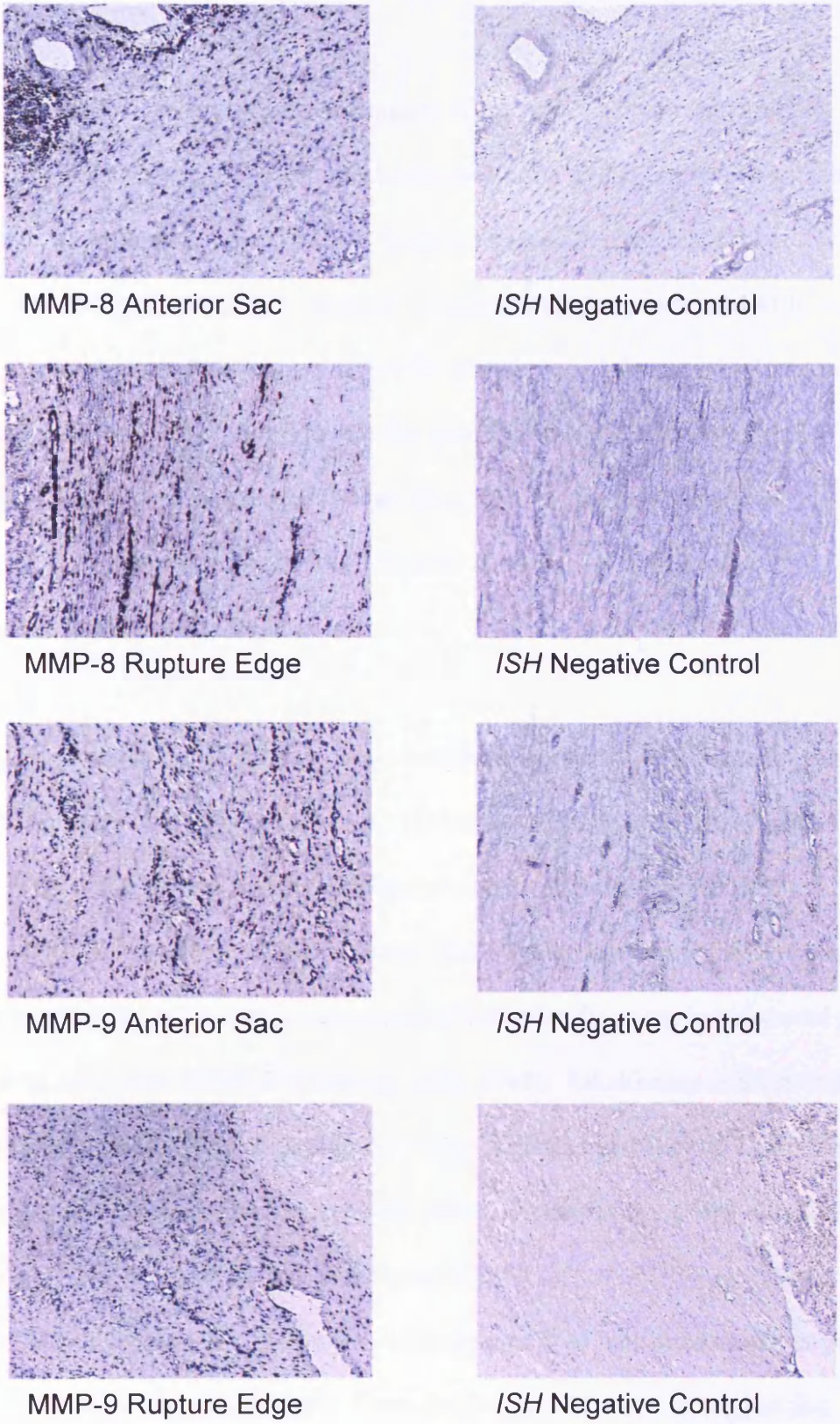


Figure 6.24
In situ hybridisation at paired anterior aneurysm sac and site of rupture biopsies in two ruptured AAA.
(magnification x100)

6.4 Summary of Findings

Aneurysm rupture occurs where the capacity of the aortic wall to resist systolic blood pressure is exceeded. This may be the result of a global or local change in aneurysm wall strength, brought about by proteolysis of the matrix proteins, elastin and collagen. Since the incidence of aneurysm rupture increases with aortic diameter, it might be expected that the global proteolytic capacity increases with aneurysm diameter. Alternatively the global proteolytic capacity of the aneurysm may remain unchanged, but an intense localised up-regulation of proteolysis, in vulnerable portions of the aneurysm wall, leads to localised collagen degradation and rupture.

This study failed to demonstrate a clear correlation between MMP concentration and the diameter of non-ruptured AAA. However, the correlation between MMP-9 and AAA diameter was negative and approached significance ($p=0.028$). Freestone et al., described more prominent MMP-9 immunostaining in aneurysms larger than 5.5cm, in contrast to aneurysms of a smaller diameter, but observed a reciprocal result with MMP-2 (Freestone et al., 1995). Sakalihasan et al., revealed that activated MMP-9 was present in AAA's with a mean diameter of 7.5cm but absent in AAA's with mean diameter 5.8cm (Sakalihasan et al., 1996). Similarly, McMillan et al., demonstrated higher transcription levels of MMP-9 in medium sized aneurysms (diameter 5 to 6.9cm) when compared to both smaller and larger aneurysms (McMillan et al., 1997). These data were interpreted to suggest that increased MMP-9 expression was related to the continued expansion of moderate

sized aneurysms, but the lower levels of MMP-9 expression in aneurysms >7cm, implied that the high rupture rates in large aneurysms were related to other factors.

There was no difference in MMP levels between non-ruptured and ruptured AAA in biopsies from the anterior aneurysm sac. Furthermore, comparing MMP levels within ruptured AAA i.e. paired anterior aneurysm sac versus edge of rupture site biopsies, the levels of MMP-1, MMP-2, MMP-3 and MMP-13 failed to show a statistical difference. By contrast the levels of MMP-8 and MMP-9 were higher at the rupture edge than the anterior sac of ruptured aneurysms. This suggested that the significant increase in MMP-8 and -9 activity at the rupture edge was a localised phenomenon. The localised elevation of MMP-8 and –9 was not accompanied by an elevation of the other MMP's, nor was there significant compensatory elevation in the levels TIMP's.

The cellular source of MMP-8 and MMP-9 throughout the abdominal aortic aneurysm is the native mesenchymal cell. The native mesenchymal cell may be a fibroblast, SMC or a myofibroblast intermediate. Previous studies support the mesenchymal cell as a source of MMP's-1, -2, -8 and –13 (Moa et al., 1999; Davis et al., 1998; Sasaguri et al., 1994) . Immunohistochemical studies localise MMP-9 to macrophages and native mesenchymal cells (Davis et al., 1998; Irizarry et al., 1993; Patel et al., 1996) . Animal models present compelling evidence supporting the macrophage as a source of aortic MMP-9 (Longo et al., 2002), but the findings from this study contradict this and demonstrate the limitations of relating animal models to human pathology.

There were a number of limitations to this study. Firstly, there was a difference in aneurysm size observed between non-ruptured and ruptured AAA. This difference did not bias the direct comparison of MMP concentrations between the groups, since MMP concentrations did not correlate with the diameter of non-ruptured aneurysms. Secondly, biopsy based observational studies are limited by a reliance on end stage disease tissue. However, the use of paired aortic biopsies to illustrate an elevated MMP concentration at the site of aortic rupture negates many of the disadvantages of using clinical material.

Studies have suggested that weakening of the AAA occurs at "hot-spots" of MMP activity (Vallabhaneni et al., 2004) or at areas of high wall stress (Fillinger et al., 2003). The presence of MMP-9 is important, not least since it appears to influence all stages of aneurysm pathogenesis (Pyo et al., 2000; Longo et al., 2002) but also because its substrate specificity, toward partially degraded fibrillar collagen fragments (Watanabe et al., 1993), complements the action of MMP-8 as a potent type I collagenase (Horwitz et al., 1977). Given the predominance of type I collagen over type III in the aneurysmal aorta (Rizzo et al., 1989) the elevation of MMP-8 at the site of rupture is indeed significant and supports Dobrin's original data (Dobrin et al., 1984).

CHAPTER 7

RESULTS

CELLULAR CHANGES IN NON-RUPTURED AND RUPTURED ABDOMINAL AORTIC ANEURYSM

7.1 Quantitative Histology – Non-Ruptured versus Ruptured AAA

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Anterior Sac of Non-Ruptured AAA versus Anterior Sac of Ruptured AAA

7.2 Quantitative Histology – Rupture Anterior Sac versus Rupture Edge

Introduction
Aims
Patient Selection
Laboratory Methods
Results - Ruptured Anterior Aneurysm Sac versus Rupture Edge

7.3 Summary of Findings

7.1 Quantitative Histology – Non-Ruptured versus Ruptured AAA

Introduction

The evidence supporting the presence of chronic inflammatory cells in non-ruptured aneurysms is well established. There is little published work pertaining to inflammation in the rupture process. A single study by Powell and Greenhalgh reported an increase in inflammatory cells in ruptured aneurysms, however, no histological plates were presented (Powell et al., 1989). No other publication has reported the cellular changes observed in ruptured aneurysms.

Aims

The aim of this initial part of the study was to quantify the cellular subtypes found in anterior aneurysm sac biopsies of non-ruptured and ruptured AAA. Quantification of immunohistochemical staining for macrophages, neutrophils, lymphocytes, smooth muscle cells and native mesenchymal cells were conducted on sections of paraformaldehyde-fixed paraffin embedded tissue.

Patient Selection

Fifty-five patients with non-ruptured AAA and 21 patients with ruptured AAA were studied. Biopsies were taken intra-operatively from the anterior aneurysm

sac of each aneurysm. Biopsies divided in two, with half frozen in liquid nitrogen and half fixed in paraformaldehyde for 24 hrs, then embedded in paraffin wax. Over the duration of the study a total of 63 patients with non-ruptured AAA were recruited. Only 55 non-ruptured AAA biopsies were fixed in paraformaldehyde. The initial 8 non-ruptured AAA patients had their biopsies frozen but not fixed in paraformaldehyde. This arose because the decision to conduct histology was taken after the initial study inception. Therefore, the number of non-ruptured AAA in this analysis (55 rather than 63) was not due to pre-stated inclusion or exclusion criteria, but rather the result of study progression. The clinical features of the 55 non-ruptured and the 21 ruptured AAA patients are described in Table 7.1. Median AAA diameter was greater in ruptured than non-ruptured AAA. There were no other differences in the characteristics of the study cohorts.

Patient Characteristics	**Non-Ruptured AAA** (n=55)	**Ruptured AAA** (n=21)	**p-value**
Age	72 (+/- 7.5) yrs	70 (+/- 5.3) yrs	0.368 *
AAA size	6.3 (5.8 - 7)	8.5 (6.3 - 10)	0.006†
Gender Distribution	50 males (91%)	18 males (86%)	0.677
Cardiovascular Event	18 (33%)	3 (14%)	0.153
Hypertension	35 (64%)	11 (52%)	0.435
Smoking History	43 (78%)	14 (67%)	0.376
Diabetes	5 (9%)	0 (0%)	0.314

Table 7.1: Characteristics of patients with non-ruptured and ruptured abdominal aortic aneurysms (AAA). (Comparison used independent t-test (*) with group mean and standard deviation in years, Mann-Whitney U-test (†) with group median and interquartile range in cm, and Fisher's Exact Test).

Laboratory Methods

Biopsies were processed for histology according to the protocols described in the methods section 4.2. In brief, anterior aneurysm sac biopsies from non-ruptured and ruptured AAA were fixed in paraformaldehyde and paraffin embedded. Sections were cut from the paraffin blocks for immunohistochemistry. Immunohistochemistry was conducted for macrophages (CD-68), lymphocytes (CD-45), neutrophils (CD-15), smooth muscle cells (α-SMA) and native mesenchymal cells (Vimentin). Quantification of the IHC staining was undertaken using a Nikon E800 microscope with an attached color video camera linked to an Apple computer. The percentage area fraction of immunostaining per high-powered field was calculated for each biopsy from a mean of 10 images taken over 5 stained sections.

Anterior Sac of Non-Ruptured AAA versus Anterior Sac of Ruptured AAA

There was no difference in the percentage area fraction of immunostaining for smooth muscle cells (α-SMA), mesenchymal cells (Vimentin), macrophage (CD-68), neutrophils (CD-15) and lymphocytes (CD-45) being comparable between AAA groups.

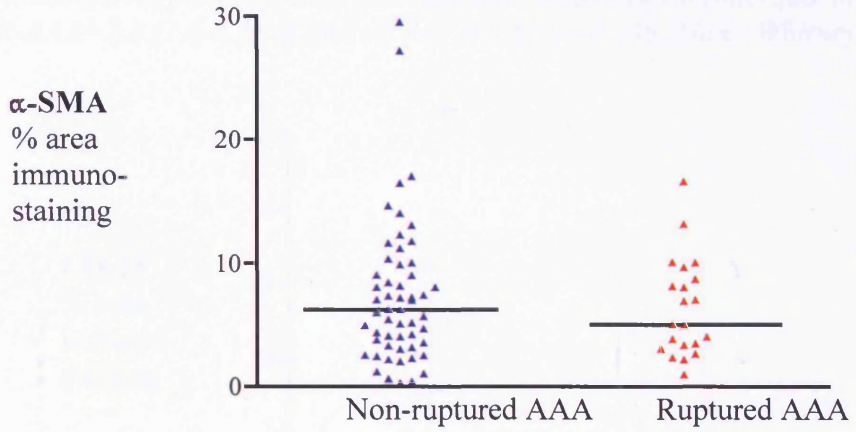


Figure 7.1: Percentage area fraction of immunostaining for smooth muscle cells (α-SMA), comparing non-ruptured and ruptured AAA (median (interquartile range) NR-AAA=6.2 (3.3-9.9), RAAA=5.0 (3.2-9.2), p=0.689, Mann-Whitney U-test).

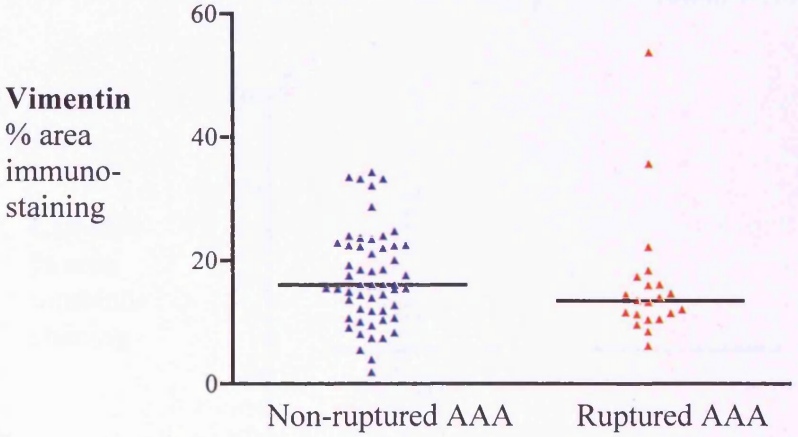


Figure 7.2: Percentage area fraction of immunostaining for mesenchymal cells (Vimentin), comparing non-ruptured and ruptured AAA (median (interquartile range) NR-AAA=16.0 (11.7-22.4) , RAAA=13.5 (10.8-16.7), p=0.152, Mann-Whitney U-test).

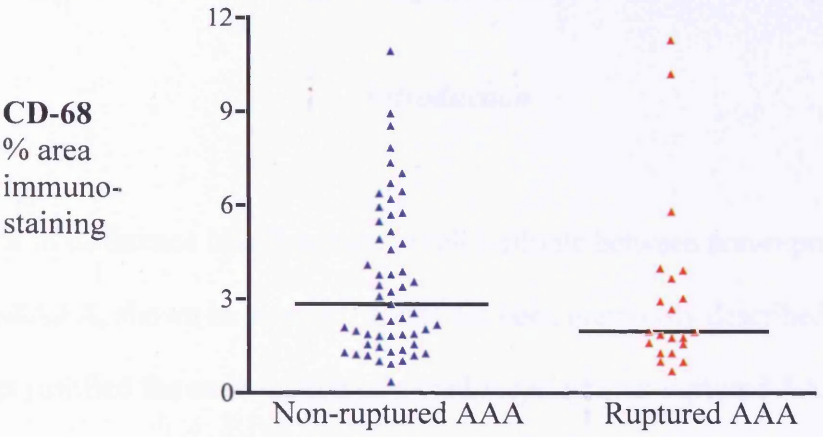


Figure 7.3: Percentage area fraction of immunostaining for macrophage (CD-68), comparing non-ruptured and ruptured AAA (median (interquartile range) NR-AAA=2.8 (1.6-5.5), RAAA=1.9 (1.4-3.8), p=0.236, Mann-Whitney U-test).

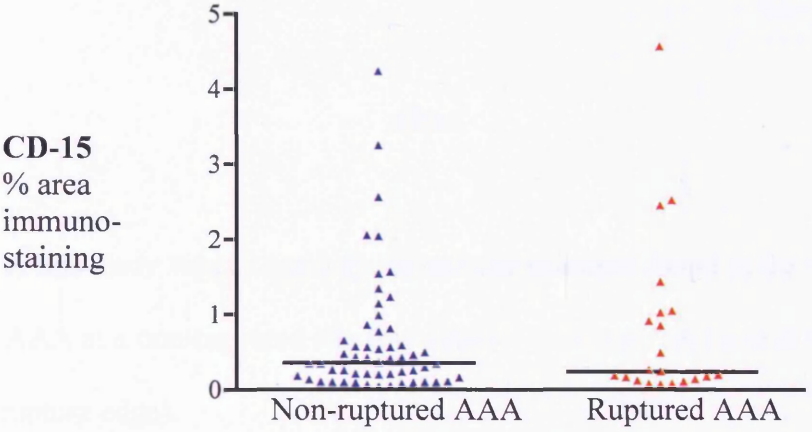


Figure 7.4: Percentage area fraction of immunostaining for neutrophils (CD-15), comparing non-ruptured and ruptured AAA (median (interquartile range) NR-AAA=0.36 (0.16-0.8), RAAA=0.24 (0.13-1.04), p=0.898, Mann-Whitney U-test).

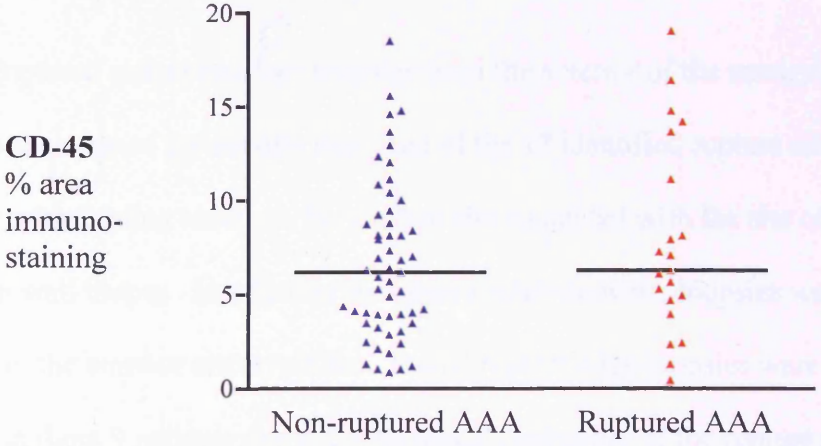


Figure 7.5: Percentage area fraction of immunostaining for lymphocytes (CD-45), comparing non-ruptured and ruptured AAA (median (interquartile range) NR-AAA=6.2 (3.5-9.2) , RAAA=6.3 (4.3-12.4), p=0.538, Mann-Whitney U-test).

7.2 Quantitative Histology – Rupture Anterior Sac versus Rupture Edge

Introduction

The lack of difference in inflammatory cell infiltrate between non-ruptured and ruptured AAA, shown in section 7.1, has not been previously described. These findings justified the examination of paired biopsies from ruptured AAA to determine a difference within ruptured AAA. The comparison of paired anterior aneurysm sac and edge of rupture site biopsies has not been reported.

Aims

The aim of this study was to quantify the cellular subtypes found in the wall of ruptured AAA at a non-ruptured site (the anterior aneurysm sac) and at the site of rupture (rupture edge).

Patient Selection

Twelve ruptured aneurysms had biopsies from the anterior of the aneurysm sac and from the edge of the rupture site. Ten of the 12 identified rupture sites were posterior with 2 being anterior. No rupture site coincided with the site of anterior aneurysm wall biopsy. In a further 9 ruptured AAA patients, biopsies were only taken from the anterior aneurysm sac. Paired rupture edge biopsies were not possible in these 9 patients due to the technical challenges of the rupture surgery.

Laboratory Methods

Biopsies were processed for histology according to the protocols described in the methods section 4.2. In brief, paired ruptured AAA biopsies from anterior aneurysm sac and rupture edge were fixed in paraformaldehyde and paraffin embedded. Sections were cut from the paraffin blocks for immunohistochemistry. Immunohistochemistry was conducted for macrophages (CD-68), lymphocytes (CD-45), neutrophils (CD-15), smooth muscle cells (α-SMA) and native mesenchymal cells (Vimentin). Quantification of the IHC staining was undertaken using a Nikon E800 microscope with an attached color video camera linked to an Apple computer. The percentage area fraction of immunostaining per high-powered field was calculated for each biopsy from a mean of 10 images taken over 5 stained sections.

The percentage area fraction of α-SMA, vimentin, CD-68 and CD-15 failed to demonstrate significant differences between biopsy sites. Of note, the percentage area fraction of CD-45 was lower at the rupture site when compared to paired anterior aneurysm wall biopsies (3.2% [1.5-7.3] vs. 6.4% [3.6-13.9], p=0.005).

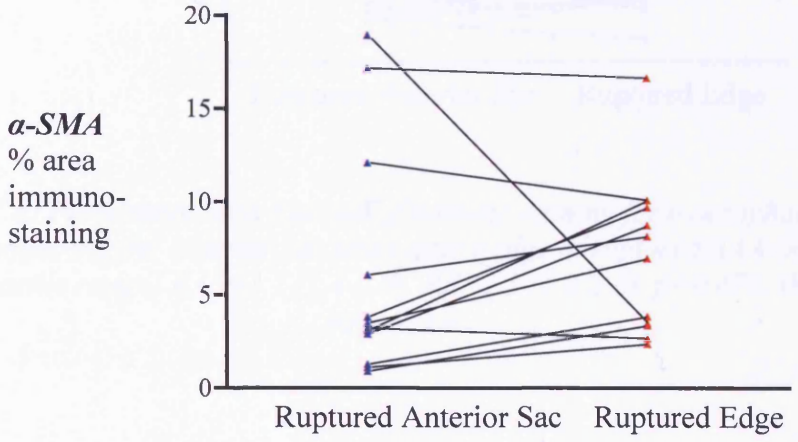


Figure 7.6: Percentage area fraction of immunostaining for smooth muscle cells (α-SMA), comparing the anterior sac and rupture edge of ruptured AAA (median (interquartile range) RAS=3.4 (2.1-9.1), RE=7.5 (3.4-9.9), p=0.129, Wilcoxon paired test).

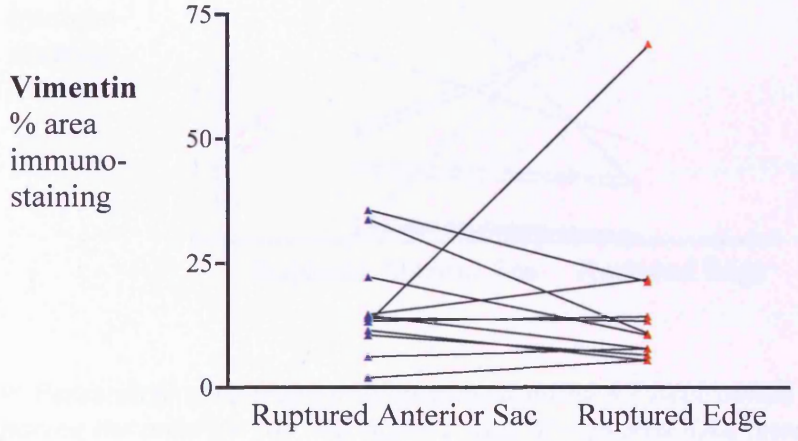


Figure 7.7: Percentage area fraction of immunostaining for mesenchymal cells (Vimentin), comparing the anterior sac and rupture edge of ruptured AAA (median (interquartile range) RAS=13.7 (10.9-18.4), RE=10.6 (7.1-17.8), p=0.301, Wilcoxon paired test).

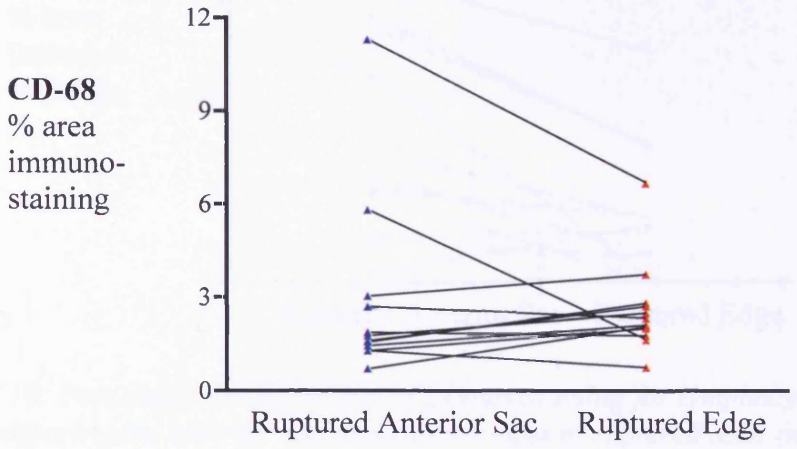


Figure 7.8: Percentage area fraction of immunostaining for macrophage (CD-68), comparing the anterior sac and rupture edge of ruptured AAA (median (interquartile range) RAS=1.7 (1.4-2.9), RE=2.3 (1.9-2.8), p=0.470, Wilcoxon paired test).

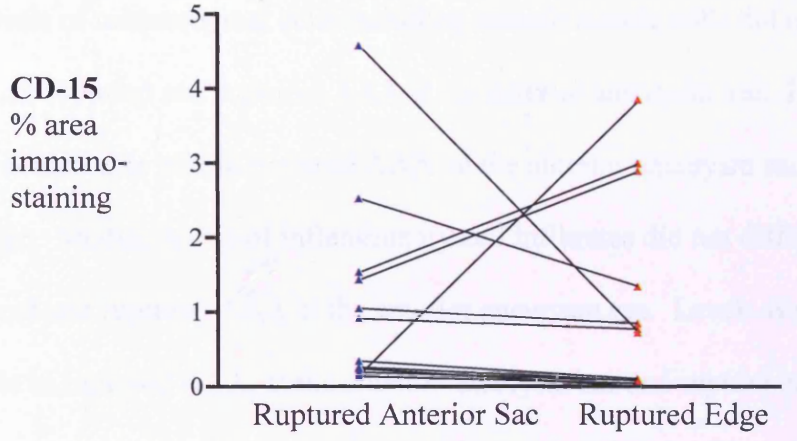


Figure 7.9: Percentage area fraction of immunostaining for neutrophils (CD-15), comparing the anterior sac and rupture edge of ruptured AAA (median (interquartile range) RAS=0.6 (0.2-1.5), RE=0.8 (0.1-2.1), p=0.519, Wilcoxon paired test).

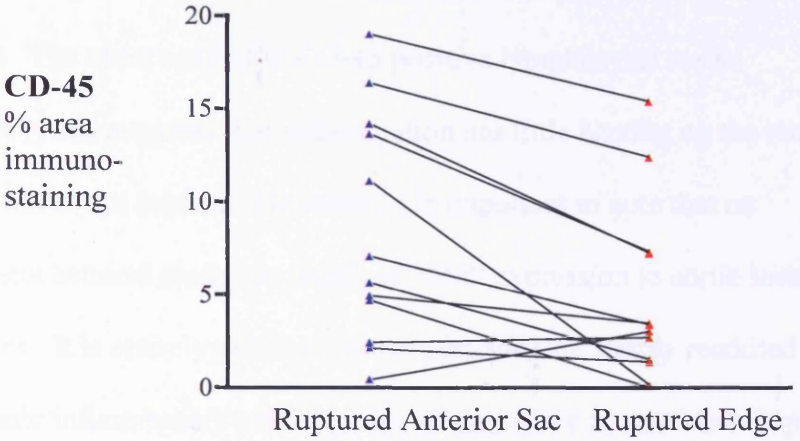


Figure 7.10: Percentage area fraction of immunostaining for lymphocytes (CD-45), comparing the anterior sac and rupture edge of ruptured AAA (median (interquartile range) RAS=6.4 (3.6-13.9), RE=3.2 (1.5-7.3), p=0.005, Wilcoxon paired test).

7.3 Summary of Findings

Median levels of mesenchymal cells including smooth muscle cells did not differ between non-ruptured and ruptured AAA at the anterior aneurysm sac. Levels were also comparable within ruptured AAA, at the anterior aneurysm sac and rupture edge. Median levels of inflammatory cell infiltrates did not differ between non-ruptured and ruptured AAA at the anterior aneurysm sac. Levels were comparable in ruptured AAA, at the anterior aneurysm sac and rupture edge, for neutrophils (CD-15) and macrophages (CD-68) however, levels of lymphocyte expression (CD-45) were decreased at the rupture edge.

This data suggests there is no global change in the cellular constitution differentiating ruptured aneurysms from non-ruptured aneurysms. By contrast,

the site of aneurysm rupture is associated with a localised decrease in lymphocytic infiltration. The observed fall in CD-45 positive lymphocytes seems contradictory, and suggests that inflammation has little bearing on the terminal process of aneurysm rupture. However, it is important to note that no immunohistochemical study has localised MMP expression to aortic located lymphocytes. It is entirely possible the lymphocytes are simply recruited as part of the chronic inflammatory process and that they serve no mechanistic purpose. More controversially, T-lymphocyte production of interferon-γ and interleukin-1 has been demonstrated to reduce monocyte-macrophage synthesis of the MMP's and increase TIMP-1 levels (Lacraz et al., 1994; Shapiro et al., 1990; Romanic et al., 1994). Though premature, it is not unreasonable to propose that the lymphocyte could be considered to have a stabilising effect on the AAA wall, with areas of low lymphocyte infiltration having a higher risk of instability.

A limitation of all histological quantification is the degree to which the observed tissue section represents the disease process as a whole. While we quantified 10 images taken over 5 stained sections per biopsy, we recognise that biopsy variation limit may this technique.

CHAPTER 8

RESULTS

HMG-COA REDUCTASE INHIBITORS (STATINS) DECREASE MMP-3AND MMP-9 LEVELS IN ABDOMINAL AORTIC ANEURYSM

Introduction

Abdominal aortic aneurysm (AAA) formation is associated with transmural aortic wall changes including loss of extracellular matrix proteins (ECM), loss of smooth muscle cells (SMC), and inflammatory cell infiltration (Dobrin et al., 1984; Lopez-Candales et al., 1997; Brophy et al., 1991). In contrast, atherosclerosis is an intimal and subintimal disease associated with endothelial cell injury, monocyte migration with insudation of lipoproteins and ECM accumulation (Gerrity et al., 1985). Imbalances in matrix metalloproteinases (MMP's), and their inhibitors (TIMP's), are implicated in both aneurysmal and atherosclerotic disease (Freestone et al., 1995; Loftus et al., 2000).

The beneficial role of 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) in patients with coronary and cerebrovascular atherosclerosis has been conclusively established by large clinical trials (Scandinavian Simvastatin Survival Study 1994; WOSCOPS 1998). The primary role of a statin is lipid lowering; however, secondary actions mediated through mesenchymal cell inhibition are also described (Bellosta et al., 1998; Guijarro et al., 1998). These include *in vitro* and *in vivo* suppression of MMP production in atherosclerotic lesions (Graf et al., 2003; Molloy et al., 2004) and *in vitro* suppression of MMP production in AAA (Nagashima et al., 2002).

Aims

The aim of this study was to observe *in vivo* MMP expression in the AAA wall of patients taking versus not taking a statin pre-operatively

Patient Selection

Sixty-three patients with asymptomatic AAA, undergoing elective open repair, were studied. Seventeen patients were taking a statin pre-operatively and 46 were not. Patient demographics and therapeutic parameters were comparable between the 2 patient cohorts (Table 8.1). The range of statin therapies used in the statin-treated cohort included; 9 patients on simvastatin (10mg n=3, 20mg n=6); 5 patients on atorvastastin (10mg n=1, 20mg n=3, 40mg n=1); 3 patients on pravastatin (20mg n= 2, 30mg n=1).

Laboratory Methods

Biopsies were processed for ELISA according to the protocols described in the methods section 4.2. In brief, anterior aneurysm sac biopsies from non-ruptured AAA were homogenised, centrifuged and dialysed in buffer overnight. The resultant protein extract underwent ELISA analysis for the quantification of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, TIMP-1 and TIMP-2.

Characteristics	Non-statin Cohort (n=46)	Statin Cohort (n=17)	p-value
Age (mean +/-std)	73 (+/-7.1)	70 (+/-7.2)	0.151†
AAA size	6.2 (4-10)	7 (5-10)	0.201‡
Gender Distribution	41 males (89%)	17 males (100%)	0.312
Cardiovascular Event	15 (33%)	6 (35%)	1
Hypertension	25 (54%)	14 (82%)	0.078
Smoking History	38 (83%)	13 (76%)	0.719
Diabetes	2 (4%)	4 (24%)	0.041
Beta-Blocker	10 (22%)	7 (41%)	0.199
Calcium Channel Blocker	11 (24%)	6 (35%)	0.523
ACE Inhibitor	11 (24%)	8 (47%)	0.120
NSAID	20 (44%)	6 (35%)	0.774

Table 8.1: Characteristics of patients taking a statin and not taking a statin prior to open abdominal aorta aneurysm repair. Comparison used independent t-test (†) with group mean and range in years, Mann-Whitney U-test (‡) with median and range in cm, and Fisher's Exact Test, $p < 0.01$).

MMP and TIMP levels in the Anterior Sac of AAA in Patients Stabilised on Statins and those not taking Statins

Elastases Levels

There was no difference in the concentration of MMP-2 in patients taking statins and those not optimised on a statin. In contrast total MMP-9 levels were significantly lower in patients taking statins than those not optimised on a statin.

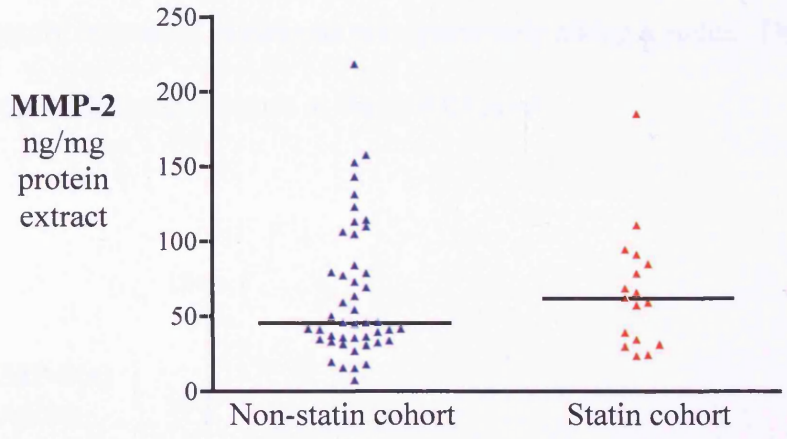


Figure 8.1: Levels of active MMP-2(total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=45.5 (33.8-94.2), AAAst=62.0 (34.2-87.8), p=0.775, Mann-Whitney U-test).

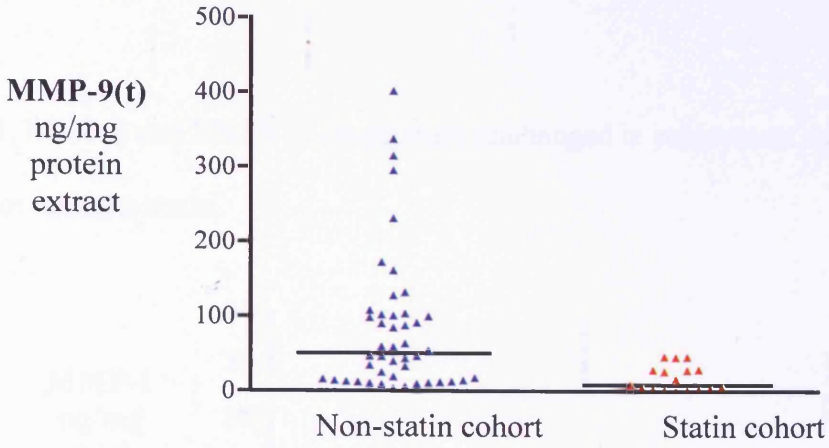


Figure 8.2: Levels of MMP-9 (total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=50.7 (14.4-103.0), AAAst=8.7 (2.7-29.3), p<0.001, Mann-Whitney U-test).

A further analysis of MMP-9 at active level was undertaken. Active MMP-9 was also significantly decreased in patients pre-operatively taking a statin. The difference approached significance at the p=0.01 level.

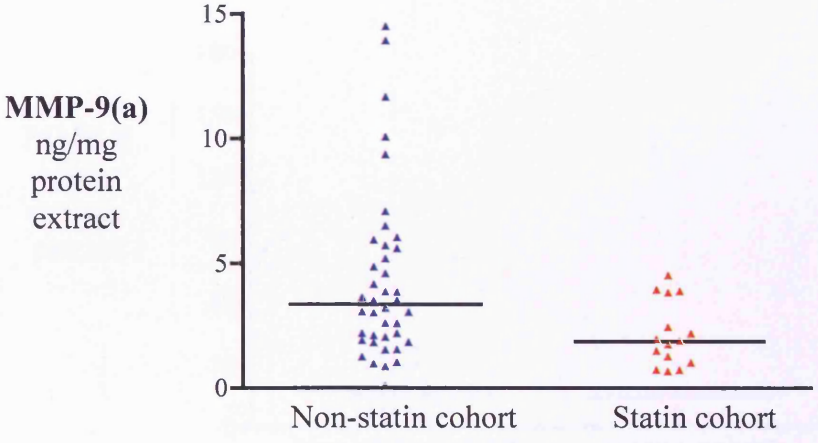


Figure 8.3: Levels of MMP-9 (active fraction) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=3.4 (2.0-5.6), AAAst=1.9 (1.0-3.8), p=0.012, Mann-Whitney U-test).

Collagenase Levels

MMP-1, MMP-8 and MMP-13 levels were unchanged in subjects on statins and those not taking a statin.

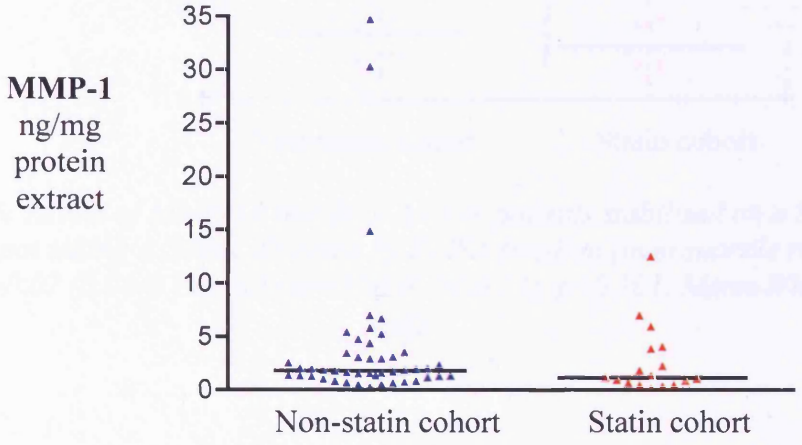


Figure 8.4: Levels of MMP-1 (total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=1.8 (1.2-3.5), AAAst=1.2 (0.6-4.0), p=0.196, Mann-Whitney U-test).

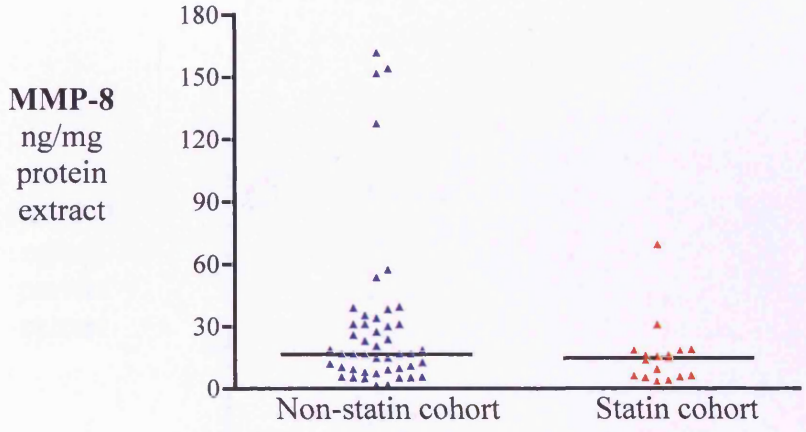


Figure 8.5: Levels of MMP-8 (total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=17.6 (8.5-34.5), AAAst=14.7 (5.5-18.5), p=0.102, Mann-Whitney U-test).

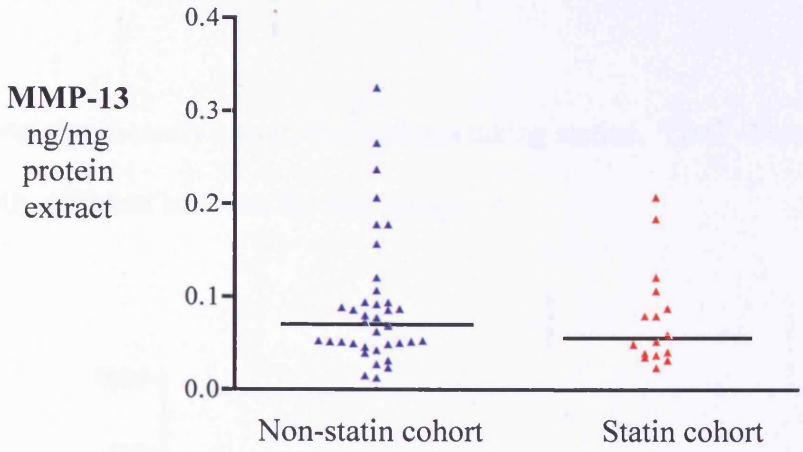


Figure 8.6: Levels of MMP-13 (total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=0.07 (0.04-0.11), AAAst=0.06 (0.04-0.11), p=0.701, Mann-Whitney U-test).

Stromelysin Levels

MMP-3 was significantly lower in patients taking statins compared to those not optimised on a statin.

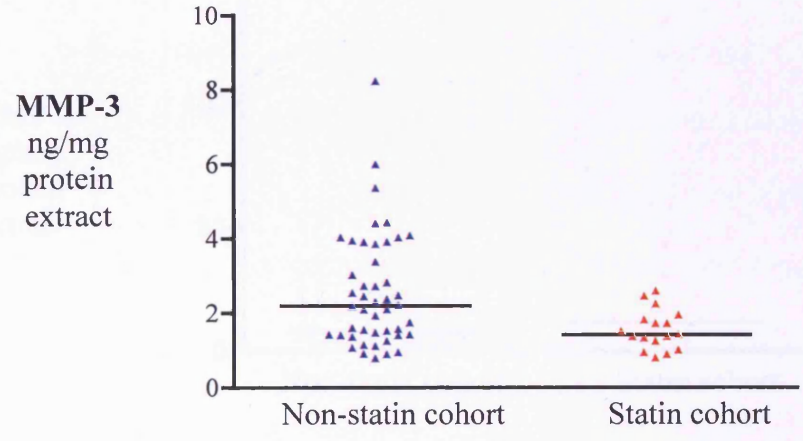


Figure 8.7: Levels of MMP-3 (total) in AAA in Patients Stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=2.2 (1.4-3.9), AAAst=1.4 (1.1-1.9), p=0.009, Mann-Whitney U-test).

TIMP Levels

TIMP-1 was significantly elevated in patients taking statins. TIMP-2 was non-significantly different between the two groups.

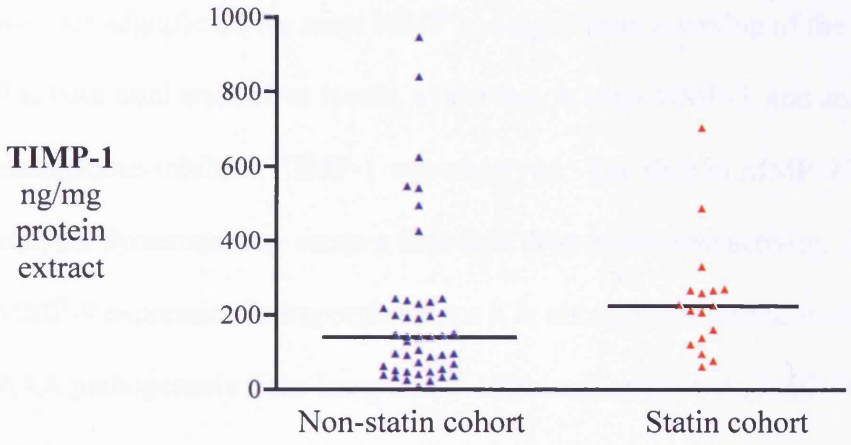


Figure 8.8: Levels of TIMP-1 (total) in AAA in patients stabilised on a Statin and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=141.0 (53.5-234.0), AAAst=226.0 (130.0-269.0) , p=0.033, Mann-Whitney U-test).

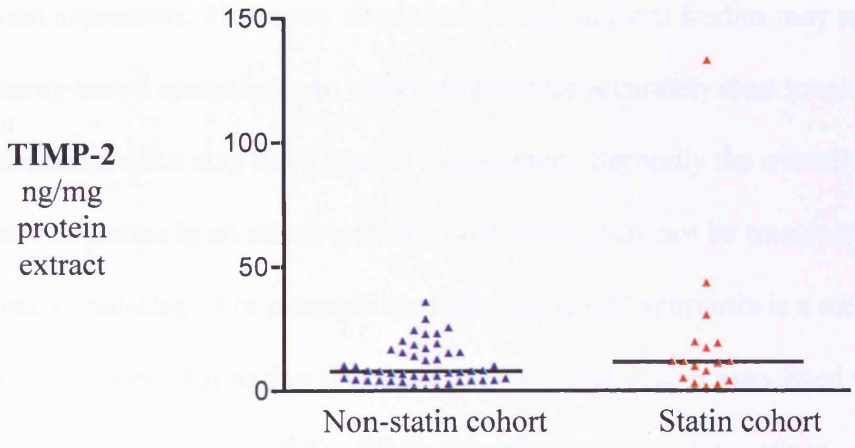


Figure 8.9: Levels of TIMP-2 (total) in AAA in patients stabilised on a Statins and those not taking a Statin, detected by ELISA (median (interquartile range) AAAnst=7.6 (4.1-15.3), AAAst=11.8 (4.5-25.0), p=0.200, Mann-Whitney U-test).

Summary

The process of aneurysm formation may be negatively influenced by statins; however, there is little published evidence to support this hypothesis. The findings from our *in vivo* study suggest that statins are associated with a generalised lowering of protease levels in established AAA. Though this effect was non-significant for most MMP's, a significant lowering of the elastase MMP-9 at both total and active levels, a decrease in total MMP-3, and an increase in the endogenous inhibitor TIMP-1 was observed. The shift in MMP-9/TIMP-1 enzyme dynamics may cause a four-fold drop in elastase activity. A reduction in MMP-9 expression is important since it is strongly implicated in all stages of AAA pathogenesis from inception to rupture (Longo et al., 2002; Petersen et al., 2002).

This study was not without its limitations. The study was based on a single time-point observation and lacked any power to predict the effects of statins on aneurysm expansion. However, single point observational studies may reflect community-based compliance to medications more accurately than longitudinal clinical trials, which may have greater compliance. Secondly the overall benefit attributed to statins in an atherosclerotic population, may not be transferable to an aneurysm population. For example smooth muscle cell apoptosis is a recognised feature of aneurysm formation (Lopez et al., 1997) but is also associated with statin use (Guijarro et al., 1998). While this effect may be of benefit in atherosclerosis (Bennet et al., 1999), it may potentially hasten aneurysm growth. Thus the commitment to a randomised, intention-to-treat study is necessary prior to the generalised acceptance of statins in the treatment of the aneurysm patient.

CHAPTER 9

DISCUSSION

9.1 A new collagenase in aortic biology – MMP-8

9.2 MMP levels in non-ruptured and ruptured AAA

9.3 Cellular changes in non-ruptured and ruptured AAA

9.4 The effect of statins on MMP expression.

9.5 Future Work

9.1 A New Collagenase In Aortic Biology– MMP-8

The results from Chapter 5 represent one of the largest age and cardiovascular risk - matched cohorts of normal aortas and AAA. The study is the first to accurately quantify protein levels of MMP-8 in aortic tissue. Significant elevations in active and total levels of MMP-8 were found in AAA tissue when compared to the normal aorta. A reciprocal decrease in TIMP-1 and TIMP–2 was observed in aneurysm tissue. A decrease in TIMP levels has been described by immunoblotting but not by ELISA (Brophy et al., 1991). The changes observed in this new study represent a crucial shift in the collagenolytic balance, a change that is capable of sustaining continued aneurysm expansion.

MMP-8 is a potent type I collagenase with a 20-fold greater proteolytic activity against type I collagen than type III (Hasty et al., 1987). Initially MMP-8 was considered to be synthesised only by bone marrow associated neutrophils, stored in specialized granules and released following stimulation of circulating neutrophils at a peripheral site (Murphy et al., 1977). This assumption has been revised and MMP-8 is now regarded as playing a central role in matrix degradation in chronic inflammatory conditions (Matsuki et al., 1996). The proteases historically implicated in aneurysm formation are the matrix metalloproteinases, MMP-2 and MMP–9. Suppression is associated with failed aneurysm formation (Longo et al., 2002), however, MMP-2 and -9 preferentially degrade intact elastins and action against fibrillar collagen is limited to partially degraded fragments (Okada et al., 1990; Murphy et al., 1991). The fibrillar collagenases MMP-1 and –13 are described at

protein level in the human aorta (Annabi et al., 2002; Mao et al., 1999). MMP-1 and –13 have substrate specificities favouring the cleavage of types III and II collagen respectively, and are much less potent against the most abundant aortic collagen - type I (Hasty et al., 1987; Knauper et al., 1996).

The site of MMP-8 production was sought by histological analysis. The subtleties of MMP-8 expression were only apparent following immunohistochemistry and *in situ* hybridisation. MMP-8 expression was significantly different in the aneurysmal and normal aorta. MMP-8 was localised to mesenchymal cells of the aneurysmal aortic wall and to macrophages within aortic plaque in normal aorta. The expression of MMP-8 in the outer media and adventitia of the aneurysm suggests a role for MMP-8 in the remodelling of this area – an area important in the rupture process. The process of atherosclerosis is different from aneurysm formation and this study serves to highlight important cellular subtypes and their topographical orientation within each process.

The difficulty in quantifying collagenase activity is well documented and we recognise the extraction of matrix bound MMP's and TIMP's is reported to be variable (Davis et al., 1998). However, our method utilised a well-established detergent based protocol (Vine and Powell, 1991). Powell et al., reported collagenase activity in non-ruptured and ruptured aortic explants (Powell et al., 1989). This study was the first to support a possible "endogenous" mechanism of aneurysm rupture, whereby an up-regulation of collagenase risks vessel rupture. The study did offer an admonition, as the difference in latent collagenase expression between ruptured and non-ruptured aneurysms was less substantial following correction for the cellular

content rather than the wet weight of the respective aneurysm biopsies. Rather than correct against cellular content, we corrected for the total protein extracted from each biopsy using a spectrophotometer, thus standardising between samples.

Elastin loss is considered an initiating event in abdominal aortic aneurysm formation. Continued aneurysm expansion and rupture is mediated through the loss of collagen (Dobrin et al., 1984). Arterial fibrillar collagen is present as two subgroups, types I and III, with type I collagen predominating over type III by a factor of 3 fold (Rizzo et al., 1989). Mutagenic studies support the role of type I collagen in the maintenance of aortic integrity, with failed synthesis resulting in gestational death from major blood vessel rupture (Lohler et al., 1984; Vouyouka et al., 2001). Given the predominance of type I collagen in the aorta, its proteolysis by a specific collagenase must be a pre-requisite to continued aneurysm expansion and rupture. The demonstration of MMP-8 expression within the adventitia of an aneurysm is potentially very important since the majority of arterial type I collagen is located there (Davidson et al., 1985). These findings indicate an *in vivo* pathway for adventitial type I collagen breakdown analogous to the animal models described by Dobrin et al. (Dobrin et al., 1984).

9.2 MMP Levels In Non-Ruptured And Ruptured AAA

Hypothetically, two mechanisms might be considered to contribute to AAA rupture, namely a global versus a local change in proteolytic activity. Since the incidence of aneurysm rupture increases with aortic diameter, it might be expected that the global proteolytic capacity increases with aneurysm diameter. Alternatively the global proteolytic capacity of the aneurysm may remain unchanged, but an intense localised up-regulation of proteolysis at vulnerable areas of the aneurysm wall leads to localised collagen degradation and rupture.

The present study did not demonstrate any positive relationship between aneurysm diameter and MMP concentration. This would suggest that there was no global elevation in proteolysis with increasing aneurysm size. The present investigation also revealed that there was no difference in MMP concentrations between the anterior aneurysm wall of non-ruptured and ruptured aneurysms. These data provide further evidence to suggest that a global up-regulation of protease activity is not responsible for aneurysm rupture. Similar findings were reported by Petersen et al., who compared MMP-9 and MMP-2 levels in stable and ruptured aneurysms (Petersen et al., 2002). Unfortunately, the biopsy sites were not described in detail by Peterson et al., but the findings did not suggest any clear difference in MMP's-2 or -9 between these two groups overall.

Data from the current investigations revealed that there was a localized increase in MMP-8 and MMP-9 concentrations at the site of aortic rupture. It is possible that

rupture may result from a localised process of elevated stress forces causing accelerated matrix degradation. Vallabhaneni et al., reported clear differences in the tensile properties of non-aneurysmal aorta in the longitudinal and transverse directions and suggested that such anisotropy exists in aneurysms (Vallabhaneni et al., 2004). A marked heterogeneity and high inter-subject variation in aneurysm wall strength suggested that there were focal areas of weakened aortic wall, pointing to localized "hot spots" of MMP hyperactivity (Vallabhaneni et al., 2004). Fillinger et al,. reported the study of 103 patients with initial aneurysm wall stress determined by finite element analysis. Follow-up for 1 year, with 42 non-interventions, 39 elective repairs and 22 emergency repairs showed initial peak wall stress to correlate with the subsequent site of aneurysm rupture (Fillinger et al., 2003). Furthermore, Vorp et al., found that wall stress increased substantially within asymmetric bulges of the aneurysm wall (Vorp et al., 1998).

The presence of MMP-8 protein in the anterior aneurysm wall and rupture site, however, conflicts with data from Carrell *et al.*, who were not able to detect MMP-8 transcripts in 8 aneurysm biopsies (Carrell et al., 2002). The high levels of MMP-8 protein in the present study may reflect the fact that MMP-8 is stored as preformed protein granules and thus messenger RNA levels may not be representative of protein concentration (Moa et al., 1999). The failure to consistently detect transcription products may be due to subthreshold levels, either from intrinsically low transcript synthesis resulting from negative feedback or loss during tissue processing. Potentially more significant is the possibility that these inconsistencies are due to variable transcript splicing, a feature that is described in other collagenase related disease states (Freije et al., 1994).

Immunohistochemical co-localisation of α-SMA to MMP-8 and –9 was slightly less consistent than that of vimentin with occasional areas of vimentin-positive but α-SMA-negative MMP staining being observed. Smooth muscle α-actin denotes smooth muscle cells, however, myofibroblasts and endothelial cells may also express α-SMA (Kazi et al., 2003). The vimentin antigen is present on mesenchymal cells including fibroblasts and smooth muscle cells. The close localisation of MMP-8 and MMP-9 staining with α-SMA and vimentin, and the failure of CD-68, CD-45, and CD-15 to co-localise, indicates the cellular source of these MMP's in the human aorta to be the native mesenchymal cells, either fibroblasts, smooth muscle cells or a myofibroblast intermediate.

These findings demonstrate that elevated MMP activity mediated by native mesenchymal cells may contribute to the rupture of an abdominal aortic aneurysm. The evidence supporting MMP-9 as a critical factor in AAA formation is strong (Pyo et al., 2002; Longo et al., 2002). The observations from this study also strongly implicate MMP-9 the terminal stages of the disease process. The role of MMP-8 in aneurysm biology has been over looked until here. Given that type I collagen is the most abundant structural protein in the aorta (Rizzo et al., 1989), the localised concentration of MMP-8 at the site of rupture suggest a clear mechanism for AAA wall weakening and rupture.

9.3 Cellular Changes In Non-Ruptured And Ruptured AAA

The cellular changes at the site of aneurysm rupture have not previously been delineated. Quantitation of the percentage area fraction immunostaining for CD-15, CD-45, CD-68, α-SMA and Vimentin failed to demonstrate any difference between site-matched biopsies of the anterior aneurysm sac from non-ruptured and ruptured aneurysms. Similarly, comparing the anterior aneurysm sac with matched rupture site biopsies, CD-15, CD-68, α-SMA and Vimentin did not differ. By contrast CD-45 was lower at the rupture site. The paucity of neutrophils observed in our study concurs with Cohen et al., who reported the absence of neutrophils, save for an occasional neutrophil caught within the vasa vasorum (Cohen et al., 1991). Powell and Greenhalgh reported an increase in inflammatory cells in ruptured aneurysms, however, no histological plates were presented and no biopsy sites were described (Powell and Greenhalgh, 1989).

Initially our observed decrease in CD-45 positive lymphocytes seems contradictory and suggests that inflammation has little bearing on the terminal process of aneurysm rupture. A conflicting role for the lymphocyte in the aneurysm wall has been proposed. T-lymphocyte production of interferon-γ and interleukin-1 has been demonstrated to reduce monocyte-macrophage synthesis of the MMP's and increase TIMP-1 levels (Lacraz et al., 1994; Shapiro et al., 1990; Romanic et al., 1994). Thus the role of the lymphocyte in the aneurysmal process is unclear; the lymphocyte may have a protective role, which fails at the point of rupture; or the lymphocyte may be an innocent bystander recruited only as part of the chronic inflammatory process.

9.4 The Effect Of Statins On MMP Expression.

The findings from our *in vivo* study suggest that 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins) are associated with a generalised lowering of protease levels in established AAA. Though this effect was non-significant for most MMP's, a significant lowering of the elastase MMP-9 at both total and active levels, a decrease in total MMP-3, and an increase in the endogenous inhibitor TIMP-1 was observed. The shift in MMP-9/TIMP-1 enzyme dynamics may cause a four-fold drop in elastase activity.

Statins decrease LDL cholesterol by competitive inhibition of the enzyme, 3-hydroxy-3-methylglutaryl coenzyme-A reductase (Grundy et al., 1988). Unanticipated cardiovascular benefits of statins may result from a build up of the intermediates of cholesterol metabolism (Nieuw et al., 2001), which have an inhibitory effect on cellular function, increasing apoptosis in smooth muscle cells (Guijarro et al., 1998), and reducing inflammation (Bellosta et al., 1998). The process of aneurysm formation may be negatively influenced by statins. There is little other evidence to support this hypothesis with only a single tissue-explant model suggesting a decrease in MMP-9 levels by immunohistochemistry (Nagashima et al., 2002). A reduction in MMP-9 expression is important since it is strongly implicated in all stages of aneurysm pathogenesis from inception to rupture (Longo et al., 2002; Petersen et al., 2002).

9.5 Future Work

This thesis raises several questions that justify further investigation. Firstly, what are the triggers of MMP synthesis and activation within the aneurysm wall? Can these triggers be down regulated at mRNA or protein level? Secondly, do lymphocytes aid or obtund the breakdown of extracellular matrix in the aortic wall? Thirdly, what are the longitudinal effects of statins in aneurysm biology? Do statins prevent the expansion of small aneurysms?

APPENDIX

Protein Extraction Solutions

Homogenising Buffer

1 liter of tissue homogenising buffer was prepared by dissolving the following in ultra pure water

	Concentration	Measurement
Urea (Sigma, Poole, UK)	2mol/l	120.12g/l
Tris HCl (Sigma, Poole, UK)	50mmol/l	7.88g/l
NaCl (Fisons, Loughborough, UK)		1g/l
EDTA (Fisons, Loughborough, UK)		1g/l
Brij 35 (Sigma, Poole, UK)		1ml/l
PMSF (Sigma, Poole, UK)	0.1mmol/l	
NaOH	for final pH of 7.6	

Dialysis Buffer

Dialysis buffer for 10 samples was derived from the following dissolved in 1.5 liters ultra pure water

	Concentration	Measurement
Tris HCl	25mmol/l	7.56g
$CaCl_2$ (Fisons, Loughborough, UK)	10mmol/l	2.425g
BRIJ 35	0.1%	1.5ml
1.74g PMSA in 5ml DMSA	0.1mmol/l	250µl
Ultra pure water		1.5ml
NaOH	for final pH of 8.5	

In Situ Hybridisation Solutions

10X PE

250ml of 10X PE was derived from the following dissolved in DEPC water

Trismethylamine (Sigma, Poole, UK)	15.15g
EDTA	4.66g
Tetra Sodium Pyrophosphate (Sigma, Poole, UK)	2.5g
Poly Vinyl Pyrrolidone (Sigma, Poole, UK)	5g
Ficoll (Sigma, Poole, UK)	5g
DEPC water	250 ml

Adjust the pH of the solution to 7.5 with concentrated HCl
The ingredients dissolve slowly with a stirrer, heat to 65°C
Autoclave and store at room temperature

Hybridisation Solution

5ml of hybridisation solution was derived from the following dissolved in DEPC water:

Reagent	Stock Concentration	Working Concentration	Volumes
NaCl	2.5molar	0.6 molar	1200µl
1X PE	10X PE	1X	500µl
Dextran Sulphate†	50%	10%	1000µl
ssDNA	10mg/ml	150µg/ml	75µl
Formamide†		30%	1500µl
DEPC Water			725µl

(†Fisons, Loughborough, UK)

Add the labeled probe cocktail at the concentration shown below.

		[Stock]	Oligo/ml Hybridisation Solution
MMP-8	200ng/ml	20ng/µl	10µl
MMP-9	200ng/ml	20ng/µl	10µl

Proteinase K (PK)

50ml of Proteinase K solution was derived from the following dissolved in DEPC water

2.5ml 1 molar Tris pH 7.6 (Sigma, Poole, UK)
47.5ml DEPC water
200µl of 1mg/ml PK (Sigma, Poole, UK)

Blocking Solution

Blocking solution was derived from the following dissolved in ultra pure water

500µl 20X TBS (Sigma, Poole, UK)
300µl Bovine Serum (Sigma, Poole, UK)
10µl Triton-X-100 (Sigma, Poole, UK)
9500µl Ultra pure water

Substrate Buffer

Substrate buffer was derived from the following dissolved in ultra pure water

1000µl 1molar tris-HCl pH 9.5
500µl 1 molar magnesium chloride (Fisons, Loughborough, UK)
400µl 2.5 molar sodium chloride
8100µl Ultra pure water

RNAse Treated Glassware

Soak interior surfaces of glassware in 3% hydrogen peroxide for 30 minutes. Rinse glassware X3 in DEPC treated water.

Other Solutions

4% Paraformaldehyde

4% Paraformaldehyde solution was derived from the following dissolved in DEPC water

Paraformaldehyde (Sigma, Poole, UK)	4grams
Phosphate buffered saline	100ml
5 molar sodium hydroxide	

Work in a fume hood. Place paraformaldehyde in a beaker, add PBS, heat to 80°C and stir. Add 5 molar sodium hydroxide solution slowly until all the paraformaldehyde has dissolved. Store for up to 2 weeks at 4°C. Protect the solution from light by wrapping the bottle in foil.

Phosphate Buffered Saline

1 liter of Phosphate Buffered Saline was derived from the following dissolved in ultra pure water.

NaCl *	7.2g
Na2HPO4.H2O *	1.48g
KH2PO4 *	0.43g

(*Fisons, Loughborough, UK)

Tris Buffered Saline

1 liter of Tris Buffered Saline was derived from the following dissolved in ultra pure water.

NaCl	7.2g
Tris base	60.6g

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