THE UNIVERSITY OF HULL

Abdominal Aortic Aneurysms;
Identification by Self-Examination and
Analysis of Variation in Matrix Metalloproteinases with Peak Wall Stress

being a Thesis submitted for the Degree of

Doctor of Medicine (MD)

in the University of Hull

by

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Thesis Summary

Thesis title

Abdominal Aortic Aneurysms; Identification by Self-Examination and Analysis of Variation in Matrix Metalloproteinases with Peak Wall Stress.

Introduction

Abdominal aortic aneurysms (AAAs) cause 5,000 deaths a year in the UK. Self-examination for AAAs may provide an economic and practical solution. Matrix metalloproteinase (MMP) concentration is elevated and levels of their natural inhibitors - Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) are reduced at sites of AAA rupture. Finite element analysis (FEA) of AAAs can define the area of peak wall stress area, the most likely site of rupture in engineering terms.

Study 1

Aim: To assess the effectiveness of self-examination for detection of AAAs compared to ultrasound measurement. The psychological consequences were also evaluated.

6,888(65%) of 10,591 male patients aged ≥65 years who were invited to participate, joined the study.

The sensitivity of self-examination for all AAAs (≥3cm) was 37% and for clinically significant AAAs (≥5cm) was 49%. Specificities were 80% and 79% respectively.

The psychological consequences of screening were assessed with the HAD (Hospital Anxiety and Depression) scale. There was no clinical anxiety or depression but there was a statistically significant reduction in both domains on completion.

Self-examination for AAAs cannot be recommended as an effective screening tool.

Study 2

Aim: To assess the tissue concentration of MMPs & TIMPs in AAAs at the site of peak wall stress.

22 patients undergoing elective AAA repair had FEA performed on their CT scans, identifying the peak stress site. Biopsies from this site and the arteriotomy site were stored at -80°C, and tested for MMP & TIMP concentrations with an enzyme linked immunosorbent assay (ELISA).

No significant difference was found between the 2 sites for MMP2, 8, 9, TIMP1 or 2.

These findings suggest either that the peak stress and rupture sites are geographically different, or that the event of rupture is temporally related to a fundamental biochemical change.

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AAA Abdominal aortic aneurysm

APMA 4-Aminophenylmercuric acetate

BMJ British Medical Journal

°C degrees Celsius

CABG Coronary artery bypass graft

CI Confidence interval

COREC Commission de la recherche clinique

CT computerised tomography

Da Dalton

DNA Deoxyribonucleic acid

DMSO Dimethyl sulfoxide

Dyn dyne (unit of force)

EAMF Elastin-associated microfibrils

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

EMMPRIN Extracellular matrix metalloproteinase inducer

EVAR Endovascular aneurysm repair

FEA Finite element analysis

HCl Hydrochloric acid

HEYT Hull and East Yorkshire Hospitals NHS Trust

HGF Hepatocyte growth factor

IL Interleukin

LREC Local research ethics committee

MASS (UK) Multicentre aneurysm screening study

MMP Matrix metalloproteinase

MRI Magnetic resonance imaging

mRNA messenger RNA

MT-MMP Membrane type matrix metalloproteinase

NAAASP NHS abdominal aortic aneurysm screening programme

NHS (UK) National Heath Service

NLR Negative likelihood ratio

NPV Negative predictive value

PCR Polymerase chain reaction

PDGF Platelet derived growth factor

PLR Positive likelihood ratio

PPV Positive predictive value

QALY Quality adjusted life year

r Pearson's correlation coefficient

r_s Spearman's r_s rank correlation coefficient [also called Spearman's ρ (rho)]

RNA Ribonucleic acid

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMC Smooth muscle cell

τ (tau) Kendall's τ rank correlation coefficient

TAA Thoracic aortic aneurysm

TIMP Tissue inhibitor of metalloproteinase

TMB 3,3',5,5'-Tetramethylbenzidine

TNF Tumour necrosis factor

UoH University of Hull

USS Ultrasound scan

Acknowledgements

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1. Introduction

1.1. Abdominal Aortic Aneurysms

For many years Albert Einstein suffered with abdominal pain. He eventually presented to Rudolph Nissen, then attending surgeon at Brooklyn Jewish Hospital, who diagnosed an abdominal aortic aneurysm. Surgery was carried out in December 1948 to wrap the anterior portion of the aneurysm in cellophane (it was thought too dangerous to mobilise the posterior aorta). Einstein was discharged 3 weeks later and returned to work 6 weeks after this.

In the early hours of Monday 18th April 1955, six and a half years later, he died in Princeton Hospital after refusing further surgery, and is quoted as saying: "I want to go when I want. It is tasteless to prolong life artificially. I have done my share, it is time to go. I will do it elegantly."(Cohen and Graver, 1990) Thus ended the life of the greatest scientist of our time.

1.1.1. Definition

An abdominal aortic aneurysm (AAA) is a permanent, localised dilatation of all 3 layers of the arterial wall in the abdominal aorta to at least 3cm.

The word aneurysm is taken from the Greek term *aneurysma* – meaning a widening. In 1975, McGregor et al proposed a definition of an abdominal aortic aneurysm as an aorta with infra-renal diameter of at least 3cm(McGregor et al., 1975). A later report by the Society for Vascular Surgery and the International Society for Cardiovascular Surgery Ad Hoc Committee on Standards in Reporting proposed as a criterion that the infra-renal diameter should be 1.5 times the expected normal diameter. However the

same report recognised that normal arterial diameter was variable, and determined by factors such as gender, age, and body habitus, and therefore a more practical definition was taken to be a 50% dilatation as compared to the adjacent normal portion of the same artery (Johnston et al., 1991). Thus there is no definite consensus on the definition of abdominal aortic aneurysm; however conventionally, infrarenal aortic diameters of 3cm or more are diagnosed AAAs.

Arterial dilatation below 50% is termed ectasia, and diffuse arterial enlargement of more than 50% above normal termed arteriomegaly. If there is dilatation but it does not affect all 3 layers of the arterial wall, then it is termed a pseudoaneurysm. Most aneurysms are fusiform – that is the entire circumference of the artery is affected, but where there is a localised bulge, this is called a saccular aneurysm. Finally an inflammatory aneurysm has extensive perianeurysmal and retroperitoneal fibrosis, with dense adhesions to surrounding organs.

1.1.2. Significance

AAAs are significant because of their propensity to rupture. They accounted for almost 5,000 deaths in England and Wales in 2005, over 95% of which were in people aged 65 and over (Office for National Statistics, 2005). AAAs are commonly asymptomatic, and rupture is often the first indication of their presence. Rupture is generally a catastrophic event associated with a highly significant overall mortality of between 65 – 85% (Kniemeyer et al., 2000, Thompson, 2003). Indeed, about 50% of patients die before arrival in theatre(Scott et al., 1991, Wilmink et al., 1999). Ruptured AAAs are responsible for 1.5% of deaths in men aged 55 and above, and are the thirteenth most common cause of death in the Western world (Hak E et al., 1996).

1.1.2.1. Historic Significance

Arterial aneurysms have been known since ancient times. One of the earliest texts containing a description was the Ebers Papyrus, a 20 metre long internal medical reference, written 2000 years before Christ. It described the association of aneurysms with trauma to the peripheries.

The Ebers Papyrus was primarily an internal medical reference, and consisted of a list of some 876 prescriptions and remedies for ailments such as wounds, stomach complaints, gynaecological problems and skin irritations. Prescriptions were made up in proportions according to fractions which were based on parts of the eye of Horus, ranging from 1/64 to 1/2. It also contained anatomical and physiological references Interestingly, and for some unknown reason, the scribe who wrote it did not finish the papyrus, and ended in mid sentence.

Galen, a second century Greek surgeon who worked with gladiators and experimented widely on animals, described an aneurysm as a localised pulsatile swelling that disappeared on pressure and wrote "if an aneurysm be wounded, the blood spouted out with so much violence that it can scarcely be arrested."

Andreas Veaslius, a 16th century anatomist, first described an aneurysm of the abdominal aorta.

In 1949, Rudolph Nissen stimulated periarterial fibrosis by wrapping cellophane around the symptomatic AAA of Albert Enstein, which eventually ruptured after 6 years.

Mr DeBakey treated a 70 year old King Edward VIII in 1965 for an abdominal aortic aneurysm. "In an operation lasting little more than an hour, DeBakey cut out the

swollen part of the aorta--normally less than an inch in diameter at this point in the body, but now the size of a large grapefruit--and replaced it with a 4-inch length of knitted Dacron tubing." The Duke of Windsor recovered without incident and lived until 1972, when he died of throat cancer aged 78.

1.1.3. Epidemiology

AAAs are predominantly a disease of the elderly male, with an incidence of between 1.3% and 8.9% (Lederle et al., 2000, Lindholt et al., 2000c, Singh et al., 2001, Vardulaki et al., 2000), increasing with age to peak incidence at 80 years of age. The disease is 4-5 times less common in women (Collin J et al., 1988) and also tends to start later at about 65 years of age. A recent study of 10,000 men and 7,500 women with a history of cardiovascular disease or a family history of AAA found an incidence of AAA of 3.9% in these men and 0.7% in these women (DeRubertis et al., 2007). One of the most important risk factors for AAA is smoking, and it is postulated that the sex ratios will be less marked in the future due to the increasing incidence of smoking in women (Lederle et al., 2001, Singh et al., 2001). It is also less common in Negro men with a 2.5 fold less incidence, and in Asian men (Spark et al., 2001).

The incidence of AAAs is increasing as demonstrated by the 20-fold increase in the age-standardised death rate over the last 4 decades (Fowkes FGR et al., 1989), and a Swedish necropsy study revealed a clear increase in prevalence over this time (Bengtsson H et al., 1992). An inverse relationship has also been shown between the decreasing prevalence of peripheral vascular disease and the increasing prevalence of AAAs (Coggon D et al., 1996).

1.1.4. Aetiology

There are a numerous causes of arterial dilatation, but specific causes of AAAs such as connective tissue disorders (Marfan's syndrome & Ehlers-Danlos type IV), inflammatory diseases (Takayasu's & Behcet disease), acute infections (brucellosis & salmonellosis), chronic infections (tuberculosis), and trauma, cause a relatively small proportion of AAAs. Most abdominal aortic aneurysms are called *non-specific* (Johnston et al., 1991).

Severe atherosclerotic disease is seen affecting the aortic wall in most AAAs, therefore atherosclerosis was considered to be an important aetiological factor (Reed et al., 1992). However, this view has been challenged due to the difficulty in consolidating atherosclerotic occlusive disease with atherosclerotic dilatational disease (Tilson, 1992, Xu et al., 2001). It is likely that atherosclerosis is an associated factor rather than an instigating factor. Atherosclerosis is primarily a disease of the intima, whereas aneurysmal disease primarily affects the medial layer of the artery. Epidemiologically, patients with atherosclerotic occlusive disease present at an average age of 55 years, contrasting with an average age of 70 for aneurysmal disease (Millis et al., 1992). It has also been suggested that patients with aneurysms tend to be taller and thinner than patients with occlusive disease, and may have generalised arteriomegaly (Tilson and Chau, 1981). Various differences have been demonstrated between levels of protease inhibitors in occlusive and aneurysmal disease suggesting the divergent evolution of atherosclerotic plaque towards one or the other (Defawe et al., 2003).

More recent evidence points to flow limitation inhibiting aneurysm dilatation and progression, with the suggestion therefore that atherosclerosis forms as a result of a change in luminal flow in AAAs rather than being an initiating factor (Hoshina et al.,

2003). Finally if the main causative factor in AAA formation was atherosclerosis, then some correlation would be expected between atherosclerotic severity and AAA development. The Tromso study examined carotid, femoral and coronary artherosclerosis and compared this to AAA diameter, with no dose-response relationship seen, forming the conclusion that there was no evidence to support the case for atherosclerosis being a causal event in AAA formation (Johnsen et al.).

Other contributing causes are wall stress, vibratory forces, and enzymatic imbalance (Millis et al., 1992).

Arterial rupture is associated with connective tissue disorders such as Marfan's Syndrome and Ehlers-Danlos type IV disease. Marfan's syndrome is associated with aortic dissection, and rupture rather than true aneurysms. A genetic defect results in the production of a defective fibrillin glycoprotein, which forms a network in the extra-cellular matrix for the deposition of elastin. These networks are particularly abundant in the aortic media where weakness may result in aortic valve incompetence, and may predispose to intimal tearing, initiating an intramural haematoma, cleaving the layer of the media to produce an aortic dissection, occasionally proceeding from the root of the aorta to the iliac arteries, and eventual rupture. This is the eventual cause of death in 30-45% of individuals with Marfan's. In Ehlers-Danlos Syndrome type IV, defective type III collagen is produced which predisposes to spontaneous rupture of large arteries and intestines. Ehlers-Danlos Syndrome type IV is a heterogenous group of disorders where 3 possible mutations affect the synthesis of type III collagen. These can affect the rate of synthesis of the pro a1 chain; secretion of type III procollagen; and the synthesis of structurally abnormal type III collagen (Cotran RS et al., 1994).

1.1.4.1. Risk Factors

Risk factors for AAA may be linked to AAA development, progression and rupture (Nordon et al.). Smoking has been found to affect at least 2 of these stages. Other risk factors for AAA include male sex, age, hypertension, chronic obstructive pulmonary disease, hyperlipidaemia, and family history of AAA (Lederle et al., 2001, Singh et al., 2001, Vardulaki et al., 2000, Brady et al., 2004, Thompson et al., 2002).

Smoking

Strong associations have been shown between tobacco smoking and the development of aneurysms (Vardulaki et al., 2000, Brown and Powell, 1999, Blanchard et al., 2000, Lindholt et al., 2001a). In fact the prevalence of AAAs in smokers is 4 times that of life-long non-smokers (Vardulaki et al., 2000). This might be perhaps attributed to elevating the general risk of atherosclerosis, but a report has also shown that the relative risk of developing an AAA in chronic smokers is 3 times higher than for developing coronary heart disease and 5 times higher than for developing cerebrovascular disease (Lederle et al., 2003). Smoking has therefore been cited as the single most important risk factor for the development and progression of aortic aneurysms (Sakalihasan et al., 2005). AAA progression is also greater in current smokers than non-smokers (2.83mm per year vs 2.53mm per year, respectively)(Brady et al., 2004). However the exact mechanism by which tobacco smoking promotes aneurysm formation is unknown, and continues to be investigated (Buckley et al., 2004).

Family history

The familial clustering of AAA was first reported in 1977 (Clifton, 1977), and patients with a positive family history have the highest risk of AAA formation. A Canadian study found that siblings of AAA patient had 8 times the incidence of AAA compared to the siblings of their spouses (Ogata et al., 2005). The incidence in first degree relatives has been shown at 15-19%, compared to 1-3% in unrelated patients (Kuivaniemi et al., 2003). This same study demonstrated two inheritance modes, with 72% of families being autosomal recessive, and 25% being autosomal dominant. A suggestion has been made for a candidate gene on chromosome 19 (Shibamura et al., 2004). Despite the genetic inheritance, this clustering is also thought to be due in some part to environmental factors (Powell, 2003).

With no clear evidence and conflicting studies on candidate-gene associations, genome-wide association studies were thought to be the way forward in finding the genetic component of AAA development (Sandford et al., 2007). This has revealed a single nucleotide polymorphism on chromosome 9p21 associated with AAA (Helgadottir et al., 2008). Further studies are awaited (Aneurysm Consortium, 2008).

Hyperlipidaemia

The association between lipid level and AAAs remains unclear. Studies have reported increased risk of AAA with increased serum cholesterol (Iribarren et al., 2007), but also the protective effect of increased serum HDL (Pleumeekers et al., 1995). Lowering of serum cholesterol levels with statins has been suggested to delay AAA formation, in a retrospective analysis of the Dutch AAA screening database (Schouten et al., 2006), however the Tromsø cohort study found that statin therapy increased the risk of AAA formation (Forsdahl et al., 2009).

1.1.5. Presentation

Most abdominal aortic aneurysms are asymptomatic and exist until found incidentally on routine examination or imaging – for example in ultrasound examination of the abdomen – or until the aneurysm ruptures.

The most common symptom is abdominal pain, mostly epigastric but very variable, constant, boring or throbbing in character, and classically radiating to the back. The location of the aneurysm can cause compression of adjacent structures, thereby causing symptoms such as weight loss due to relative gastric outlet obstruction secondary to duodenal compression. Other presentations are flank and groin pain perhaps due to ureteric compression.

Thrombus within the aneurysm can cause symptoms by distal embolisation, for example, acute lower limb ischaemia, or blue toe syndrome. As most AAAs are asymptomatic it is no surprise that in the 1960s only about 50% presented at an asymptomatic stage. However with improved imaging, and perhaps more accessible healthcare, about 80% are now diagnosed in the asymptomatic stage (Millis et al., 1992).

On examination, an AAA is palpated as an expansile mass midway between the umbilicus and the xiphisternum. Occasionally a pulsatile mass is felt in this area due to transmitted aortic pulsation, overlaying mass, or perhaps due to the slight built of the patient.

1.1.6. Investigations

Physical examination

Physical examination has been the main method of AAA detection for centuries, however it can very be inaccurate with sensitivities quoted from 29% in AAAs from 3-3.9cm, through 50% in AAAs from 4-4.9cm, up to 76% in AAAs above 5cm in diameter (Lederle and Simel, 1999). It often over-estimates size by 20% compared to intra-operative measurement (Brewster et al., 1977).

Radiographs

Lateral spine radiographs can be useful in the 75% of patients with some calcification in the aorta. It does however involve a dose of radiation, and skilled interpretation by a radiologist.

Ultrasonography

Ultrasonography was reported to be reliable and useful as far back as 1977, and comments at that time related to the fact that it would under-estimate AAA size due to the difficultly in delineating the external wall. Although this problem still exists to some extent, it is much improved due to improvements in technology particularly resolution and grey-scale displays, and does not present much of a problem to a skilled ultrasonographer. Ultrasound examination does not involve radiation, is quick and painless and widely acceptable. The ultrasonographer in most cases also can report it instantly. The main problems with this technique include bowel gas and excessive obesity – both of which can prevent adequate visualisation of the aneurysm.

Aortography

Aortography involves the use of radio-opaque dye, and radiation, is an invasive technique and requires time-consuming radiology reporting. The radio-opaque dye is also nephrotoxic, although some centres use carbon dioxide angiography. It is of value in operation planning, but as it demonstrates mainly the lumen of the AAA, it is limited in determining AAA size based on external wall dimensions, as both intra-luminal thrombus and aneurysm wall thickness may hinder the acquisition of external dimensions.

Computerised tomography

CT imaging is more expensive than plain radiographs or ultrasonography, but provides detailed information regarding aneurysm geometry, internal and external diameters, thrombus, and relationships to neighbouring structures including the renal arteries. The information obtained can be reprocessed to form 3-D images, and can help to plan endovascular procedures.

Magnetic resonance angiography

This involves the use of gadolinium as contrast, but no xrays, and can be a problem in claustrophobic patients as it is commonly performed in a tunnel configuration. It provides excellent soft tissue definition, and does not have a problem with penetrating bone, therefore giving particularly good images of the iliac arteries. The scans are more expensive than CT scans, but these are increasingly being used in the planning stage prior to endovascular AAA repair.

1.1.7. Histology

1.1.7.1. Structure of an artery

The walls of large and medium sized arteries have 3 discernable layers – the *intima*, *media*, and *adventitia*. Smaller arteries also have these layers but they tend to merge and are less discrete.

The intima is the thin inner layer of the artery, and its main function is to facilitate the smooth, friction-free passage of blood in the arterial lumen. It therefore mainly consists of flat endothelial cells. These cells lie on a basement membrane and just beneath this is a layer of elastic fibres – the internal elastic lamina. This is clearly seen in muscular arteries, but less clearly seen in elastic arteries like the aorta. The other functions of the intima are to be watertight, but selectively permeable, and to withstand the shearing, tangential forces experienced by the walls with systolic flow. The endothelium also modulates vascular tone via the synthesis and release of factors such as nitric oxide, prostacyclin, endothelin, and angiotensin II. It is also responsible for the production and release of various growth factors, and cytokines. The oxidation of low density lipoprotein (LDL) also occurs here.

Depending on the position of the artery within the circulatory circuit, the media has different characteristics. Large proximal arteries, have elastic characteristics which enable them to firstly withstand the systolic pressure wave without rupturing, and secondly to conserve the potential energy and retransmit this at the end of systole. The distal arteries (about 1cm in diameter), have a more muscular aspect, controlled by the autonomic nervous system, which helps to control the preferential supply of blood

depending on conditions such as fright or flight, when blood is diverted to the musculature, and diverted from the gut.

In an elastic artery – i.e. the aorta, the media is thick and consists of sheets of elastin fibres concentrically arranged throughout the thickness of the media (about 28-32 layers in the infrarenal aorta). The fibres are arranged circumferentially to best counteract the pulsatile blood flow. Return of the artery to its un-stretched state during diastole results in a pressure of 60-80mm Hg within the larger vessels. These medial elastic fibres take most of the stress applied to the arterial wall at physiological pressures (McMillan et al., 1997, Clark and Glagov, 1985). Interposed within these elastin layers are smooth muscle cells and collagen fibre bundles.

In a muscular artery (figure 1, below), the media is composed almost entirely of smooth muscle, which are arranged circumferentially at right angles to the long axis of the vessel. In large muscular arteries there may be 30 or more smooth muscle layers, which can decrease to 2 or 3 layers in a small peripheral artery. These arteries are therefore highly contractile. A few fine elastic fibres are scattered amongst the smooth muscle cells, but are not arranged into sheets. These are most numerous in the larger of these vessels, where they have continued from an elastic artery.

The adventitia is composed largely of collagen, which takes the load when extra-physiological pressures are experienced in the vessel (McMillan et al., 1997). The adventitia of larger vessels also contains the *vasa vasorum*, small blood vessels from which small nutrient branches penetrate into the media. In smaller vessels oxygen simply diffuses through the thinner walls to supply the media (Stevens and Lowe, 1992b). The adventitia is demarcated from the media in arteries by a condensation of elastic fibres that forms the variably distinct external elastic lamina.

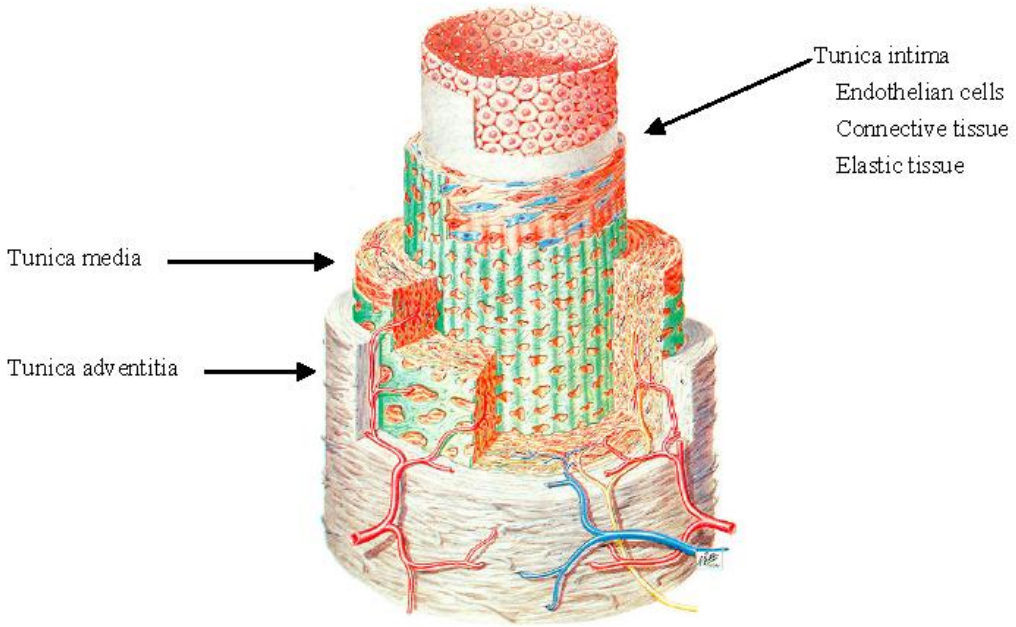


Figure 1. A medium sized artery wall (Kangasneime and Opas, 1997)

1.1.7.2. The Extracellular Matrix

The extracellular matrix (ECM) is a complex structural network that provides cell and tissue support, helps form different tissue compartments, and provides a surface for molecular sieving and cellular regulation. Extracellular barriers are formed by elements of the matrix;

- Basement membrane: separating parenchyma & stroma
- Interstitial stroma: separating blood vessels and lymphatics from organ parenchyma.

The components of the ECM include the fibrous proteins, proteoglycans, and several glycopeptides.

The 4 major proteins that form fibrils in the extracellular matrix are;

- Collagen;
- Fibrillin;
- Elastin;
- Fibronectin.

The ECM is normally impermeable to cells, but focal penetration can occur during healing, remodelling, inflammation, and neoplastic development. Continual mechanical stresses require the maintenance of structural integrity and physical properties to prevent weakening of the vessel wall. The processes that can be affected in order for permeability to occur are;

- Cell/basement membrane attachment via cell surface receptors
- Excess cellular secretion of proteolytic enzymes or the reduction of inhibitors
- Tumour cell migration into the area modified by proteolysis.

The degradation of the ECM is an essential step in the process of tumour metastases. ECM degradation is also a feature of some benign conditions including the arthropathies, vascular aneurysms, and atherosclerosis.

1.1.7.3. Collagen

The collagens type I – XI are a family of closely related proteins concerned with providing tensile strength to tissues. They are the most abundant of the extracellular proteins, and are constructed of precursor proteins (α chains, of which there are more than 20 different types) wound together to form rigid linear triple helix structures in long filaments. They are produced by fibroblasts

Collagen type I-III are the main forms of fibrillar collagen. This is formed from 3 polypeptide (α chains), which are initially secreted with both carboxyl (COOH) and amino (NH_2) terminals to prevent the formation of collagen in cells. These chains form a triple helix (procollagen), and cleavage of the carboxyl and amino extensions results in the functional mid regions (tropocollagen), which can now form long filaments (collagen microfibres). Microfibres form fibres (figure 2 below) and fibres form bundles by tight cross-linking lysine residues between molecules. Collagen molecules are 300nm long and are arranged with a 67nm overlap between adjacent molecules. The main forms of fibrillar collagen maintaining structural integrity in arterial vessel walls are types I and III (Melrose et al., 1998), but tensile characteristics are mainly due to type III collagen (Menashi et al., 1987).

Type I collagen is found primarily in the adventitial layer and to a lesser extent in the medial layer of arteries, and consists of large, well-structured fibres providing structural integrity (Millis et al., 1992).

Type III collagen, the predominant collagen in blood vessels, and is finer, more distensible then type I collagen, and forms a banded collagen fibre. It is found mainly in the medial layer of arteries. It also occasionally forms reticulin, thin fibres of type III collagen which form a mesh or scaffold for other components of the extracellular matrix.

Type IV collagen is the predominant collagen seen in the basement membrane of blood vessels, and it forms a meshwork rather than fibrils.

Finally type VIII collagen is seen in the endothelium of vessels.

Collagen is a highly stable protein, but it is in a continuous state of synthetic and degradative flux.

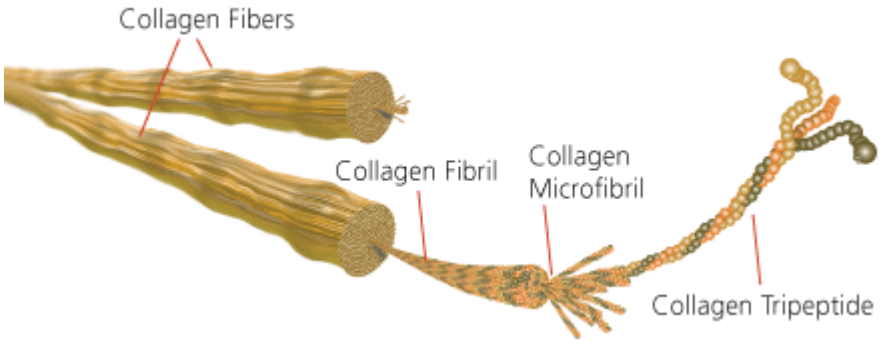


Figure 2. Collagen (www.SigmaAldrich.com)

1.1.7.4. Fibrillin

The glycoprotein fibrillin is one of the components of elastin-associated microfibrils (EAMF), which are ubiquitous connective tissue structures, thought to act as a scaffold for elastin deposition, and to mediate adhesion between different components of the extracellular matrix. It has been noted to decrease with moderate age in human aorta (Godfrey et al., 1993).

1.1.7.5. Elastin

Elastin (figure 3, below) is the main component of elastic fibres (which it forms with fibrillin) and has a random coil structure in a relaxed state that can stretch, but reforms in a different coil shape when relaxed again. This hydrophobic protein is produced by fibroblasts, and covalently linked molecules form fibres or sheets of elastin. As elastin is secreted, fibrillin microfibrils organise it so as to form distinct fibres between the microfibrils (Stevens and Lowe, 1992a). The half-life of elastin is 40-70 years, and it

is not synthesized in adults, which accounts for the reduction in elastin often seen with age.

It is a major structural component of the abdominal aorta, mainly contained in the medial layer. It is one of the most durable proteins of the extracellular matrix and requires the presence of specific proteinases (eg certain MMPs) for dissolution.

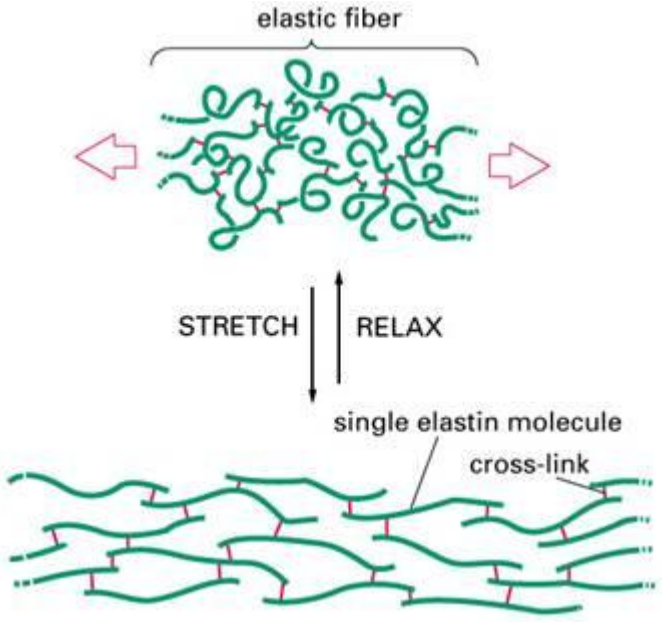


Figure 3. Elastin – relaxed and stretched states (Alberts et al., 2002)

1.1.7.6. Fibronectin

This is a glycoprotein with the ability to bind a variety of substances including collagen, heparin, and specific fibronectin receptor proteins in cell membranes. It can exist in a plasma protein form, a protein form that can transiently attach to many different cells, and an insoluble fibril form, with disulphide bond cross-links, which can form part of the extracellular matrix(Stevens and Lowe, 1992a).

1.1.7.7. The abdominal aorta

The abdominal aorta is the continuation of the thoracic aorta, emerging from the diaphragmatic aortic hiatus, in front of the 12th thoracic vertebra, descending anterior to the lumbar vertebral bodies to the 4th lumbar vertebra where it bifurcates into the 2 common iliac arteries (Snell RS, 1992). It is about 12-15cm in length, with support from the vertebrae posteriorly, the renal arteries superiorly (and to a lesser extent the suprarenal arteries and aortic hiatus of the diaphragm), anteriorly, the 3 visceral branches (celiac, superior and inferior mesenteric arteries), and the aortic bifurcation inferiorly. In addition there is some support from the surrounding tissue/organ matrix.

The normal diameter of the abdominal aorta varies with age, sex and bodyweight, and decreases rapidly from its entry into the abdomen at the aortic opening of the diaphragm progressively to the bifurcation to become the iliac arteries, due to the size of its branches. Studies from cadavers have reported superior diameters between 9-14mm, and inferior diameters between 8-12mm, with little differences between the sexes (Borley 2005). In elderly men the diameter is between 15 and 24mm (Liddington and Heather, 1992). The average diameter of the aorta is taken to be about 2cm in an average 65 year old man. Following on from the definition of an aneurysm – at least 50% larger in diameter compared to normal segments – a size of 3cm is taken as the minimum diameter of an aneurysm. This would be expected to be smaller in women, and therefore a lower threshold may be applied.

1.1.7.8. Structure of the abdominal aorta

As with other arteries, the abdominal aorta has 3 main layers, with the main strength of the vessel coming from the medial layer. This is composed of around 30 layers of

circumferentially arranged elastin fibres, together with collagen and smooth muscle – occasionally referred to as a lamellar unit. These help the aorta to withstand the pulsatile nature of systolic blood close to the left ventricle, and also help to retain the energy contained in the blood flow, transferring kinetic energy from the blood flow to potential energy in elastin fibres and back again to the blood flow. There is also some support from the external elastic lamina within the adventitial layer of the aorta, but hardly any support from the internal elastic lamina in the intimal layer. Allowing the aorta to dilate or pulsate with systolic pulsation also enables the aorta to better withstand the systolic pressures of the blood transmitted within it as a rigid lumen would have to be stronger to withstand the same pressures. However the strength of a normal healthy aorta has in-built redundancy, with wall strength much greater than required.

The main mechanical properties of the aorta including its visco-elastic properties are determined by elastic and fibrillar collagens within the medial and adventitial layers.

Elastin and collagen are arranged within the aortic wall to convey resistance to stretch. Most of the load at physiological pressures is taken up by the elastin within the medial layer of the arterial wall, and elastic deformation is seen, with the return to the pre-stretched state after each pressure cycle (McMillan et al., 1997, Clark and Glagov, 1985)

Aortic wall also has to withstand wall shear stresses – the stresses that occur due to the flow of blood along the vessel wall. 2 main features ameliorate this stress, the smooth internal layer of the vessel – the intima, and the laminar flow of blood through the vessel. This allows blood flowing through the middle of the lumen to flow more

quickly than blood along the periphery of the vessel – blood acting as its own lubricant.

The blood supply to the medial layer of the aorta is via small vasa vasorum penetrating the adventitial layer(Millis et al., 1992).

1.1.7.9. Abdominal aortic aneurysm

The normal abdominal aorta dilates with age, and is the commonest site for an aneurysm.

The normal histological architecture of the aorta is lost in aneurysms, with characteristic destructive remodelling of the elastic media, and outer aortic wall. The media has scattered smooth muscle cells, fragments of collagen and elastin fibres, and thickened, less pliable type 1 collagen replaces the normal adventitial collagen (Millis et al., 1992). In addition, the intimal layer is replaced by debris, with mural thrombus, cholesterol and calcified lipid.

Investigations have emphasized disease mechanisms involving chronic wall inflammation, and the progressive degradation of fibrillar matrix proteins (Dobrin and Mrkvicka, 1994, Shah, 1997).

Elastin content has been shown to decrease through a decrease in elastin cross-links(decrease in high performance liquid chromatography (HPLC) demonstrated desmosines & isodesmosines). The number of collagen cross-links seems to increase (HPLC demonstrated pyridinoline collagen cross-links), but the tissue content of collagen as demonstrated by characteristic amino acids 4-hydroxyproline, and 5-hydroxylysine was shown to decrease, suggesting that old collagen accumulates cross-links whilst new collagen biosynthesis is defective or reduced(Carmo et al., 2002).

Medial neovascularisation is more common in aneurismal aortas than in normal aorta or aortas involved in occlusive disease. Furthermore this seems to correlate with areas of elastin degradation and chronic inflammation (Holmes et al., 1995).

A study analysing the histological features of aneurysms, took biopsies from 21 small (4-5.5cm) and 45 larger aneurysms. The histological feature most associated with enlarging diameter was a higher density of inflammatory cells in the adventitia. This inflammation was non-specific with mostly macrophages and B lymphocytes. Fibrosis of the adventitia provided compensatory thickening of the aortic wall as the aneurysm diameter increased (Freestone et al., 1995).

It has been noted that the average number of lamellar units in human aorta is 28, which is far fewer than seen in other mammals. In addition the number of vasa vasorum supplying the medial layer is less than expected from comparison of similar thickness arterial wall in comparable mammals. It has been postulated that this results in greater tension per lamellar unit, and relative arterial wall ischaemia, with a propensity for degeneration and aneurysm formation (Zatina et al., 1984). Porcine studies suggest a critical value of less than 40 lamellar units (porcine normal = 75) and an average tension of greater than 3 times normal (porcine) (4087 dyn/cm= 40mN/cm) lead to aneurysm formation. Aneurysm formation was directly proportional to tension per unit and inversely proportional to number of lamellar units present(Zatina et al., 1984).

Elastin associated microfibrils (EAMF) are ubiquitous connective tissue structures, thought to provide tensile strength and flexibility to numerous tissues by providing a framework for elastin deposition. One of the components of EAMF is fibrillin, and a study looking at EAMF in normal human aorta by identifying fibrillin through immunohistochemical methods and mRNA levels, found decreasing levels with

increasing age in organ donor patients from 11 months to 44 years (Godfrey et al., 1993).

1.1.7.10. Thrombus

The role of thrombus in AAA is uncertain, although in general the development of abdominal aortic aneurysms is associated with a mural thrombus, and it is seen in 75% of AAAs. In contrast to arterial occlusive disease, blood flow is maintained in aneurysms leading to a constant remodelling of the thrombus and its components. Thrombus diameter has been potentially linked to the change diameter of aneurysms, through correlation with circulating plasmin-α_2-anti-plasmin (Lindholt et al., 2001b), together with plasma markers of fibrin formation and degradation (Yamazumi et al., 1998). A rapid increase in relative intraluminal thrombus (ILT) volume may be a better predictor of AAA rupture than AAA diameter (Stenbaek et al., 2000).

Opinion varies regarding the effect of thrombus on aneurysm wall stress and strength and thus on aneurysm rupture potential. Earlier studies showed that ILT decreased the peak stress by up to 30% (Inzoli et al., 1993), and other studies suggested that thrombus was mechanically protective (Vorp et al., 1996a), reducing mechanical stress transmitted to the arterial wall through a diffusing or blanketing mechanism (Mower et al., 1997, Wang et al., 2002). However the failure to reduce the transmission of pressure and pressure transmission by a different mechanism have also been demonstrated (Schurink GW et al., 2000). Wall stress is influenced by many factors including AAA diameter, length, wall thickness, luminal pressure, thrombus thickness, volume ratio, surface area ratio, elastic modulus and homogenicity. Of equal importance to AAA wall stress is AAA wall strength. It has been demonstrated that

ILT reduces wall tensile strength, which is postulated to be due to a hypoxic environment, compensatory inflammation and local proteolytic activity (Vorp and Vande Geest, 2005). Local hypoxia of the arterial media has been observed, with increased medial neovascularisation and inflammation (Vorp et al., 2001). More recent work has provided a constitutive model for ILT derived from biaxial testing but clearly the impact of ILT rupture risk is a complex one and further work is required on AAA (Vande Geest et al., 2006b).

ILT is also a source of proteases, with enrichment of MMP9 (gelatinase B) in the thrombus (Sakalihasan et al., 1996). Polymorphonuclear neutrophils (PMN) within this, trap and store MMP9, which may represent another important role for ILT in AAA development and progression (Fontaine et al., 2002). In addition, ILT contains plasminogen and its activator (u-PA), which in turn could result in local generation of plasmin, an MMP activator.

1.1.7.11. Atherosclerotic plaque

Inzoli (Inzoli et al., 1993) demonstrated calcified atherosclerotic plaque to be associated with elevated stress concentrations and an elevation in peak wall stress. This is presumably due to the higher stiffness of the plaque in comparison to the adjacent ‘normal’(without plaque) aneurysmal tissue, and the abrupt change in tissue characteristics at the boundary.

1.1.8. Pathophysiology of AAA

The primary abnormality in the formation of abdominal aortic aneurysms is alteration of the connective tissue in the vessel wall, namely the loss of elastin and smooth muscle cells from the medial layer of the artery, together with fragmentation of elastic

fibres (Powell, 1990, Baxter et al., 1992, Sakalihasan et al., 1993). This loss of elastic fibres seems to be an early event in aneurysm formation (Dobrin and Mrkvicka, 1994). White et al agreed, but they thought that the primary layer involved was the adventitia (White et al., 1993).

The previously accepted hypothesis associated with these aneurysmal changes in the medial layer was postulated to be atherosclerotic related degeneration. The intimal atherosclerotic plaque was thought to impair the medial blood supply resulting in muscle and elastin loss. It was suggested (Mower et al., 1993), that vasa vasorum and other blood vessels in regions of increased stress may tend to be compressed and not provide nutrition to the vessel wall. However this could apply to stress due to atherosclerotic plaque or to wall stress due to intraluminal pressure.

Other evidence suggests that the loss of elastin is primarily due to an increase in proteolytic capacity. Specifically, increased elastolytic activity has been observed in aneurysmal aortic wall compared to normal aorta (Busuttil et al., 1982), and in ruptured versus non-ruptured AAA regardless of size (Cohen et al., 1987). The matrix metalloproteinases are an important group of proteases with elastase activity amongst others, and they will be discussed in detail later. This proteolytic process is hastened by the prostaglandins and proteolytic enzymes from infiltrating leucocytes, which also secrete growth factors stimulating angiogenesis and chemotaxis within the arterial wall. Elastin loss leads to other components of the aortic wall being subjected to greater forces, and the tensile strength of the vessel is decreased. The natural compensatory response is the laying down of extra *excessive* adventitial collagen, and thickening of the aortic wall. The disruption of the extracellular matrix is associated

with a widespread inflammatory infiltrate (Koch AE et al., 1990), and decreased proteoglycan synthesis (Tamarina NA et al., 1998).

In AAA, medial elastin loss results in arterial wall strength being dependant upon adventitial collagen and therefore final rupture occurs due to failure of this collagen layer (Dobrin and Mrkvicka, 1994). Indeed increased collagen turnover has been demonstrated in human AAA (Satta et al., 1995), possibly consistent with the demonstration of collagen repair processes in animal models (Huffman et al., 2000). An imbalance in collagen homeostasis could lead to a catabolic state and aneurysm rupture.

Vine and Powell demonstrated significantly increased gelatinase activity in the luminal aspect of the aortic media compared to control aorta. Interestingly collagenase and stromelysin could be detected in homogenates of aneurysmal aorta (n=10), but rarely in atherosclerotic aorta (n=10) and never in control aorta (n=6), and collagenase activity was highest in the adventitial aspect of the aortic media (Vine and Powell, 1991).

1.1.8.1. Natural History

Expansion

The natural history of large abdominal aortic aneurysm is gradual expansion, which can lead to rupture (Vardulaki et al., 1998).

An expension rate of 0.4cm/yr in untreated AAA until rupture was reported (Cronenwett et al., 1990). A similar expansion rate of 0.33cm/yr was reported by the (UK Small Aneurysm Trial Participants, 1998b), in patients with AAA diameter 4.0-5.5cm under surveillance. (Vega de Ceniga et al., 2006) reported an expansion rate of

0.21cm/yr in AAAs 3-3.9cm in diameter and a rate of 0.47cm/yr in AAAs 4-4.9cmm in diameter. Of the smaller group (3-3.9cm), they estimated that 2.4% of patients would reach 5cm within 2 years, and 17.6% within 5 years. Of the larger group (4-4.9cm) 55.8% would reach 5cm within 2 years, and 81.6% within 5 years. They also admitted however that aneurysm growth wasn't necessarily uniform with 25% of the smaller group and 18% of the larger group experiencing no expansion (0cm) at all during the mean follow-up time of 3.5 years. This finding is in line with work by (Brady et al., 2004) which showed an AAA growth pattern characterised by growth spurts, stasis and aneurysm regression in 6.4% of cases.

Rupture

(Cronenwett et al., 1985) followed up a homogenous group of 67 patients with AAAs (diameter 2 to 7 cm) selected for conservative management for a mean time period of 36 months, and found an annual rupture rate of 6%.

Small aneurysms (4.0-5.5cm diameter) were found to have a 1% annual rupture risk (UK Small Aneurysm Trial Participants, 1998b), and AAAs at least 5.5cm in diameter (median 6.3cm, IQR 6-7cm) had an annual rupture risk of 9% (172 patients observed in the no-treatment arm of EVAR II had 23 ruptures over a median 3.3 year follow up)(EVAR trial participants, 2005a). The overall annual risk of rupture in AAA with diameters > 6cm is estimated at 25% (Brown and Powell, 1999). Independent factors associated with aneurysm rupture were found to be; female sex (3 to 1), larger initial AAA diameter, lower FEV_1, current smoking status, and higher mean blood pressure.

AAA repair is generally recommended when maximum diameter approaches / exceeds 5-5.5cm. At this size, the annual rupture risk is estimated to be 5%, thus roughly

equating to the mortality associated with open surgical repair (Dryjski et al., 1994). However studies have shown that this is often unreliable (Darling et al., 1977, Geroulakos and Nicolaides, 1992), and indeed small aneurysms have been shown to rupture (Choksy et al., 1999, Hall et al., 2000) and some large AAAs may not rupture given the life expectancy of certain patients (Darling et al., 1977). The present situation is therefore not ideal and more accurate patient specific assessments of rupture risk are continuously being evaluated (Vorp, 2007).

An autopsy study (Darling et al., 1977) at the Massachusetts General Hospital analysed 24,000 consecutive, non-specific autopsies performed over a 23-year period (1952-1975). 473 non-resected AAAs were found, of which 118 were ruptured. Of 265 AAAs ≤5cm in diameter, 34 had ruptured (12.8%), and of 194 AAAs >5cm, 78 had ruptured (40%). In addition 14 had no size recorded, and 6 (43%) of these had ruptured. Interestingly they found that aneurysms of diameter between 4.1cm and 7.0cm had a similar rupture rate of about 25% (4.1-5.0cm 23.4%, n=64; 5.1-7.0cm 25.3%, n=83). Aneurysms of diameter 7.1-10.0cm had a rupture rate of about 45%, and aneurysms with a diameter of more than 10.0cm, had a mortality rate by aortic haemorrhage of 60%. It could however be postulated that measuring the size of these AAAs post rupture may have lead to an incorrectly low measurement for the diameter, however the repost suggested by the authors was that the AAA size during a surgical procedure after proximal aortic clamping was rarely less than the diastolic size (2-4mm)(which they put down to calcification of the aortic wall).

By retrospectively going through the patient notes, it was possible to ascertain survival times after onset of symptoms consistent with AAA rupture (severe back pain, acute abdominal pain, or collapse). 46% died within 6hrs, 57% within 24hrs, but 25%

survived longer than 6 days, and 6% survived longer than 6 wks (total group n=118, of which 14 length of survival not determined).

Also of interest, there was no clear predilection for the site of rupture, but anterior and posterior surfaces seemed to experience the same number of events (18% each), and left and right, also about the same number (36% and 30% respectively). However if the circumference of the AAA was split into thirds, the anterior third (roughly correlating with ruptures that may rupture into the peritoneum), would only account for 18% of ruptures (Darling et al., 1977).

1.1.8.2. Progression of an aneurysm

Hydraulic stress

The angle of the aortic bifurcation varies widely, particularly in the elderly, and there has been speculation that the relationship between aortic size and shape is a possible causative factor in the development of AAA (Newman et al., 1971). This is possibly because of reflected pressure waves, which occurs at junctions between vessels. Animal and post-mortem studies, have demonstrated that the magnitude of the wave was proportional to the diameter of the proximal aorta and inversely proportional to the diameter of the iliac vessels (Gosling et al., 1971). In this way the wave increased in intensity as the aneurysm increased in size. This continued succession of waves increasing in magnitude with time would potentially result in progressive dilatation of the aortic wall. (Shah et al., 1978) found no evidence to support the theory of distal aortic intimal trauma through pressure oscillations and turbulence associated with change in vessel diameter at the aortic bifurcation. A ‘water hammer’ theory of direct

tangential lateral aortic wall stress has also been proposed where a retrograde, reflected wave hits an advancing, antegrade wave (Blakemore and Voorhees, 1954b).

Vibratory forces

Turbulent flow generated distal to the renal arteries was also proposed as a reason for aneurysm initiation. This turbulent flow results in low frequency vibrations in the vessel wall (200 cycles per second), and this was demonstrated to be damaging to the medial layer of the artery in an experimental setting (Roach, 1963).

Wall stress

The law of Laplace states that wall tension is directly proportional to intraluminal radius and pressure in a perfect sphere with infinitely thin walls (Laplace, 1806). Using this principle, and applying it to the imperfect situation of an AAA, it would be reasonable to associate increased wall stress with increased rates of AAA expansion.

1.1.9. AAA management

1.1.9.1. Historical treatments

Ligation

The first AAA ligation was performed by Astley Cooper, a student of John Hunter. He found that applying ligatures to proximal or distal ends alone was ineffective, but that ligating both proximal and distal sides of the aneurysm was better. Not surprisingly however, the extremities were vulnerable to ischaemic damage.

Embolization with intraluminal wire

In 1864 Moore inserted and left lengths of silver wire in a thoracic aneurysm to induce clot formation. Corradi (1879) passed galvanic current through this wire, and this combined technique was adapted by various investigators including Blakemore & King (1938) who devised an accurate means of electrothermic coagulation. A deposit of protein coagulum formed on wire, which stimulated clot production throughout the aneurysm. Power demonstrated the same method in AAAs (1921).

Periarterial fibrosis with cellophane

The stimulation of periarterial fibrosis was attempted by using cellophane or other types of plastic film. First developed by Harrison and Chandy (1943) to an aneurysm of the subclavian artery, then applied by Poppe and De Oliviera (1946) to syphilitic thoracic aneurysms, both with some success, but the method was very unpredictable. Rea introduced this technique to AAAs (1948), wrapping cellophane around the neck and anteriolateral surfaces of the AAA, and it was used by Nissen (1949) to treat the symptomatic AAA of Albert Enstein, who eventually ruptured after 6 years.

Endoaneurysmorrhaphy (obliterative / restorative / reconstructive)

Rudolph Matas, a New Orleans surgeon revived endoaneurysmorrhaphy (which simply means suturing of the inside of the aneurysm whilst in place) in 1888, when he treated a plantation worker (Manual Harris) who had sustained a traumatic brachial artery aneurysm after being shot with buckshot. Proximal ligation or amputation was the usual treatment for false aneurysms at that time. After initial intermittent compression therapy for 3 weeks, proximal then distal ligation, the aneurysm had not improved. "Being loath to subject a young strong man to amputation," Matas eventually decided

to follow Antyllus' procedure. He opened the aneurysm, with an Esmarch tourniquet in place, and carried out intima-to-intima approximation of the 3 feeding vessels in the aneurysm with fine silk sutures. Harris subsequently did well, and was still in manual plantation occupation 10 years later. This was Matas' first obliterative endoaneurysmorrhaphy. This form of endoaneurysmorrhaphy still precluded application to situations where the artery in question was necessary to prevent ischaemia.

Matas also devised restorative endoaneurysmorrhaphy which he used for saccular aneurysms, and reconstructive endoaneurysmorrhaphy where he excised the diseased portion, and created a tunnel through the remaining normal portion. Both of these allowed the artery to continue working, and allowed application to the aorta or other major arteries.

Jose Goyanes (Madrid 1906) ligated popliteal aneurysms, then mobilised the adjacent popliteal vein which was used as an interposition graft via an end-to-end anastomosis. Alexis Carrel (1873-1948) was awarded the Nobel Prize in 1912 by formulating a technique in animals of replacing a segment of aorta with artery or vein. Charles DuBost (1951) was the first to successfully replace aorta with freeze dried homograft (Dubost et al., 1952). In 1953, Voorhees used a new synthetic fibre fabric graft called Vinyon-N (Blakemore and Voorhees, 1954a). This proved to be a major innovation over the unreliable homograft, and heralded the start of the modern era of aneurysm repair.

King Edward VIII was treated for an abdominal aortic aneurysm by DeBakey. "In an operation lasting little more than an hour, DeBakey cut out the swollen part of the aorta--normally less than an inch in diameter at this point in the body, but now the size

of a large grapefruit--and replaced it with a 4-inch length of knitted Dacron tubing." The Duke of Windsor recovered without incident and lived until 1972, when he died of throat cancer at age 78.

The principle of relining rather than excising the diseased portion of aorta, led to endoaneurysmorrhaphy with intraluminal graft placement. This was popularised by Creech and DeBakey.

1.1.9.1. Medical management

The medical management of AAA includes medical therapy to treat conditions that can contribute to aneurysm growth (eg hypertension, smoking, hyperlipidaemia) and medical therapy to treat conditions that can affect the health of patients and the risk of intervention for AAA (eg diabetes, heart failure, ischaemic heart disease).

Some studies have demonstrated theoretical methods of reducing or decreasing the rate of aneurysm expansion, including by the use of Doxycycline, MMP inhibitors, and statins.

Statins

Hydroxymethylglutaryl-Coenzyme A reductase inhibitors (statins) are known to have pleiotrophic effects in addition to their lipid lowering function. They have been shown to reduce aortic diameter and the number of AAAs in mice (Steinmetz et al., 2005). Retrospective analysis of the Dutch AAA screening database also suggests that statins delay AAA growth (Schouten et al., 2006). An observational study of conservatively managed medium-sized AAA patients treated with or without statins also demonstrates reduced growth at 2 years and reduced AAA rupture rate at 4 years in those treated with statins (Sukhija et al., 2006). Analysis of AAA tissue samples in patients treated

pre-operatively has shown a decrease in tissue MMP9 giving a possible mechanism to its protective effect on AAA development and progression (Evans et al., 2007). In contrast however, the Tromsø cohort study suggested that statin therapy increased the risk of AAA formation (Forsdahl et al., 2009).

1.1.9.2. Endovascular Aneurysm Repair (EVAR)

1.1.9.2.1. The history of EVAR

In 1992, Laborde in collaboration with Parodi reported a feasibility study of "intra-luminal bypass of AAA" in 8 dogs. An artificial aneurysm was created in each dog with Dacron, and a Dacron tube graft with integrated balloon expandable stents at both ends was inserted trans-femorally and expanded within the artificial aneurysm. Early occlusion was seen in 2 dogs due to torsion of the graft, but 6 remained patent for 6 months, with 4 showing kinking resulting from shrinkage of the artificial aneurysm. Trans-luminal bypass of AAA was concluded as possible in a dog model (Laborde et al., 1992).

Parodi carried out the first endovascular abdominal aortic aneurysm repair by using balloon expandable stents sutured to the ends of a tubular knitted Dacron graft. The friction seals established at the ends fixed the ends of the graft to the AAA wall and excluded the aneurysm from the circulation. His study reported the treatment in 5 patients, but recognised that further study and development were necessary before widespread use was possible (Parodi et al., 1991).

1.1.9.2.2. The aim of EVAR

Endovascular AAA repair (EVAR) attempts to isolate the aneurysmal aortic segment from the circulation by deploying a covered stent-graft, which is inserted in a narrow deployment system via the femoral artery. Once the aneurysmal segment is isolated then the aneurysm is depressurised and thus should not increase in size or have any propensity to rupture.

1.1.9.2.3. The EVAR procedure

EVAR is commonly carried out in a sterile operating theatre with radiological facilities. This may be within the theatre suite or in a suitably equipped radiology department. General or regional anaesthesia is administered with or without prophylactic antibiotics and a surgeon carries out vascular cut down to allow access to one or both femoral arteries, and achieves control of the vessel(s). Occasionally the brachial artery can be used if access to the femoral arteries is difficult. An endovascular catheter is then introduced and an on-table angiography carried out to establish and locate the desired landing zones for the stent-graft. The deployment system with the appropriate pre-ordered stent-graft is then introduced under fluoroscopic guidance, and once in position, is deployed, again under close fluoroscopic scrutiny. Further limbs of the stent-graft are inserted and deployed as necessary (eg for a bifurcated graft). Completion angiography is performed to exclude primary type 1 endoleaks (see below). The femoral artery/arteries are then repaired surgically and the incision closed.

1.1.9.3. Operative intervention

1.1.9.3.1. Open AAA repair

Current guidelines suggest that elective repair of AAA be offered at a diameter of 5.5cm or above to patients in reasonable health (Brewster et al., 2003, Dryjski et al., 1994). Open surgery, consisting reconstructive aneurysmorrhaphy, with the insertion of an in-line synthetic vascular graft is associated with a mortality rate of 4-5% (Katz et al., 1992), but many patients have extensive or advanced co-morbid conditions, and will have mortality rates in excessive of the above quoted. It is common practice to have a short period of high dependency nursing following surgery due to the high-risk nature of the surgery, and the general frailty of the patients involved.

The average length of hospital stay following open AAA repair was 11 days (UK Small Aneurysm Trial Participants, 1998a). The only factors associated with a longer length of hospital stay was lung function (positive association) and pre-operative physical function (negative association).

1.1.9.4. Conservative management

Some patients with significant co-morbidities are considered to be too high risk to undergo elective surgery, and are therefore managed conservatively. In a series of 52 such patients, 40% were dead at 1 yr, 75% were dead at 5 yrs, and all had died by 10 yrs. The cumulative percentage of ruptures increased as patients survived longer with 43% dying of rupture in the first year, 46% at 5 yrs and 52% at 10 yrs. There was significant association between rupture and size, in the 5-7cm AAA group approximately 50% of patients died of rupture whilst in the >10cm AAA group, 93% died of rupture (Darling et al., 1977).

1.2. Screening & Abdominal Aortic Aneurysms

1.2.1. Principles of screening

Screening has the aim of intervening at an early stage in disease, where significant improvement can be achieved, in order to prevent undesirable normal disease progression. It thus seeks to improve the quantity and quality of life of the screening programme's participants.

Several standards are required to support the introduction of a screening programme (Wilson and Jungner, 1968, Gavin et al., 2007);

- The disease should be an important health problem
- The natural history of the disease should be reasonably understood
- There should be a recognisable pre-disease or early disease stage
- There should be an effective, safe, and acceptable test
- The treatment should improve outcome and there should be additional improvement with treatment at the pre-disease or early disease stage
- There should be evidence of reduction in mortality and morbidity in participants
- The benefit from the screening programme should outweigh possible physical and psychological harm from the programme itself, diagnostic tests, and treatment
- Costs and facilities needed have to be considered in context of other resource needs.

The health and well being of patients is perhaps the most important consideration of any screening programme. Patients may be subjected to substantial physical and psychological harm from the most well intentioned screening programme. Raising the possibility of a disease that had not been previously considered by the participants may induce significant anxiety, which may be prolonged by the time lag between test and final result.

Diagnostic tests may be both invasive and traumatic (eg colonoscopy or fine needle aspiration (FNA) biopsy), thus careful consideration of the risk / benefit profile should be mandatory.

The management of disease positive subjects needs specific consideration. A proportion of such patients will merit urgent treatment, which may be associated with substantial morbidity, occasional mortality, and perhaps a prolonged incapacitated recovery period. A second group of disease positive patients may not have sufficiently advanced disease to warrant treatment, but require continuous surveillance, which may be associated with its own specific problems i.e. anxiety / quality of life impairment (Lindholt et al., 2000a). Finally there is a group of disease positive patients that are unsuitable for intervention, in whom diagnosis raises the permanent spectre of an untreatable disease. Ideally, such patients should be excluded prior to commencement of the screening process, however precise selection of patients beyond a general target group can be extremely difficult.

1.2.2. The case for AAA screening

AAAs affect 5% of men aged 65 to 74 years old (Ashton et al., 2002) and are generally asymptomatic until they rupture or leak. AAA rupture is associated with an 80%

overall mortality (Scott et al., 1995), accounting for 6800 deaths / year in the UK (Vardulaki et al., 2000). In addition 4-5% of sudden death is due to ruptured AAA (O'Sullivan, 1996). In contrast, elective AAA repair is associated with a 5-7% 30-day mortality (Bayly et al., 2001, EVAR trial participants, 2005b, National confidential enquiry into patient outcome and death, 2005).

The main case for AAA screening therefore, lies with the fact that emergency AAA repair is dramatically more dangerous in terms of mortality and morbidity compared to elective AAA repair. An AAA screening programme would result in the detection of AAAs at an asymptomatic stage when elective surgery is an option.

The other main argument for an AAA screening programme is that AAAs are easily and reliably detected given the appropriate staff and equipment, utilising a test that is non-invasive and widely acceptable to patients.

1.2.3. Problems with AAA screening

Problems with AAA screening pertain to effects on the psychological health of all screening participants – in particular anxiety;

- Healthy participants - between being invited to the programme (this raising the possibility that they have an AAA) and to having the test (patients can be informed straight away if they do not have an aneurysm)
- Participants found to have an asymptomatic AAA < 5.5cm in diameter – undergoing lengthy surveillance of their aneurysm before potentially undergoing intervention

- Participants found to have a large (>5.5cm diameter) or symptomatic AAA having to await investigations before undergoing major surgery to repair their AAA.
- Participants found to have a large >5.5cm AAA who are unfit to undergo elective repair and are thus burdened with the permanent spectre of an untreatable, unpredictable and potentially lethal disease.

There are also substantial resource implications;

- The screening programme involves the need for skilled ultrasonographers or vascular technologists, and ultrasound machines. The test is relatively quick and simple, and the teams can be mobile, carrying out testing at local and accessible settings, but the number of patients involved still results in the need for substantial resources.
- Screening for AAA would bring extra work to the vascular surgeons in terms of increasing the elective AAA repair workload. This may be particularly intensive to begin with, but should reach a plateau level once the screening programme is established. This increased workload has all kinds of service implications including the need for extra theatre space, equipment & staff, extra vascular wards, beds and staff, and extra clinics, vascular, anaesthetic and cardiology. However, in time the screening programme should reduce the number of unpredictable emergency AAA repairs necessary, which require relatively more resources than elective surgery.
- AAA screening results in the detection of a significant number of AAAs <5.5cm which require surveillance, a further resource consideration.

1.2.4. AAA screening studies

The Birmingham Community Aneurysm Screening Project (Smith et al., 1993) invited 3500 men aged 65-75 years for AAA ultrasonographic screening at their own general practitioner's surgery. 2669 (76%) attended (52% inner city areas vs 90% suburbs), and 97.3% of scans successfully imaged the abdominal aorta. 219 patients (8.4%) had an aortic diameter of >2.9cm, and 79 (3.0%) with diameter >4.0cm were referred for vascular surgical assessment.

Scott (Scott et al., 1995), randomised over 15,000 men and women aged 65-80 years to AAA ultrasound screening or non-intervention. Of 7887 invited to screening 5394 (68.4%) accepted, and AAA was detected in 218 (4%) participants and 7.6% of men. The relative risk of rupture was reduced by 55% in the screened group compared to controls.

Lindholt (Lindholt et al., 2005) carried out a randomised controlled trial of AAA screening involving 5 hospitals within the county of Viborg, Denmark, between 1994 and 1998. They randomised more than 12,000 men aged 64-73 years to either ultrasonic AAA screening or non-intervention. AAAs with diameters > 5cm were referred to vascular surgeons, whilst AAAs with diameters between 3-5cm, and 2.5-3cm were rescanned at 1 and 5 years respectively. Outcome measures included AAA specific mortality, overall mortality, number of elective and emergent operations for AAA, and number of ruptured AAAs. Over 6,300 patients (50.1%) were randomised to screening, of which 76% attended. 44 scans (0.7%) were unsuccessful in measuring the aorta and identifying or excluding an AAA. The incidence of AAA in the screened group was 191(4%), of which 24 (0.5%) had an AAA≥5cm. There were 9 deaths due to AAA in the screened group compared to 27 in the unscreened group (p=0.003), and

total deaths were 939 in the screened group compared to 1019 in the unscreened group (p=0.053). The number of ruptured AAAs was also reduced in the screened group (8 versus 29 ruptures, p=0.001). Mean follow up was 52 months. During the first 18 months aneurysm related deaths were the same in both groups. It was calculated that 32 life years were gained from AAA related deaths after offering screening to 6333 men during the first 5 years, and this was estimated by extrapolation to 107, and 158 life years at 10 and 15 years respectively. The number of patients required to be screened to save 1 life was 352.

A study carried out in Western Australia (Norman et al., 2004, Spencer et al., 2004) randomised 41,000 men aged 65-83 years of age to either ultrasonic AAA screening or no intervention. Attendance was 70% in the screening group, the incidence of AAA >3cm was 7.2% and of AAA >=5.5cm was 0.5%. Elective AAA repair was twice as common in the screened group than in the no-intervention group (107 vs 54, p=0.002). Between screening and end of follow up (5 years) 18 men in the screened group and 25 in the control group died from AAA (mortality ratio 0.61, 95% CI, 0.33 to 1.11). Benefit (mortality ratio 0.19, 95% CI, 0.04 to 0.89) was confined to the younger age group cohort (65-75 years). The authors concluded that national screening for the age group 65-83 years was not effective, and that a more select age group, with exclusion of inappropriate patients may make screening effective.

The Multicentre Aneurysm Screening Study (MASS) involved more than 67,000 men aged 65-74 years of age and demonstrated that AAA screening with abdominal ultrasonography results in a relative reduction in AAA rupture of 49%, and in AAA related mortality of 42% (from 0.33%) through early detection and surgical intervention (Multicentre Aneurysm Screening Study Group, 2002a). Cost

effectiveness analysis of the MASS study (Multicentre Aneurysm Screening Study Group, 2002b), showed the cost of a quality-adjusted life year (QALY) to be £36,000 over the four years of the study although they estimated that the cost would reduce to £10,500 per QALY after 10 years of running the screening programme at national level (full costs of screening analysed including costs for elective and emergency intervention). They stated that although the costs as calculated at 4 years were at the margins of acceptability to the NHS, the estimated costs at 10 years would be acceptable. The latest analysis from the MASS group states the cost per QALY at 10 years to be £7,600 (95% CI: £5,100 - £13,000) (Thompson et al., 2009), and that screening continues to have a relative risk reduction in AAA related deaths of 48% compared to the control group at 10 years. The incremental cost per man recruited to the programme at this stage is £100.

A more recent Danish study calculated the cost per QALY of AAA screening to be £43,500 (Ehlers et al., 2009), and concluded that AAA screening was not cost effective and would probably not be acceptable to health systems valuing a QALY at £30,000 (the UK NHS). Their calculations were based on a computerised analytical model which used data from other studies, and AAA outcome data from the Danish vascular registry. Data from their study suggested that at 5 years after the introduction of screening, the screened group would still have higher aneurysm related mortality compared to the non-screened group. It would be 8 years after the introduction of screening when aneurysm related mortality would be similar between the groups. Their conclusions however were still based on were also based on 5 years of screening, rather than the more recent report of the MASS study which looked at 10 years of screening data. Increased screening time is associated with ongoing potential benefit

from increased survival in the intervention group, and also discounts on screening and service costs. In fact the earlier cost effectiveness analysis of the MASS study (Multicentre Aneurysm Screening Study Group, 2002b) carried out at 4 years of screening showed a QALY as costing £36,000 – not too dissimilar to this study.

1.2.5. Screening for AAA in the US

Routine screening for AAA has been recommended by various organisations in the United States including the United States Preventative Services Task Force, American Heart Association / American College of Cardiology, the Society for Vascular Surgery and Society for Vascular Medicine in the United States (Pande and Beckman, 2008). The prevalence of AAA amongst smokers is up to 5 time that amongst non-smokers (Lederle et al., 2000), a much stronger association than that of coronary heart disease (Lederle et al., 2003). Smoking and AAA also demonstrates a dose dependent relationship, which increases with number of years smoked, and number smoked per day, and decreases with number of years after quitting (Lederle et al., 1997). The US Preventative Task Force modelled the potential benefits of screening based on smoking status and determined that screening a hypothetical group of men aged 65 who were smokers would detect 89% of AAAs in this group (Fleming et al., 2005). Based on this data, the US Preventative Task Force recommend one time duplex screening for AAA in men aged 65-75 years of age who have ever smoked. Despite the possible benefits in mortality reduction in similar aged men who have never smoked, due to the reduced prevalence of AAA, there is no recommendation for screening for AAA in this group. In contrast there is a specific recommendation against screening for AAA in women from the Preventative Services Task Force (Fleming et al., 2005).

The Centres for Medicare and Medicaid Services in the United States have thus agreed to fund one time duplex screening for AAA in men aged 65-75 years of age who have ever smoked, or men or women more than 60 years of age with a family history of AAA (Pande and Beckman, 2008).

1.2.6. The NHS AAA screening programme (NAAASP)

The NHS AAA Screening programme (UK national screening committee, 2009) was announced on 4 January 2008 by the Secretary of State for Health, and has started at early implementation sites. The programme will continue to roll out throughout the UK until 2012/3 at which time it is anticipated there will be up to 60 operational centres, covering all 270,000 men aged 65. The aim of this screening programme is to reduce the mortality from ruptured AAA in the population of men aged 65 and over. It will seek to identify asymptomatic AAA through ultrasonography of all 65 year old men, with older men able to self refer via their general practitioner. All men with aortic diameters below 3cm will be discharged. Ultrasound surveillance will be carried out on all participants with aortic diameters from 3-5.4cm, and participants with aortic diameters 5.5cm or above will be referred to a vascular surgeon.

1.2.7. Methods of AAA detection

Physical examination by doctor

Physical examination by a doctor is the traditional method by which AAAs are detected, however sensitivity & specificity varies between 33-100% (Allardice et al., 1988, Arnell et al., 1996) and 44-100% (Allardice et al., 1988, Cabellon et al., 1983) respectively. The largest study of physical examination to date, involves patients in a general practice setting, and consists of 4171 patients. This quotes a sensitivity of

42%, but has no details of specificity, and has a rather low prevalence of AAA of 0.6% reflecting the fact that a rather young age range was involved - 45-69 years (Zuhrie et al., 1999). A meta-analysis involving 2955 patients with an AAA prevalence of 6.5% despite the age range of 17-90 years, demonstrated a sensitivity of 39%, which improved to 76% in AAAs ≥5cm in diameter (Lederle and Simel, 1999).

We studied the value of doctor and directed self-examination via a simple 4 stage process in a group of patients with known AAA, controlled with a group of patients attending the vascular laboratory for carotid duplex scanning. This showed a sensitivity of 88% on blinded examination by a doctor, and 86% in un-blinded patients following an instructional self-examination sheet. In AAA >5cm the sensitivity of doctor examination was 98%, and patient examination 93%, with specificities 64% and 69% respectively (Venkatasubramaniam et al., 2004c).

1.2.8. Study aim

Our aim was to assess the effectiveness of directed self-examination in the detection of clinically significant (≥5 cm) AAAs in a blinded community population, and to assess the psychological consequences of this study. Our secondary aim was to assess the effectiveness of tandem 2nd person examination in the detection of clinically significant AAAs, and the overall effectiveness of self and tandem examination in the detection of all AAAs (≥3cm).

1.3. Biomechanics & Abdominal Aortic Aneurysms

1.3.1. Biomechanics

Biomechanics is the study of the mechanics of living organisms with the application and derivation of engineering principles.

In order to examine the biomechanics of AAA, it is necessary to understand some of the engineering principles involved in material and structural science.

1.3.1.1. Stress

Stress is a measure of force per unit area. It is normally separated into normal (also called axial) and shear components. Normal stress is defined as the force perpendicular to the cross sectional area of the member divided by the cross sectional area (figure 4, below). It is often symbolised by the Greek letter sigma (σ).

$$\sigma \text{ [normal stress]} = F \text{ [force]} / A \text{ [area]}$$

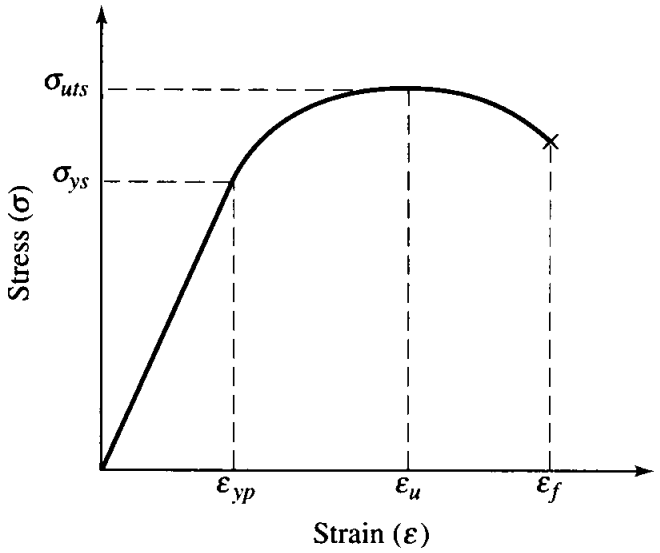
Figure 4. Definition of stress

Axial stress is the equivalent of pressure in a liquid or gas, and the units are force / unit area (Newtons/m^2, also known as pascals (Pa)). As with force, stress cannot be measured directly and is therefore inferred from strain measurements, and knowledge of the elastic properties of the material.

Static fluids can support normal stresses, but will flow if subject to shear stress. In solids, ductile materials will fail under shear stress, and brittle materials will fail under normal stress.

1.3.1.2. Strain

When a load is applied to a material, it experiences a force (or stress when measured per unit area), and deforms. This deformation initially occurs in a reversible fashion which is often nearly proportional, (figure 5, below) up to the yield strength, later in a non-reversible, non-proportional fashion up to the ultimate strength, and finally undergoes fracture. This deformation - the change between initial and final state - is referred to as strain. The actual shape of the stress/strain curve depends on the characteristics of the material involved.



ε_{yp} yield point, ε_u ultimate strain, ε_f fracture, σ_{ys} yield strength, σ_{uts}, ultimate strength

Figure 5. Stress/strain curve

1.3.1.3. Material strength

The definitions of strength are;

- Yield strength: The stress a material can withstand without permanent deformation.
- Ultimate strength: The maximum stress a material can withstand.
- Breaking strength: The stress coordinate on the stress-strain curve at the point of rupture.

1.3.1.4. Deformation

Elastic deformation

The initial deformation of a reversible, manner is referred to as *elastic* deformation. In the aorta this reversible deformation would be due to elastin fibres in the medial layer stretching from its resting random coil structure before returning to a different random coil structure of about the same initial length once relaxed.

Plastic deformation

The second phase of deformation that occurs is referred to as *plastic* deformation where there is non-reversible deformation of the material. In the aorta this would be mainly due to collagen contained in the outer adventitial layer.

1.3.2. Stress Distribution and the AAA

Law of Laplace

Early estimations of AAA wall stress used the law of Laplace, also referred to as the Young-Laplace equation, $\sigma=P*r/t$ where σ, circumferential wall stress; P, intraluminal pressure; r, vessel radius; t, thickness of vessel wall (Stringfellow et al., 1987). This stated that the stress experienced by the wall was proportional to the stress within, the radius (or diameter) of the vessel, and inversely proportional to the thickness of the wall.

However the Law of Laplace was developed to describe the pressure difference over a meniscus between two fluids (Marquis de Laplace, 1806). Thus it assumed a single radius of curvature – which would equate to a sphere or perhaps the longitudinal walls of a perfect cylinder.

The main two problems with using this equation to determine wall stress, is the required assumption of an idealised cylinder for the shape of the aneurysm, and the assumption of axisymmetric geometry, neither of which are true. AAA geometry is complex with many major and minor wall curvatures. In addition, this equation assumes the cylinder involved is thin-walled. Although the walls of most aneurysms probably fall within this category, if intra-luminal thrombus plays a role in altering wall stress, then the equation would be even less accurate. Finally only an estimate of average stress experienced by the arterial wall was produced. A review article by Dobrin (Dobrin, 1989) also quoted studies using the law of Laplace.

Finite element analysis

Finite element analysis (FEA) is an established engineering method of predicting stresses involved in various structures by dividing complex structures into a large number of simpler elements, and allowing these to interact via equations derived from the study of the material properties of the structure. (Mower et al., 1993) first claimed that FEA predicted stress distributions better than aneurysm diameter in hypothetical aneurysms. They assumed linear elasticity for the tissue characteristics i.e. a linear relationship between stress and strain. A few other papers continued to work on this principle, and although results were more reliable than using the law of Laplace, newer studies on aneurysm tissue ex-vivo showed that large strains (20-40%) were experienced by the tissue prior to failure (He and Roach, 1994, Raghavan et al., 1996), demonstrating that the tissue was in fact hyperelastic. Thus a more complex constitutive relation (the stress/strain relationship in the material, or the deformation or stretch that a particular stress produces) was necessary. Yamada et al, using a hyperelastic material model and other parameters initially designed for normal aortic

wall, first carried this out. However the mechanical properties and thus constitutive behaviour was demonstrated to be markedly different for AAA tissue compared to normal aortic tissue (Raghavan et al., 1996, He and Roach, 1994). (Raghavan and Vorp, 2000) then mechanically tested 69 samples of AAA tissue to determine its mechanical properties and constitutive behaviour. This was building on previous work adding 5 longitudinal and 3 circumferential specimens to bring the numbers up to 50 and 19 respectively. Because of their previous assumption that the material was isotropic, they thought it fit to combine the groups to make a single group of 69 specimens. Their main aim was to develop a constitutive model for AAA tissue and determine the effect of variation of aneurysm specific parameters on the aneurysmal wall stress following finite element analysis. They developed a constitutive model which apparently had good agreement with all the experimental results (min $R^2 > 0.9$). They determined that varying the parameters within the 95% confidence interval varied the computed stress up to 4%.

No aneurysm is an ideal cylinder, and most AAAs are not axisymmetric due to a difference in the anterior and posterior support to the aneurysms, ie the vertebral column in posterior support. (Vorp et al., 1998) looked at artificially constructed aneurysms, and varied posterior to anterior diameter (a measure of axisymmetry) and diameter. The results showed that altering the posterior to anterior diameter to 0.3:1 could increase the local wall stress by 85% whilst increasing the diameter from 4 to 8cm would only increase the wall stress at the same area by 33% (figure 6, below). Interestingly peak wall stress increased 137% on increasing diameter from 4cm to 8cm, but only 45% on increasing the asymmetry as above. This was also shown in an earlier study by (Elger et al., 1996).

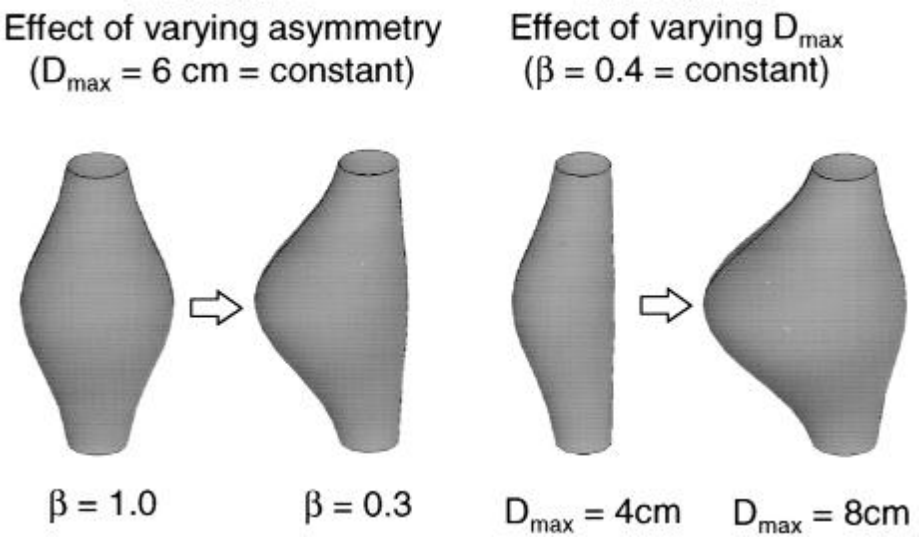


Figure 6. The effect of varying AAA asymmetry versus AAA diameter (Dmax - maximum diameter of AAA, β – ratio of posterior to anterior radii) (Vorp et al., 1998)

Raghavan et al (Raghavan et al., 2000) analysed Spiral CT scans of 7 patients (6 AAAs and 1 control) and reconstructed 3-D models of the aortas to calculate peak wall stress using a finite element analysis method. They found that the 6 aneurysms had a higher peak wall stress than the control aneurysm although no statistical significance was mentioned presumably due to the small numbers involved. They did conclude that volume seemed to correlate best with peak wall stress in this study. Interestingly maximum AAA diameter, AAA height, and systolic pressure all had about the same correlation coefficient (about 0.56), but aneurysm volume had a correlation coefficient of 0.7 with peak wall stress (control patient was removed for this analysis).

Isotropy versus anisotropy

Another element involved in the assumption that peak wall stress sites are the sites most likely to rupture when the critical wall strength is reached is the assumption that

all areas of the wall are the same or similar in strength and the wall is homogeneous. This may not be the case. Initial FEA studies assumed the wall was isotropic in character meaning that the mechanical characteristics are the same in both circumferential and longitudinal directions. However, evidence in general suggests that AAA tissue is anisotropic (mechanical characteristics are different in circumferential and longitudinal directions). Canine abdominal aortic tissue has been proven to be anisotropic (Patel, 1972), but studies have consistently failed to show a statistically significant difference between circumferential and longitudinal tissue elasticity in humans. These tend towards a difference but none has been demonstrated conclusively. (Vande Geest et al., 2006a) demonstrated a significant reduction in circumferential strain in AAA compared to normal abdominal aortic tissue during biaxial testing, suggesting that aneurysm formation increases circumferential tissue stiffness, but still did not conclusively prove anisotropy within AAA tissue. When Raghavan developed his isotropic (mechanical characteristics same in circumferential and longitudinal directions) constitutive AAA model in 2000, he speculated that AAA tissue may be anisotropic, but due to technical considerations, and the desire to simplify things, the model was left with isotropic characteristics (Raghavan and Vorp, 2000). He pointed out the similarity in mechanical behaviour between longitudinal and circumferential samples, but confirmed that he could not prove isotropic behaviour either.

Other studies have demonstrated that the tissue of the normal aortic wall is heterogeneous (Sumner et al., 1970) and bi-phasic – undergoing large strains prior to failure (He and Roach, 1994, Raghavan et al., 1996) after presumably a more conventional, perhaps even linear, initial stress-strain constitutive relationship. Studies

have modelled non-aneurysmal arterial tissue as hyperelastic (Vito and Hickey, 1980, Fung, 1967, Patel and Fry, 1969, Chuong and Fung, 1984, Vorp et al., 1995), some have assumed isotropy (characteristics not directionally dependent)(Vito and Hickey, 1980, Fung, 1967), and others assumed anisotropy(characteristics directionally dependent)(Chuong and Fung, 1984, Vorp et al., 1995). It has also been shown that vascular tissue is incompressible (Carew et al., 1968).

1.3.3. The Formation and Progression of an Aneurysmal Aorta

The formation and progression of aneurysmal growth is linked to plastic (non-reversible change of shape in response to an applied force) deformation of the collagen in the aortic wall. Collagen fibres generally take up excess load placed on the arterial wall after elastin fibres have exceeded their capacity (McMillan et al., 1997). This may be due to extra-physiological forces being applied to the arterial wall (eg with geometry change resulting in localised high stress areas despite physiological blood pressures), resulting in stress being taken up by collagen after the load bearing capability of elastin has been exhausted. It may also be due to the degradation of elastin (eg via increased elastase activity), resulting in lower load-bearing potential for elastin, and collagen being subject to stress at physiological wall stress levels. A combination of the 2 factors is also possible. The concentration of elastin has been seen to decrease, and the concentration of collagen increase in the formation of AAAs (Baxter et al., 1994).

In an EVAR group of 48 successfully excluded aneurysms, a median reduction in aneurysm sac size of 8mm ($p<0.0001$) was recorded at 18 months post-procedure, using regular CT imaging. Interesting in the 16 (n=64) that had persistent sac

perfusion (identified as type 2 endoleak – side-branch), there was no statistically significant difference in diameter of aneurysm sac after 18 months, where an increase might have been expected. Since the numbers involved were small, there may have been a type II statistical error (Resch et al., 1998).

Another study comparing CT and US follow-up of EVAR patients showed successfully excluded aneurysms underwent a median reduction in diameter of 4mm per year (Thompson et al., 1998).

EVAR repair of AAAs also seems to change the length of the aneurysm, which presents a problem in particular to modular stent-grafts – leading to an increased risk of modular dislocation, and stent kinking or distortion (Harris et al., 1999).

1.3.4. The Rupture of an Aneurysmal Aorta

Risk of rupture is related to maximum AAA diameter. This is true simply because maximum diameter is an important component of AAA geometry. It is also true that diameter is not the most important determinant of rupture risk as we know that certain small aneurysms do rupture, and some large aneurysms have been found in autopsy studies that must have been so called "clinically significant" for years, but not ruptured (Darling et al., 1977). Biomechanically, aneurysm rupture is due to gross mechanical failure of the aneurysm wall. This occurs when the mechanical stress on the wall exceeds its failure strength. Thus either increasing levels of stress on the aneurysm wall or a decreasing strength of the wall tissue or both could lead to eventual AAA rupture. Ex vivo studies have shown that the failure strength of aneurysmal wall tissue is lower than normal aortic wall tissue (Raghavan et al., 1996, Vorp et al., 1996b). They demonstrated that failure strength decreases from 1.21MPa in healthy aorta,

down to 0.65MPa in AAA tissue. This would seem to be in agreement with the compensatory process by which AAA forms and grows. The loss of elastin and replacement by collagen, albeit excessive but disordered, wound result in this lower failure strength (Petersen et al., 2000). Rupture would then occur either because of this lower failure strength and/or probably a localised peak wall stress area.

(Raghavan et al., 2000) also calculated that the lower wall stress and higher strength within the wall of a normal aorta results in the peak wall stress being only about 10% of failure strength, whereas an AAA seems to run between 45-69% of its failure strength.

1.3.5. The Future

The mechanical properties of AAA tissue differ in each aneurysm and in each site within a particular AAA. Since ex-vivo destructive mechanical testing is not possible for an intact non-ruptured AAA, it is usually necessary to use average characteristics for AAA tissue. However it has been postulated that as imaging improves, in vivo deformations under physiological stresses can be identified, measured and used to calculate aneurysm specific material properties. This would enable more accurate modelling of specific aneurysms and more accurate prediction of rupture risk in the future.

1.3.6. Finite Element Analysis

Finite element analysis is a method of analysing complex structures by breaking them down into a number of simpler elements. The elements involved can thus be more accurately modelled and analysed in turn, and separate rules governing the interaction of elements put into force thus leading to an equilibrium state. Differential equations

describing the physical problem considered are taken to hold over this region or element, and although this is an approximation, this is more valid then when applied globally over the entire structure. Once the behaviour of these elements is determined, the elements are patched together with specific rules to form the entire region or structure, and this enables an approximate solution for the behaviour of the entire body.

The accuracy of the finite element analysis with the resultant equilibrium state, including stress distribution, peak and low wall stress sites, together with the values for the stresses experienced at these sites are mainly dependent on the quality of the mathematical model for tissue characteristics. Clearly, the information on which the model is based is also important including constraints (character and position), applied pressures, wall thickness, and finally thrombus. There is on-going debate regarding the biomechanical influence of thrombus on the arterial wall. There is some evidence that it protects or buffers the wall against tangential stress. However it may also reduce the wall strength by creating a hypoxic environment or acting as a matrix metalloproteinase reservoir which degrade collagen and elastin.

The approximation for a single region is usually a polynomial equation, this allows for the changing of the variable over the element, and it is an interpolation over the element, with the assumption that the value is known at certain points within the element. These points are often boundary or nodal points

Steps in FEA

- Establishment of stiffness relations for each element. Material properties and equilibrium conditions for each element are used in this establishment

- Enforcement of compatibility – the connection of the elements
- Enforcement of equilibrium conditions for the whole structure
- Assembling – the construction of the system of equations for the entire structure
- Enforcement of boundary conditions
- Solution of the system of equations.

Identifying an area rather than a single point at risk is more realistic, because in a symmetrically shaped aneurysm, the same or very similar peak stress will occur over an area, or even at a number of locations, and small changes in the local geometry, wall thickness or constraint conditions will shift it within that area. On the other hand, a highly asymmetric shape will have a more focussed region of high stress, a steeper local stress gradient, and therefore be relatively insensitive to local variations in the geometry or wall thickness. The surface of a purely cylindrical aneurysm will have a uniform peak stress with all points having the same probability of failure. A finite element analysis of such a cylindrical shape will always identify a single point with the maximum stress because of rounding errors in the post-processing.

1.4. Matrix Metalloproteinases

1.4.1. Background

There are substantial physical and structural obstacles to many processes, both physiological, and pathological, which require the dissolution or at least co-operative permeability of substantial biological barriers, commonly collagenous membranes, or material. These processes include physiologic mechanisms such as the involution of

the female uterus, eruption of teeth, fetal development and pathologic mechanisms such as the progression of cancer, rheumatoid arthritis, and arterial aneurysmal development and progression.

Collagenases

Collagenases are enzymes, which have the ability to cleave intact collagen molecules, and are seen in many tissues, in both in procaryotic, and eucaryotic organisms. Since collagens represent the major structural proteins of all tissues, and are the chief obstacle to cell migration, collagenases play a pivotal role in many processes. These are important in normal development, and also in certain disease states (Grillo and Gross, 1967, Harper, 1980). In normal physiological conditions, collagenases are present in very low quantities in mammalian tissues and are therefore difficult to detect. They are more prominent in connective tissue cell cultures and tissues that undergo rapid resorption (eg involuting uterus)(Cawston and Murphy, 1981). Most mammalian collagenases appear to be metalloproteinases (Seltzer et al., 1977), but collagenases from micro-organisms, and invertebrates come from metalloproteinases, and other classes of proteinases including the serine proteinases, and thiol proteinases (Keil, 1979).

Mammalian collagenases

Mammalian collagenases are zinc dependent enzymes, often also requiring calcium for activation. They are seen in culture media of connective tissue cells (e.g. fibroblasts & chondrocytes). The collagenases are usually latent and require activation. In vitro methods of activation include the addition of mercurial compounds, chaotropic agents (which disrupt the 3-dimentional stability of macromolecules, e.g. Urea 6-8mol/l,

lithium perchlorate 4.5mol/l), short treatments with trypsin or trypsin-related enzymes, or prolonged incubation at 37°C. The nature of the latent collagenase is uncertain; it is unclear whether it is a pro-enzyme which needs proteolytic cleavage, or whether it is complexed with inhibitors that are removed by proteolysis or adding chemical agents (Murphy and Sellers, 1980).

The specificity of mammalian collagenases is very limited. They cleave the collagen triple helix at a point of weakness, three-quarters of the way along from the N-terminal residue. The susceptible bond is the Gly-Leu or Gly-Ile bond (Harper, 1980, Cawston and Murphy, 1981).

Earlier reports that these collagenases do not work on non-collagen proteins are incorrect, as studies have shown proteins like casein undergo limited cleavage, however the products can be difficult to detect as they are not soluble in trichloracetic acid (Murphy and Sellers, 1980). Type I collagen is most rapidly cleaved followed by type III then type II collagens.

1.4.2. History

The first matrix metalloproteinase (MMP) to be discovered was interstitial collagenase (MMP1), which was discovered in 1962 during analysis of the collagen remodelling necessary for tadpole tail metamorphosis (Gross and Lapiere, 1962). It was later found in human skin as 'Human Skin Collagenase' (Eisen et al., 1968). Subsequently further MMPs were discovered, many of which were not limited to collagen as their preferred substrate. These MMPs shared common functional domains, a dependency for zinc, and between them had the ability to degrade all components of the extracellular matrix. These MMPs were numbered according to their order of discovery.

1.4.3. Definitions

Metalloproteinases & Metzincins

There are 4 major classes of proteolytic enzymes, the metalloproteinases, serine proteinases, cysteine proteinases, and the aspartic proteinases. Each class of proteinase has a common mechanism of action dependant upon the amino acid at the active site. Amongst these enzymes, the metalloproteinases are most abundant with over 200 genes listed in the human genome (Sterchi, 2008).

A large number of enzymes seem to require metal ions, with the assumption that the metal ions aid activation of the enzyme or that the action of the enzyme is inhibited by metal chelating agents. For true metalloproteinases however, the metal ion should be at the centre of the active site, and involved in the catalytic event(Hofmann, 1985).

The metzincins are a super family of zinc endopeptidases which account for almost half the metalloproteinases (Sterchi, 2008). These have a highly conserved motif, which contain 3 histidines that bind to zinc at the catalytic site, and a conserved methionine that sits beneath the active site. The metzincins are subdivided into 4 multigene families; seralysins, astacins, ADAMs/adamalysins, and MMPs (Cao and Zucker, 2008).

Matrix metalloproteinases

The matrix metalloproteinases are a family of zinc dependent endopeptidases that have the ability - between them - to degrade all components of the extracellular matrix. More recently it has been recognised that MMPs cleave many other types of peptides and proteins, and have important abilities that may not be related to their proteolytic activity (Overall and Lopez-Otin, 2002). They are initially synthesized in a latent form

known as a zymogen, or ‘pro’ form, and require activation through the removal of a pro-peptide domain and the cysteine switch (Van Wart and Birkedal-Hansen, 1990). Certain sequence homologies are shared within the group. Their activity is regulated by gene expression (controlled by pro- and anti- inflammatory cytokines and growth factors) and also inhibitors, including a matrix metalloproteinase specific family called “tissue inhibitor of matrix metalloproteinases” (TIMPs).

The MMPs are numbered according to their order of discovery (table 1, below), and also grouped according to their substrate specificity;

- Collagenases; degrade only collagen type I to III
- Gelatinases (or type IV collagenases); specificallydegrade basement membrane components, and partially degraded collagen
- Stromelysins; which have a broad substrate specificity
- Matrilysin
- Membrane type MMPs
- “Others” (eg MMP12 macrophage metalloelastase).

However despite this classification most MMPs are not specific for one substrate, and will display activity for a number of substrates. Their distinct but overlapping substrate specificities lead to a lack of clear phenotypes in MMP knockout mice with lack of specific MMPs. For example, mice deficient in MMP14 (MT1-MMP), whose substrates include collagen I, II, III, gelatin, MMP2 and MMP13, demonstrate dwarfism, arthritis, osteopenia, and craniofacial dysmorphism suggesting the importance of soft connective tissue modelling, and collagen turnover by resident cells

in the development and maintenance of the hard tissues of the skeleton (Holmbeck et al., 1999). MMP9 (gelatinase B) deficient mice demonstrate abnormal skeletal growth plate vascularisation and ossification, with progressive lengthening of the growth plate to about 8 times normal. Transplantation of wild-type bone marrow cells rescues vascularisation and ossification suggesting that these processes are mediated by Gelatinase-B expressing cells of bone marrow origin, designated chondroclasts (Vu et al., 1998).

Table 1. The major MMP subtypes and their substrates

MMP	Enzyme	Principal substrate
Collagenases		
MMP1	Collagenase-1 (Interstitial collagenase)	Collagens I, II, III, VII, VIII, X, gelatin, aggrecan, MMP2 and 9
MMP8	Collagenase-2 (Neutrophil collagenase)	Collagens I, II, III, V, VII, VIII, X, gelatin, aggrecan
MMP13	Collagenase-3	Collagens I, II, III, IV, gelatin, aggrecan, PA12
MMP18	Collagenase-4 (Xenopus collagenase)	Collagen I
Gelatinases		
MMP2	Gelatinase A (72 kDa gelatinase)	Gelatin, collagens I, IV, V, VII, X, XI, XIV, elastin, Fibronectin, aggrecan
MMP9	Gelatinase B (92 kDa gelatinase)	Gelatin, collagens IV, V, VII, X, elastin
Stromelysins		
MMP3	Stromelysin-1	Collagens III, IV, IX, X, gelatin, aggrecan, MMP1, 7, 8, 9, 13
MMP10	Stromelysin-2	Collagens III, IV, V, gelatin, casein, MMP1, 8
MMP11	Stromelysin-3	Gelatin, Collagen IV, fibronectin, casein, proteoglycans
Matrilysins		
MMP7	Matrilysin-1 (PUMP-1)	Collagen IV, X, fibronectin, gelatin
MMP26	Matrilysin-2	Collagen IV, fibronectin, gelatin, proMMP9, fibrinogen
Membrane type MMPs		
MMP14	MT1-MMP	Collagens I, II, III, gelatins, MMP2 and 13
MMP15	MT2-MMP	MMP2, gelatin
MMP16	MT3-MMP	MMP2
MMP17	MT4-MMP	Gelatin, proMMP2
MMP24	MT5-MMP	Proteoglycans, proMMP2, collagen I, gelatin, fibronectin, laminin
MMP25	MT6-MMP	Collagen IV, proMMP8, proMMP9
Others		
MMP12	Macrophage metalloproteinase	Collagen IV, gelatin, elastin, fibronectin

MMPs have a complex range of targets / effects which extend beyond classical remodelling. They are known to precisely regulate the action of bioactive molecules by proteolytic processing. MMPs also regulate cell-surface receptor cleavage and release, cytokine activation and inactivation and release of apoptotic ligands (Egeblad and Werb, 2002). These cellular processes regulated by MMPs therefore may have tumour growth promoting potential (proliferation, adhesion, dispersion, migration, differentiation, angiogenesis, apoptosis, and host defence evasion), and some will suppress normal tissue function and host defence mechanisms. Thus the effect of MMP suppression is not straightforward. Some studies have suggested that MMPs may play a pivotal role in early disease despite the classical idea that their role is in disease progression and malignant metastases (Egeblad and Werb, 2002, Coussens et al., 2002).

1.4.4. Structure

Several highly conserved domains are common to various MMPs (figure 7, below)

- The signal peptide domain directs the MMP towards the secretory or plasma membrane insertion pathway.
- The prodomain, found in the zymogen, has the highly conserved sequence PRCGVPNPD. The cysteine residue within this sequence coordinates with the zinc ion within the active site, occupying the active zinc site, and therefore conferring latency to the enzyme by making the catalytic enzyme inaccessible to substrates. This is cleaved during activation of the MMP, the so-called cysteine switch.

- The catalytic domain contains the zinc-binding area, called the metal ion binding domain (MBD). Another highly conserved sequence HExGHxxGxxH is found in this domain in all MMPs. It contains 3 histidine residues which interact with the zinc ion.
- The C-terminal domain has structural similarities to the serum protein hemopexin, and is therefore often termed the 'hemopexin domain'. It has a 4-bladed B-propeller structure. B-propeller structures provide a large flat surface, thought to be involved in protein-protein interactions. The hemopexin domain determines the specificity of the enzyme through mediating interactions with substrates, and this is also the site for interactions with TIMPs. Certain MMPs do not have this domain including the short MMP7, and another MMP in the Matrilysin subtype, MMP26 (or matrilysin 2), together with MMP23a and MMP23b.
- The hinge region links the catalytic and hemopexin domains.

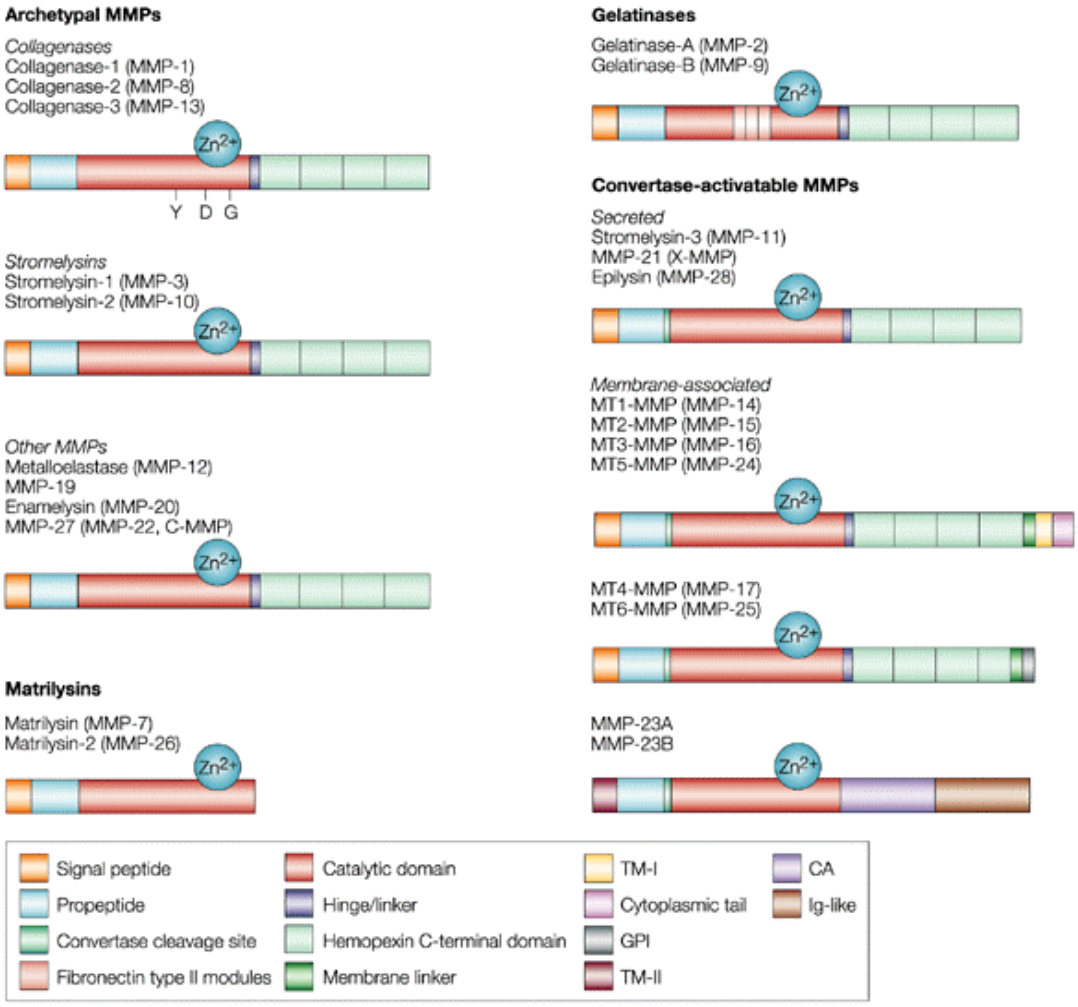


Figure 7. The structure of MMPs

(University of British Colombia website:

http://www.clip.ubc.ca/archive/mmp_timp_folder/mmp_schematic_master.gif)

Key: Y tyrosine, D aspartic acid, G glycine – amino acids that are present in the catalytic domain of all collagenases.

Certain differences exist, for example the smallest MMP (MMP7), lacks the hemopexin domain, however it still has substrate specificity. The membrane type MMPs (MT-MMP) contain an additional 20 amino acid trans-membrane domain, and a small cytoplasmic domain (MMP14, MMP15, MMP16, MMP24), or a glycosylphosphatidyl inositol linkage (MMP17, MMP25), which attaches these proteins to the cell surface. MMP2 and MMP9 (gelatinases), contain fibronectin-like domain repeats, which aid in substrate binding(Strongin et al., 1993).

1.4.5. Control of MMP activity

The activity of MMPs is tightly controlled in vivo at several levels.

- Transcription is tightly regulated in both directions by cytokines and growth factors including interleukins (IL-1, IL-4, IL-6), transforming growth factors (EGF, HGF, TGFβ), or tumour necrosis factor alpha (TNFα). Of these, IL-1, PDGF, TNF-α are known to stimulate or induce MMP synthesis, and TGF-β, heparin and corticosteroids have an inhibitory effect. Some of these can be activated or inactivated proteolytically by MMPs (feedback effect). The relationship between cytokines and MMP and TIMP expression is complex (Wang et al., 1996).
- Latency is conferred by the prodomain group occupying the active site zinc in the newly synthesized zymogen.
- Activation of the MMP depends on disruption of the interaction between the prodomain and the active site. This may occur through conformational changes, or proteolytic removal of the prodomain. In vivo the actual mechanism of activation is poorly understood. Certain MMPs (MMP11, MT-

MMP, MMP28) contain furin-like recognition domains within the propeptides, and can be activated in the trans-Golgi network by members of the subtilisin family of serine proteases. MMP14 plays a role in the cell surface activation of MMP2 from pro-MMP2. Other protease groups, such as the serine protease plasmin can activate extracellular secreted MMPs. This suggests some co-operation between groups of enzymes in remodelling of the extra-cellular matrix. Some active MMPs can activate other pro-MMPs, eg MMP3 (stromelysin) can activate MMP9 and MMP1, and in fact proteolytic activity is potentiated 5-8 times by cleavage of collagenase by stromelysin at the carboxy terminal. It has been suggested that there are similarities between this system and the coagulation cascade as for example activation of stromelysin by plasmin results in further activation of other MMPs with potentiation of effect as detailed above (Dollery et al., 1995).

- Post activation control of MMPs may be achieved by endogenous inhibitors, autodegradation, and selective endocytosis. MMP2, MMP9, and MMP13 have been found to undergo endocytosis via a low density lipoprotein receptor related protein mechanism(Yang et al., 2001). MMP9 is also able to bind to the cell surface following cell secretion, and here it is somewhat protected from local inhibitors.

- Tissue Inhibitors of MMPs (TIMPs) are a 4-member-family of homologous MMP inhibitors (TIMPs 1-4) (figure 8, below). The N-terminus of all 4 TIMPs bind to the catalytic domain of most activated MMPs therefore inhibiting function. The C-terminus of TIMP1 and TIMP2 bind to the hemopexin domain of pro-MMP2 and pro-MMP9 respectively; this also regulates MMP function

(Cao and Zucker, 2008). TIMPs are found in much higher concentrations than MMPs in tissue and extracellular fluid thus limiting MMP activity to focal peri-cellular areas. Other, more complex interactions of TIMPs have also been discovered eg low concentrations of TIMP2 enhance MMP14 activation of MMP2 by forming a triplex on the cell surface. Growth promoting activities of TIMPs, independent of their MMP inhibiting actions, have also been observed, and TIMP-3 has apoptosis-inducing properties. Control of TIMPs is similar to MMPs, with cytokines and growth factors promoting TIMP transcription eg TGFβ, TNFα, IL-1, IL-6.

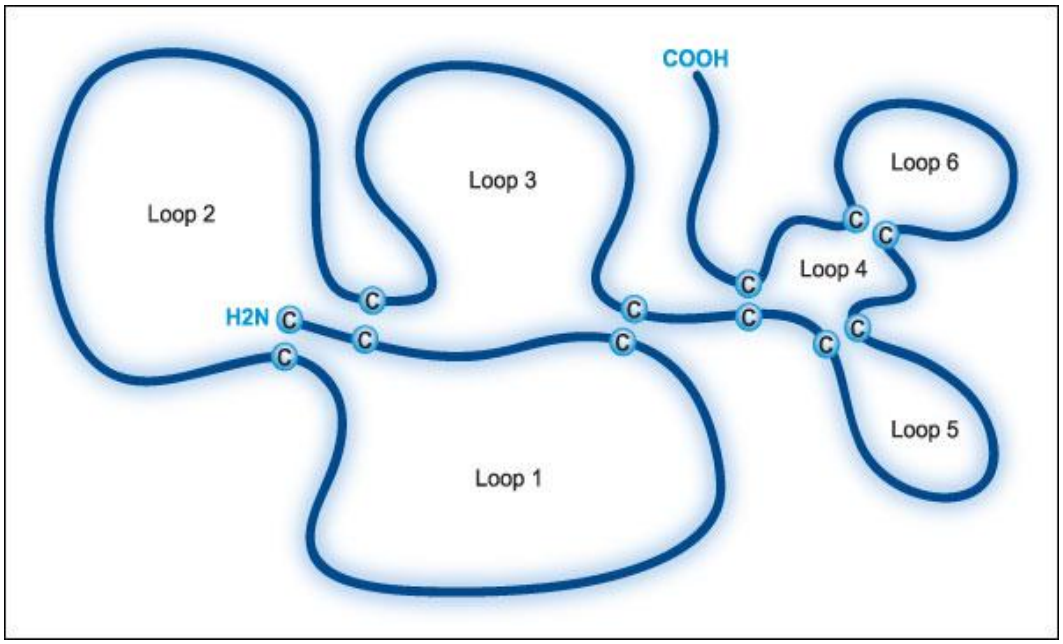


Figure 8. Schematic representation of TIMP1 structure (www.abcam.com)

- Other inhibitors of MMPs include the endogenous factor plasma protein α_2-macroglobulin. This is a large plasma protein, part of the α_2 band in protein electrophoresis, and is produced by the liver. Its effects are limited by the large size of the molecule. It is able to inactivate a wide variety of proteinases

including serine, cysteine, aspartic and metalloproteinases. It functions by attracting proteinases to a 35 amino acid 'bait' region, and when the proteinases binds and cleaves the region, it becomes bound to α_2-macroglobulin, forming a complex which can be recognised by macrophages, and removed. A study of MMPs in diseased aorta tissue found that gelatinase activity increased 3-5 fold after the destruction of α_2-macroglobulin with potassium thiocyanate (KSCN) (Vine and Powell, 1991).

- exogenous factors such as heparin.
- a surface inhibitor, the Reversion-Inducing Cysteine rich protein with Kazal motifs (RECK inhibitor), is a membrane-anchored glycoprotein, which is vital for normal vasculogenesis, and has been found to inhibit membrane type 1 MMP (MMP14), MMP2 and MMP9. Down regulation of RECK has been implicated in tumour angiogenesis and progression (Span et al., 2003). The 'Kazal Motif' refers to three pairs of conserved cysteine residues (Morris and Carruthers, 2003). The figure below demonstrates schematically TIMP1. The N-terminus of all 4 TIMPs bind to the catalytic domain of most activated MMPs therefore inhibits function. The C-terminus of TIMP1 and TIMP2 binds to the hemopexin domain of proMMP2 and proMMP9 respectively; this also regulates MMP function (Cao and Zucker, 2008).

1.4.6. Natural history

MMPs are generally produced locally, with stimulation by local factors, but seepage into the blood stream accounts for changes in serum or plasma MMP concentration (Zucker et al., 1995).

Studies have shown that levels of MMP9 increased 30-fold after healthy volunteers were injected low dose bacterial lipopolysaccharide to simulate endotoxin in septic shock. These levels were achieved within 2 hours, but fell within 4-6 hours suggesting rapid inactivation through some yet unidentified mechanism. The rapid release of MMP9 was presumably due to storage in neutrophils. MMP2 levels in contrast did not alter dramatically (Albert et al., 2003). In further studies on septic shock, plasma MMP9 levels were noted to be significantly higher in non-survivors compared to survivors or control patients (Nakamura et al., 1998).

1.4.7. Natural occurrences

The MMPs are involved in normal physiological remodelling, such as occurs during tissue development and repair in wound healing, ovulation, bone and growth plate remodelling, post-partum involution of the uterus, and embryonic development (Zucker et al., 1992a). These processes involve cell motility, the release of growth factors bound in tissues, and remodelling of the extracellular matrix (ECM).

1.4.8. Pathological associations

Over-expression and activation of MMPs, or an imbalance of active MMPs and inhibition by TIMPs has been associated with disease states involving the breakdown and remodelling of the extracellular matrix eg, rheumatoid arthritis, periodontal disease, tumour invasion & metastasis, and vascular diseases such as atherosclerosis, angiogenesis and aneurysms. It may also play a part in Alzheimer's disease. The more recent recognition of roles other than that of matrix degrading enzymes for the MMPs, and inhibitors of MMPs for the TIMPs, is leading to the exploration of fields not previously visited for these enzymes.

Some uncertainly remains regarding the role of MMPs in disease – as to whether it is part of the disease process, and initiates tissue damage, or indeed whether it is part of the repair mechanism.

In a similar way, the increased production of TIMPs may be a response to MMPs in an attempt to inhibit protease function, but may also be independently produced with other effects in mind (Zucker et al., 1999).

As regards to the timing of MMPs in a particular situation, stromelysin-3 (MMP11), and matrilysin (MMP7) are released early in tumour development (colonic adenomas and breast carcinoma in situ), prior to the development of metastatic potential and this suggests that these MMPs are not involved in metastasis (Zucker et al., 1999).

There has been interest for a long time on MMPs in cardiovascular disease, mainly in 4 areas, the role of MMPs in development of atherosclerotic plaque, aneurysms, release of smooth muscle cells after angioplasty, and the role of MMPs in cardiac failure.

1.4.8.1. Atherosclerosis

Atherosclerosis relates to the build-up of intimal atheroma and one of the earliest events to occur in process is the adhesion of circulating monocytes to the vascular endothelium. Cellular migration has been studied in rat vascular SMC (smooth muscle cell), and MMP2 (gelatinase A) has been determined to be necessary for cells to cross the basement membrane layer (composed of type IV collagen) (Pauly et al., 1994).

Commonly an atherosclerotic plaque consists of a thickened intimal layer and an asymmetrical lesion that encroaches into the lumen. These lesions can be fibrous or consist of a lipid core with a fibrous cap. They contain mainly macrophages and SMC (smooth muscle cells), but there are also “foam cells” frequently seen - macrophages

containing lipid within cytoplasm, and giving these cells a foamy appearance. Levels of MMP, particularly MMP9 (gelatinase B), MMP3 (stromelysin-1), and MMP1 (interstitial collagenase) were over-expressed in areas of foam cell accumulation in coronary atherosclerotic lesions (Galis et al., 1994). The lipid cores are often unable to bear the circumferential tension from systole, and redistribution of the stress to the fibrous cap occurs. Regions of high stress may develop (Richardson et al., 1989), and certain factors contribute to an increase in stress across these caps including the loss of content and structure of the connective tissue matrix, and increase in the size of the lipid core (Davies et al., 1993c). It has been postulated therefore that the most common event initiating coronary thrombosis is intimal tearing, which occurs in regions of cap weakness. This is correlated with accumulation of macrophages and seems to be found in the shoulders of the plaque. Although there is no direct evidence of association of MMP activity with increased risk of vessel occlusion, MMP9 (gelatinase B) has been shown to be elevated in arterectomy samples from patients with unstable angina compared to patients with stable angina (Brown et al., 1995).

MMP in atherosclerotic plaques has been shown to be localised to smooth muscle cells, and macrophages by in situ zymography. In situ hybridization has indicated that the MMPs are also produced by these cells (Henney et al., 1991, Galis et al., 1994). MMP3 (Stromelysin) mRNA transcripts have been localised to macrophages and SMC in fibrous and lipid filled atherosclerotic plaques through examination of frozen sections. Since the accumulation of large numbers of macrophages and foam cells in the arterial wall is not normal, it is reasonable to assume that the production of these MMPs is also pathological. In contrast, radioimmunoassay has demonstrated that TIMP1 is normally present within the matrix of aortic wall (Brophy et al., 1990). This

further raises the possibility that the fine balance between MMPs and TIMPs is disrupted in certain pathological situations. Vine and Powell demonstrated that MMP9 (gelatinase B) was raised in homogenates made from the luminal aspect of atherosclerotic but not normal aortas (Vine and Powell, 1991). It is postulated therefore that certain cytokines (eg TNF-α) induce the production of MMP3 (stromelysin) and other MMPs in response to a localised area of mechanical stress, and this triggers the cascade-like response resulting in plaque rupture and vascular occlusion (Dollery et al., 1995). High levels of TIMP1 and MMP9 have been located at the vasa vasorum (which may be involved in the maintenance and genesis of the plaque) of human AAA specimens examined with Immunoprecipitation (Herron et al., 1991).

In porcine coronary arteries there was a higher intrinsic gelatinolytic activity and rapid cell outgrowth in the adventitia, whereas the media had slower cell outgrowth, with a preferential expression of TIMPs. The impairment of TIMP synthesis may thus contribute to the pathogenesis of coronary lesion formation (Shi et al., 1999).

Angina

In a study looking at unstable and stable angina patients, both groups had positive staining for MMP9 (83% of each group), but interestingly 10/10 patients in the unstable angina group but only 3/10 patients in the stable angina group had intracellular localisation of MMP9 suggesting more active synthesis in the unstable angina group, and the potential contribution of MMP9 to acute coronary ischaemia, perhaps through degradation of matrix and rupture of atherosclerotic plaque(Brown et al., 1995).

Myocardial infarction

At the DNA level a single nucleotide polymorphism (SNP) for MMP9 has been shown to be associated with the level of severity of coronary atherosclerosis, but no association with myocardial infarction. This study looked at 584 male patients with myocardial infarction and compared genotypes with 645 age-matched healthy male controls. No significant difference was found in genotype, however on sub-group analysis, in 374 patients with available angiographic data, 26% with the SNP had >50% stenosis in 3 coronary vessels compared to 15% of patients without the sequence variation (Zhang et al., 1999).

Another study used sandwich ELISA to demonstrate an increase in MMP1 in infarcted heart tissue from coronary heart disease patients compared to heart tissue from donor patients (75 +/- 11 ng/mg vs 9 +/- 2ng/mg, $P<0.01$), and a decrease in TIMP1 (12 +/- 5 ng/ml vs 37 +/- 8ng/ml, $P<0.001$). Northern blot analysis in the study showed that mRNA levels for both MMP1 and TIMP1 were elevated 3-4 fold in the infarcted tissue, suggesting up-regulation of MMP and TIMP gene transcription following infarction, however since the level of TIMP1 is actually decreased, post-translational regulation of TIMP1 was assumed. This study therefore suggests that post-translational modification was important in this situation. Other bands seen during zymographic analysis were at 66kDa and 92kDa and were thought to be MMP2 and MMP9 respectively, although it described the probability of part of the 66kDa band belonging to plasmin, and hence perhaps tPA converting plasminogen to plasmin, in turn activating MMPs and inactivating TIMP1 post-translationally (Tyagi et al., 1996).

During myocardial infarction, MMP9 deficient mice seem to be partially protected against ventricular enlargement, collagen accumulation, and cardiac rupture, which can

all complicate myocardial infarction. Mice deficient in urokinase-type plasminogen activator were completely protected against cardiac rupture, but showed impaired scar formation and infarct revascularisation, even after treatment with vascular endothelial growth factor. These mice died of cardiac failure due to depressed contractility, arrhythmias and ischaemia. However temporary TIMP1 administration to wild-type mice completely prevented cardiac rupture and did not abort infarct healing (Heymans et al., 1999). Mice lacking one MMP9 allele showed a reduction in ischaemia reperfusion induced expression of pro-MMP9(78%), and active MMP9(69%) compared to wild-type mice after the LAD coronary artery was occluded for 30 minutes followed by 24 hours of reperfusion. TIMP1 was elevated 4.7 fold in these mice. Immunohistochemical methods revealed that neutrophils were the primary source of MMP9, and less neutrophils were seen in the ischaemic region of the heart following ischaemia reperfusion injury but only in mice with both alleles of MMP9 absent compared to wild-type mice. Myeloperoxidase activity (a marker enzyme of neutrophils) also demonstrated a reduction (44%) in neutrophils infiltrated into the ischaemic myocardium in MMP9 absent mice compared to wild-type mice (Romanic et al., 2002).

1.4.8.2. Post-angioplasty restenosis

Ischaemic heart disease is a major cause of mortality and morbidity in the UK, and percutaneous transluminal coronary angioplasty is widely used in its treatment. A balloon catheter is used to compress an atheromatous narrowing into the vessel wall. The initial success rate is >80%, but unfortunately up to half these patients have recurrence of their symptoms within 6 months due to restenosis at the same site (Landau et al., 1994, Editor the Lancet, 1987). This is due to migration and rapid

proliferation of SMC at the 'site of injury' causing the pattern of fibrocellular intimal hyperplasia (Waller et al., 1990). Also adding to the problem is that a mature angioplastied lesion is associated with increased extracellular matrix, and that the overall dimensions of the vessel change (Glagov, 1994). Studies on a rat model have suggested that certain growth factors contribute to the proliferation of extracellular matrix; fibroblast growth factor controlling SMC replication, and PDGF (platelet derived growth factor) controlling cell migration. Intimal hyperplasia is present to some extent in all cases after angioplasty, and restenosis is thought to be an exaggeration of this effect. Restenosis can be remarkably resistant to treatment, but manipulation of the healing process of the vessel wall may prevent accumulation of occlusive neointima.

Mechanical damage has been shown to stimulate gene expression of collagenase, and stromelysin in vascular SMC (James et al., 1993), this supplies a possible "on-off switch" for post-injury stenosis. Studies of rat carotid artery has demonstrated a sequence, with MMP9 expressed the day after angioplasty, and expression of gelatinase A 4-5 days later (Bendeck et al., 1994). The authors speculated that MMP9 may induce the migration of SMC from the media to the intima. There is some evidence of up-regulation of plasminogen activator after balloon injury in both rat and rabbit models, which may activate the MMP cascade (Clowes et al., 1990, More et al., 1995, Tyagi et al., 1996). Porcine studies also show initial induction of MMP9 (day 3), followed by gelatinase A (day 7) on zymography, with elevation of levels until day 21 (Southgate et al., 1996). Administration of a systemic matrix metalloproteinase inhibitor resulted in a 97% reduction of early migration of SMC into the intima. Although this may have potential for treatment of post-injury stenosis lesions, arresting

migration itself may not in fact lead to a reduction in stenosis since compensatory proliferation may indeed itself cause restenosis, and 80% of restenosis lesions are made up of connective tissue rather than SMC.

1.4.8.3. Abdominal Aortic Aneurysms

In 1991, Vine and Powell found elevated gelatinase activity in atherosclerotic and aneurysmal aortas highest in at luminal portion of the aortic media. The gelatinase was principally 92kDa in atherosclerotic aorta but covered a spectrum from 55 to 92kDa in aneurysmal aorta in zymographic studies. α_2-macroglobulin removal by potassium cyanate resulted in a 3-5 fold increase in gelatinase activity. Collagenase and stromelysin was also tested for and found via immunoblotting in aneurysmal aorta, rarely in atherosclerotic aorta and never in control aorta. Activities of these were low, but increased after destruction of TIMP by the order of 2-3. The highest activities were found on the adventitial aspect of the aortic media in contrast to gelatinase (Vine and Powell, 1991).

Thompson et al used zymography and immunohistochemical methods and found elevated levels of MMP9 in the aortic wall tissue samples of athero-occlusive disease, but the most elevated levels in aortic aneurysmal wall tissue samples. These localised to macrophages within the damaged wall of aneurysmal aortas suggesting chronic release of this MMP contributing to the degradation of the extracellular matrix (Thompson et al., 1995).

Further studies showed high levels of MMP2 in smaller aneurysms (4.0-5.5cm) through zymography & immunoblotting, but reducing levels in larger aneurysms, with an increasing activity of MMP9. Tissue homogenates prepared from both groups

showed a similar total activity against gelatin or type IV collagen. The concentration of MMP2, determined by immunoassay was highest for small aneurysms; median concentrations 385, 244, 166ng/mg protein for small aneurysms, large aneurysms and atherosclerotic aorta respectively. Immunolocalisation studies demonstrated concentrations of MMP2 along fibrous tissue of the acellular medial layer, and the atherosclerotic plaque. The recruitment of inflammatory cells into the adventitia, with subsequent higher levels of metalloproteinases (including MMP9) may contribute to the rapid growth and rupture of larger aneurysms. MMP9 has therefore been suggested to be an important factor in the transition to large aneurysm (Freestone et al., 1995).

Sakalihasan et al, studied gelatinase (MMP2 and 9) activity by examining samples of aortic wall, thrombus and serum in 10 patients with AAA, and 6 aged-matched controls. Thrombus was found to contain up to twenty times more gelatinase activity than serum (luminal thrombus twenty-fold activity, parietal thrombus ten fold activity), with the predominant found as MMP9. Although total gelatinase activity was in the same range in AAA and control tissue, a significantly higher proportion of MMP9 was found in the aneurysmal wall samples. Also a significant proportion of MMP9 was in its active form, which was not seen in control samples. A higher proportion of active MMP2 was also seen in aneurysmal wall samples (Sakalihasan et al., 1996).

MMP9 mRNA has also been shown in higher levels in AAA than normal aortic tissue (0.855 +/- 0.180 vs 0.046 +/- 0.23, $P<0.02$), with aorto-occlusive disease in between at a non-significant difference from either. The mRNA localised to adventitial macrophages in areas of neovascularisation (McMillan et al., 1995).

Davis et al investigated the levels of MMP2 and 9 mRNA, and protein, and found significantly higher levels of MMP2 mRNA and protein levels in AAA tissue compared to aortic tissue from patients with aorto-occlusive disease (AOD) or non-diseased control tissues. Protein and mRNA levels for MMP9 however were raised in both AAA and AOD samples compared to control tissue, but there was no significant difference between AAA and AOD samples (Davis et al., 1998). Analysis of MMP mRNA levels was by quantitative competitive reverse transcription-polymerase chain reaction (QCRT-PCR) and by gelatin zymography.

Pyo et al as part of Rob Thompson's St Louis group used MMP9 and MMP12 deficient mice, together with wild-type mice, and experimentally induced aneurysms using a porcine pancreatic elastase aortic infusion at laparotomy, with a control group being infused heat-inactivated porcine elastase. In the wild-type mice, there was an increase in the aortic diameter of both control and elastase groups immediately after the infusion (at least 5 minutes after restoration of lower limb perfusion), but the difference between groups was statistically significant (mean percentage change in aortic diameter: 54 +/- 3% vs 74 +/- 5% $P<0.05$). There was no further significant change in aortic diameter until measurement at 14 days when 91% (21 out of 23) of elastase infused mice had developed aneurysms (arbitrary study definition set at aortic diameter increase of 100%). None of the wild-type control group developed aneurysms (mean percentage change in aortic diameter: elastase group 134 +/- 8% vs control group 41 +/- 6%, $P<0.05$). A subgroup was treated with doxycycline (known to be a non-selective MMP inhibitor)(n=8) for 14 days, and this showed a significant reduction in change in aortic diameter compared with non-doxycycline treated wild-type mice(n=15) at the end of the fourteen days(89 +/- 17% vs 134 +/- 9%, $P<0.05$),

and 50% versus 93% reached study aneurysm threshold. MMP9 and double deficient MMP9/12 mice both had experienced less aortic dilatation compared to the wild type mice when these were exposed to elastase infusion (87 +/- 10% vs 79 +/- 9% vs 134 +/- 8%, significant difference between either deficient group and wild-type mice, $P<0.05$). The number of mice developing aneurysms was 40%, 20%, and 94% respectively. MMP12 deficient mice however were not significantly different to wild-type mice with an increase of aortic diameter of 134 +/- 7%, and an aneurysm development rate of 100%. Gelatin zymography demonstrated only level of MMP2 prior to infusion of elastase, but levels of MMP2 and 9 immediately after infusion, and when animals were sacrificed on days 2, 7 and 14. Reverse-transcriptase PCR with Southern blot demonstrated elevated levels of MMP9 and 12 on days 2, 7 and 14. When these same tests were done on MMP deficient mice, the results were as expected with no MMP9, 12 or both as per genotype. Interestingly there was no 'compensatory' increase in any other elastolytic MMP (MMP2, 9 or 12) in the absence of MMP9 or 12 or both. In the final arm of the study, wild-type mice, and MMP9 deficient mice were subject to lethal irradiation and underwent bone marrow transplantation 6 hours later with either bone marrow from wild-type mice or MMP9 deficient mice, giving 4 groups. 10 weeks later, elastase infusion was carried out as above, and the wild-type mice that had bone marrow transplantation from the MMP9 deficient group had significantly smaller aortic diameter increases than those transplanted with bone marrow from wild-type mice. In a similar fashion MMP9 deficient mice that had bone marrow transplantation from the MMP9 deficient group had significantly smaller artic diameters than mice with bone marrow transplants from wild-type mice (table 2, below). This suggests that the important factor in aneurysm promotion is expression

of MMP9 in infiltrating inflammatory cells rather than developmental abnormalities in aortic wall structure or the absence of MMP9 expression by resident aortic wall cell types (Pyo et al., 2000).

Table 2. Effects of bone marrow transplantation on elastase-induced aneurysmal dilatation

			Mouse aortic diameter (mm)		
Donor	Host	n	Pre-elastase	Post-elastase	14 days
Wild-type	Wild-type	7	0.50 +/-0.01	0.83 +/-0.02	0.90 +/-0.04
MMP9(-/-)	Wild-type	10	0.52 +/-0.01	0.87 +/-0.02	0.79 +/-0.02[c]
MMP9(-/-)	MMP9(-/-)	9	0.52 +/-0.01	0.83 +/-0.02	0.78 +/-0.02
Wild-type	MMP9(-/-)	12	0.50 +/-0.01	0.87 +/-0.02	1.00 +/-0.03[c]

Pyo et al., 2000. [c] $p<0.05$, student's t test

Traditionally, elastases (including MMP2 & 9) were thought to have a role in the formation and growth of AAAs. More recently MMPs with collagenase activity (including MMP8, 13) are thought to contribute to AAA rupture. Complicating the issue is the fact that most MMPs have more than 1 function. For example, MMP9 is principally an elastase, but is also known to have collagenase activity on partially degraded forms of collagen.

A study carried out in 2002, demonstrated that ruptured AAAs have a higher level of tissue MMP9 than non-ruptured AAAs. The same paper, also showed that larger AAAs had a higher level of MMP2 compared to smaller AAAs (Petersen E et al., 2002). Another study found a localised tissue increase in MMP8 at the site of rupture compared to the arteriotomy site within the same aneurysm (Wilson et al., 2004). Interestingly this study looked at the other MMPs which were considered to be

collagenases (MMP1 & 13), and found no difference in those between rupture and arteriotomy sites.

In summary then, studies dating back to 1994 demonstrated elevated tissue levels of MMP2 and 9 in human AAA, and these were thought to be implicated in the formation of AAAs. The MMPs involved were elastases, and the mechanism of aneurysm formation and growth was thought to be the loss of elastase in the medial wall of the aorta. The importance of these MMPs was confirmed by the studies in gene manipulated mice, where genetic deficiencies in the genes coding for MMP2 and 9 resulted in the rapid formation of aneurysms. More recent studies have shown elevated tissue levels of MMP8 at sites of aneurysm rupture compared to paired samples taken from an arteriotomy site in the same aneurysm. However it is not certain whether the elevated levels of MMPs predate and contribute to rupture, or whether indeed they are a result of the actual process of AAA rupture.

Blood

Plasma MMP9 has been found to be elevated in AAA patients(n=22) compared to aorto-occlusive disease(AOD)(n=9) or healthy controls(n=8) (86 +/-12ng/ml vs 26 +/-4ng/ml($P<0.001$) vs 13 +/-2ng/ml($P<0.001$). The healthy controls were organ donors, and thus there was a significant age difference between the groups (mean age 73yrs(AAA), 61yrs(AOD), 35yrs(healthy). It is plausible that these age differences may account for some of the variation in MMP levels. Tissue explants from patients were also cultured (smaller groups less difference in mean age between groups), and supernatants taken from these cultures at 48hrs were analysed for MMP9. These showed significantly higher levels of MMP9 from diseased aorta (combination of both AAA and aorto-occlusive groups) compared to normal aorta, suggesting that the aorta

was the source of the MMP9. In an attempt to counter controversy regarding the age effect in the three patient populations, the authors went on to demonstrate higher levels of plasma MMP9 in patients with multiple aneurysms in comparison to patients with an isolated infrarenal aortic aneurysm (McMillan and Pearce, 1999). They suggested that the extent rather than the size of an aneurysm might have a direct relationship with the level of plasma MMP9.

Lindholt et al suggested that plasma MMP9 and plasma antitrypsin may predict the natural history of patients with aneurysms after demonstrating that increased plasma MMP9 levels were significantly related to the size and expansion of small AAAs, and plasma antitrypsin was significantly associated with AAA expansion. This group also found a difference between serum and plasma levels of MMP9, which they attributed to release of MMP9 from platelets (Lindholt et al., 2000b).

Open and endovascular surgery for AAA has been shown to reduce plasma MMP3 and 9 levels to less than half their original level. There was also an interesting and significant difference in these levels in patients with and without endoleak following EVAR (MMP9: 44.3+/-20.7 vs 14.6+/-7.0ng/ml, 2P<0.005; MMP3: 25+/-11.5 vs 10.3+/-5.4ng/ml, 2P<0.005) (Sangiorgi et al., 2001). These findings highlighted the potential for MMP levels to be used as an indicator for the success or otherwise of individual EVAR procedures.

Wilson et al (Wilson et al., 2008) studied MMP and TIMP1 levels in patients with elective (n=52) and ruptured AAA (n=16) and found significantly higher levels of MMP1 (median 8.9, [IQR 5.57-15.69] vs 20.18 [16.09-28.68], P<0.001) and MMP9 (17.54 [10.3-34.24] vs 59.11 [20.82-123.70], P=0.006), in the ruptured AAA group.

1.4.9. Specific Metalloproteinases

1.4.9.1. Matrix Metalloproteinase 2 (Gelatinase A)

Matrix metalloproteinase 2 is a 72 kDa gelatinase/type IV collagenase. It is also known as Gelatinase A or 72kDa Gelatinase. It cleaves a variety of substrates including gelatines, collagen types IV, V VII, X XI, fibronectin, proteoglycan and elastin.

MMP2 is derived from fibroblasts (Mannello et al., 2003).

It is secreted in precursor form (72kDa, pro-MMP2) and can be activated in vitro by organomercurial compounds such as p-aminophenylmercuric acetate (APMA). It can also self activate (Bergmann et al., 1995). The active form has a molecular weight of 62kDa or 59kDa(Davies et al., 1993b, Davies et al., 1993a). Pro-MMP2 can be inhibited by binding of the C-terminus of TIMP1 to its hemopexin domain (Cao and Zucker, 2008). Pro-MMP2 and pro-MMP2 complexed with TIMP2 are specifically activated in vivo by active MT-MMP on the surface of tumour cells. Cao confirmed that MT1-MMP (MMP14) could activate MMP2 and that its propeptide domain was necessary for this (Cao et al., 1998). This occurs in benign and malignant conditions.

MMP2 is secreted as a latent pro-enzyme complexed with TIMP2. This complex has been found to be capable of activation as a complex, and without MMP2 being separated from it. It is speculated that the reason that MMP2 is secreted as a complex is that this complex is more stable. MMP2 would auto-activate and undergo rapid degradation. MMP2 contains a stabilisation site that is separate from the inhibition site. This stabilisation site can be occupied by various MMP inhibitors including MMP2 and 1,10-phenanthroline, but not TIMP1. The occupation of the stabilisation site by

MMP2 does not inhibit gelatinolysis – thus it is possible to have an MMP2 / TIMP2 complex that is active (Howard et al., 1991b). Certain studies have shown the possibility of this MMP/TIMP complex being activated by cellular mechanisms rather than the more frequently seen organomercurial mediated activation (Brown et al., 1993).

Further studies by Howard et al show greater inhibitory effect of TIMP2 compared to TIMP1. In studying auto-activated MMP2 - which produces 2 active peptides and various inactive fragments – TIMP2 had greatest effect on the 42.5kDa active fragment – up to 10 times more effect than TIMP1. It was also more than 2 times more effective than TIMP1 on MMP2 / TIMP2 complex activated by APMA. It was also noted to be more than 7 times more effective than TIMP1 on MMP9 activated by APMA. Radio-labelled TIMP1 and TIMP2 incubated in equal quantities with MMP9, autoactivated MMP2, and 42.5kDa fragment (from auto-activated MMP2) showed TIMP2 / TIMP1 ratios of 4.4, 10, and 33 respectively. TIMP1 was however noted to be more than 2 times more effective compared to TIMP2 in inhibition of MMP1 (interstitial collagenase) (Howard et al., 1991a).

Plasma MMP2 is not found to be increased in many cancers (including advanced gastrointestinal cancer, breast cancer, gynaecological cancer, lung cancer, and lymphoma-leukaemia), and on the basis of in vitro studies, this is thought to be due to the sizeable contribution made by endothelial, and other normal cells hiding the MMP2 increase from solid tumour cells (Zucker et al., 1992b). Fujimoto used home made kits, and found a significant decrease in MMP2 levels in osteoarthritis, rheumatoid arthritis, gastric and pancreatic cancer compared to normal patients. They

also found significantly increased levels in hepatocellular cancer, hyperthyroidism and biliary cirrhosis (Fujimoto et al., 1993).

Garbisa et al looked only at stage IV lung cancer, and did find a markedly significant difference between serum levels of MMP2 in these patients versus normal patients ($P<0.0001$). They also found a less significant difference in MMP2 levels in patients with versus patients without distant metastases($P<0.01$) (Garbisa et al., 1992).

Note: Gelatin is an irreversibly hydrolysed form of collagen (Wikipedia, 2008)

Studies have suggested that MMP2 and 9 are similar in various ways including having identical substrate specificities (type IV and V collagen and gelatin), and sharing 4 highly homologous domains with the exception of an extra collagen-like domain in MMP9 (Okada et al., 1990). They do however have different gene expressions, and have different activation mechanisms.

1.4.9.2. Matrix Metalloproteinase 8 (Neutrophil collagenase)

MMP8 is known as neutrophil collagenase due to the fact that it was thought to be confined to polymorphonuclear leukocytes (neutrophils, PMN), stored in granules and secreted upon activation. MMP8 has been demonstrated in osteoarthritic chondrocytes, synovial fibroblasts, and endothelial cells.

MMP8 is known as neutrophil collagenase due to the high content found in normal neutrophils.

It is secreted in a pro-form 75kDa in the case of MMP8 secreted from neutrophil, and 50-55kDa when secreted from other cells. The difference in size is due to glycosylation of neutrophil MMP8.

Pro-MMP8 can be activated by organomercurial compounds such as p-aminophenylmercuric acetate (APMA), oxidative agents such as oxygen radicals, hydrogen peroxide, or hypochlorite, and proteinases such as trypsin, α-chymotrypsin, cathepsin G, tissue kallilrein, and MMP13. MMP8 activity can be inhibited by TIMP1 and TIMP2 in a 1:1 molar ratio.

The active form of MMP8 is 68kDa.

1.4.9.3. Matrix Metalloproteinase 9 (Gelatinase B)

Matrix metalloproteinase 9 is a 92 kDa gelatinase/type IV collagenase in its initially secreted 'pro'-form. It is also called or 92kDa Gelatinase. It cleaves a variety of substrates including gelatines, collagen types IV, V VII, X, and elastin.

It can be activated from its precursor form by organomercurial compounds in vitro (e.g. APMA) and in vivo by proteinases such as trypsin, α-chymotrypsin, cathepsin G, and others including MMP3 and hypochlorous acid. Pro-MMP9 can be inhibited by binding of its hemopexin domain by the C-terminus of TIMP2 (Cao and Zucker, 2008). The active form of MMP8 is 83kDa. Activation is inhibited by TIMPs in a 1:1 molar ratio.

MMP9 is produced in a variety of cells, in particular fibroblasts, and has been demonstrated to be elevated in serum samples of patients with hepatocellular carcinoma. It has also been shown to play in role in other disease processes including Alzheimer's disease.

MMP9 is derived from neutrophils (Mannello et al., 2003, Romanic et al., 2002).

Work carried out on human prostatic tissue has shown that MMP26 (Matrilysin-2) has the ability to activate MMP9 (Zhao et al., 2003). This has also been shown in oesophageal cancer cells (Yamamoto et al., 2004).

1.4.10. Specific Tissue Inhibitor of Matrix Metalloproteinase

The regulation of such powerful enzymes as MMPs is crucial and after control of gene expression, and post-translational control in the extra-cellular space, active MMPs are inhibited by substances including α_2-macroglobulin and TIMPs (tissue inhibitors of MMP), a family of MMP-specific inhibitors. These complex with active MMPs in order to inhibit them. In addition some TIMPs can inhibit the activation of pro-MMPs. In general this inhibition seems to occur in a 1:1 molar ratio.

TIMPs 1 to 4 all form physiologically irreversible complexes with activated MMPs at the amino terminal of the MMP, in order to inhibit these enzymes. In addition to this, TIMP1 and 2 also form complexes with MMP9 and 2 respectively at the carboxyl terminal which stabilizes the MMP, but does not actually convey latency.

TIMPs also act as growth factors for euthyroid precursors (Dollery et al., 1995).

Expression studies suggest that there are different roles for the TIMPs. TIMP1 seems to be inducible and is stimulated by cytokines and hormones, whereas TIMP2 is constitutive, following the expression of MMP2, with which is interacts specifically.

There is only 43% sequence homology between TIMP1 and 2, but they are essentially interchangeable in their ability to inhibit MMPs. TIMP1 and 2 are however

distinguished by their interactions with the pro-gelatinases. TIMP-3 shares 37% sequence homology with TIMP1 and 42% with TIMP2. It seems to be localized specifically to the extracellular matrix unlike the other TIMPs (Leco et al., 1994). TIMP1 to 3 have 6 disulfide bridges in order to maintain their 3-D structure.

1.4.10.1. Tissue Inhibitor of Matrix Metalloproteinase 1

TIMP1 is a 184 amino acid glycoprotein of 28.5kDa. TIMP1 inhibits all the MMPs and is the most widely distributed TIMP. The inhibition is via high affinity, reversible non-covalent binding to form a 1:1 complex. It is however thought that in vivo, the binding is irreversible (Dollery et al., 1995). When reversible, TIMP1 is not cleaved by this binding and has been shown to maintain full functionality after recovery from complexes with MMP3 (Stromelysin-1) (Murphy et al., 1989).

TIMP1 has been shown to have erythroid potentiating activity.

TIMP1 contains 12 cysteine residues, which form 6 loop structures through disulphide bonds. This is demonstrated schematically in the diagram above (see previous section on TIMPs).

It is known that platelets contain some TIMP1, and that serum samples have higher levels of TIMP1 than plasma samples from the same patient due to the release of TIMP1 from platelets during the clotting process. The majority of reports however still seem to describe the use of serum for TIMP analysis (Zucker et al., 2004).

TIMP1 is produced by most types of connective tissue cell, as well as macrophages and acts against all members of the collagenase, gelatinase and stromelysin classes of MMP (Dollery et al., 1995).

TIMP1 is produced by transformed fibroblasts, together with TIMP2 (Howard et al., 1991a).

TIMP1 has been shown to be produced by hepatic stellate cells, activated when the liver is under stress (Iredale et al., 1992).

It is highly expressed in resorbing tissue, as its role is to regulate enzyme activity, in activation from latent form and inhibition of catalytic activity (Leco et al., 1994).

1.4.10.2. Tissue Inhibitor of Matrix Metalloproteinase 2

TIMP2 is a 21kDa, 194 amino acid unglycosylated protein with some similarity to TIMP1 and TIMP-3 (43% and 44% amino acid sequence homology respectively). It inhibits the activity of all active MMPs, and regulates the activation of pro-MMP2 by binding to the C-terminal region of pro-MMP2. This reduces the risk of spontaneous activation (which can occur in MMP2 and 9) and also the MMP2/TIMP2 complex whether activated or not, is more susceptible to inhibition by other TIMPs (Denhardt et al., 1993). MMP2/TIMP2 complex can be activated, but is 20 times less active than uncomplexed activated MMP2 (Fridman et al., 1993). Fridman et al also demonstrated an 86% decrease in activity of active MMP2 when TIMP2 was added in a 1:1 molar ratio.

TIMP2 has also been shown to have erythroid potentiating activity, and cell growth-promoting activity. However TIMP2 complexed to pro-MMP2 has no cell growth-promoting activity at all.

1.4.11. Hypothesis and aim of study

Matrix metalloproteinases (MMPs) have been shown to be elevated in AAA tissue compared to normal aortic tissue (Vine and Powell, 1991, McMillan et al., 1995). Furthermore certain MMPs are also increased within the same aneurysm at the site of aneurysm rupture compared to anterior aneurysm wall (Wilson et al., 2006). It is thought that the increase in MMPs brings about a state of proteolysis which initially causes the development of aneurysms through the breakdown of elastin, but eventually causes catastrophic rupture mediated by the collagenases (Dobrin and Mrkvicka, 1994).

Finite element analysis (FEA) is an engineering tool which identifies stress levels within a structure (Fagan, 1992). It has been shown that stress levels as identified by FEA is higher in ruptured AAAs as compared to AAAs of a similar size but non-ruptured and undergoing elective repair (Venkatasubramaniam et al., 2004b). Peak wall stress sites have been anecdotally linked to rupture sites by observation in theatre, and on CT scans. Theoretically the site in a structure with the highest stress would be the most likely to rupture in a situation where the stresses involved are of the same magnitude as the failure strength of the structure. It makes sense therefore that the peak stress site undergoes the most remodelling, and that this remodelling is mediated by MMPs.

Since in engineering theory the peak wall site is most likely to rupture, and biochemically, the site highest in collagenase is most like to rupture, it is probable that this site is in fact the same.

The hypothesis of this study is that at peak stress sites, the level of MMPs would be elevated compared to other sites within the aneurysm. This difference would be greatest when comparing peak stress sites with minimum or low stress sites.

Due to the desire to minimise risk to the patient, the site chosen to compare to the peak stress site is the arteriotomy site, where the incision is made in order to carry out the elective AAA repair.

Aim of study

The aims of the study were;

- To compare the levels of tissue MMP2, MMP8, MMP9 (both active and total concentrations) and their natural inhibitors TIMP1 and TIMP2 at peak wall stress sites and arteriotomy sites in elective patients undergoing abdominal aortic aneurysm repair.
- To compare the levels of tissue and circulating MMP2, MMP8, MMP9, TIMP1 and TIMP2.

2. AAA Screening

2.1. Materials & Methods

2.1.1. Design

We designed a community prospective cohort study, and obtained full institutional (Hull and East Yorkshire NHS Trust Research and Development approval number: ELSY2599) and local regional ethics committee approval (Corec Ethics Committee approval number: LREC/12/01/253).

2.1.2. Sample size

The statistical objective of the study is to estimate the proportion of true positives detected by the various examinations out of the total number of patients with AAAs (sensitivity). In our pilot study, 93 % of patients with an AAA ≥ 5cm were able to feel a pulsation. To have clinical significance we expect 80% sensitivity, within 10% of the correct value, for which 35 patients with AAA ≥ 5cm will be needed (using the negative binomial distribution). As the prevalence is about 0.7% (The Multicentre Aneurysm Screening Study Group, 2002), 5100 patients will have to be screened. The response rate in most of the AAA screening programmes is around 60%, hence targeting 9,000 patients for the study. The above calculations were done with the kind help of Dr. E.D. Gardiner, independent consultant medical statistician.

2.1.3. Study participants

Inclusion criteria

All male patients aged 65 and above (n=10,591), enrolled in 15 general practices located in 10 separate small semi-rural towns and villages, and 1 large city in north of England were invited to participate.

Exclusion criteria

All patients reported by the general practice to have a known AAA were excluded. Patients subsequently reporting a known AAA via the screening form were also excluded. In addition, the general practice was asked to indicate anyone that they thought was too unwell to participate in the study. In an attempt to avoid inviting recently deceased patients, up to date patients lists were obtained from the GP practice prior to each batch of invitations being dispatched.

2.1.4. Invitation and self-examination

Patients were invited via a 2 sided letter from the practice, with the second side detailing the study (see appendix). Enclosed with this letter was another 2 sided sheet with self-examination instructions (previously validated (Venkatasubramaniam et al., 2004c)), a medical history enquiry section, and a study consent section (figures 9 & 10, below). The self-examination instruction sheet was 1 side of A4, and contained both self-examination and 2nd person examination instructions. Self-examination instructions were a simple 4-step process, detailed alongside 2 hand-drawn line illustrations. Second person examination was an optional additional part if somebody was available. The answers required in both sections consisted a simple tick box. Contact telephone numbers were included for queries.

Also included was a stamped addressed envelope. These invitational packs were sent in batches of 100-200.

The entire cohort from one practice was also sent a questionnaire to assess the psychological aspect of being selected for this study (hospital anxiety & depression scale – HAD scale – see appendix).

Those that did not respond after 4 weeks were sent 1 reminder letter. No further reminders were sent after this. All responses and non-responses of participating patients were recorded in a database (Microsoft Access XP).

Self-Examination for AAA Instruction Sheet

Academic Vascular Unit, University of Hull & Hull Royal Infirmary

Part 1
We would like you to do this simple examination.

1. Lie on bed or floor.
2. Overlap hands just above your belly-button.
3. Relax. Press firmly down for a few seconds (This shouldn't hurt).
4. Can you feel a pulse in your tummy? **YES** ☐ **NO** ☐

(Don't worry if you can or cannot feel a pulse)

Part 2 (this part is optional)
Do this part if you have a relative/friend to feel your tummy.

1. Lie on a bed or floor.
2. Request your friend/relative to sit to one side of you.
3. He/she should place their overlapped hands flat on tummy just above your bellybutton (as shown below).

4. Ask them to press firmly down for a few seconds.
5. Can they feel a pulse in your tummy? **YES** ☐ **NO** ☐

(Don't worry if they can or cannot feel a pulse)

Thank you for your kind co-operation.

If you have any difficulty in carrying out the examination, please contact Mrs Bryce on 01482 674703 or Ms Tennison on 01482 675784 between 9am to 5 pm.

V1.2 14/9/2005

Figure 9. AAA Screening: Self-examination instruction sheet

Self-examination for Screening Abdominal Aortic Aneurysms
Instruction leaflet & Questionnaire

Please complete both sides & return within 2 weeks

Some questions about you *(please fill in)*

Your full name?				
Your height?				ft/m *(please delete)*
Your weight?				stone/kg *(please delete)*
Your waist size?				inches
		Yes	No	How many cigarettes /day ?
Do you smoke?				
Are you an ex-smoker?				

Some questions about your health *(please tick)*

Do you have / have you had	Yes	No	Don't know
An Abdominal Aortic Aneurysm (AAA)?			
Any other aneurysms?			
High blood pressure?			
High cholesterol?			
Diabetes?			
Angina?			
A heart attack?			
A stroke?			
Are you on any medication? (if yes, please enclose a list)			

Questions about your family

Does anyone in your family (blood relatives only please) have	Yes	No	Don't know
An Abdominal Aortic Aneurysm (AAA)?			
Your relationship to this person?			

OFFICE USE ONLY Aorta size: Girth:
Unit no:

Consent to trial

I understand that;
- This screening programme is part of a study.
- I will be offered an ultrasound scan.
- I can ask questions when I come for my ultrasound scan.
- My participation is voluntary and I may withdraw at any time.

I am willing to take part in this study.
Full Name:

Date of Birth:
Signed:

Date:

Now please complete the instructions on the reverse side of this sheet, then use the stamped addressed envelope enclosed to return this leaflet.

Please turn over.

This study is being carried out by the Academic Vascular Unit, The University of Hull & Hull Royal Infirmary, in collaboration with your GP.
Postal address: Vascular Laboratory, Alderson House, Hull Royal Infirmary, Anlaby Road, HU3 2JZ.
Corec ethics committee approval number: LREC/12/01/253
Hull and East Yorkshire NHS Trust Research & Development approval number: ELSY2599

SE leaflet v2a.4.6 12/7/05

Figure 10. Medical History enquiry sheet

2.1.5. Abdominal ultrasound

Appointments were sent out to all participating patients within 3 months of receipt of returned participant pack for an abdominal ultrasound scan, purely to ascertain abdominal aorta size. Scanning took place at the GP practice in one cohort and at a local cottage hospital (within the town) in the other (simultaneous scanning in 2 rooms by 2 vascular technologists and a vascular research fellow). All scans were performed on one of;

- SonoSite TITAN, C60/5-2MHz convex probe, mainly B-mode
- Philips ATL HDI 5000, 3.5MHz convex probe, abdominal aorta settings, mainly B-mode
- Fukuda Denshi (FF sonic UF-4000), FUT-C111A (convex 3.5 MHz 60R) probe, B-mode.

All patients with abdominal aortas < 3cm were reassured and discharged. A vascular surgical research fellow counselled patients with abdominal aortas from 3 to 5cm, a standard printed information sheet on AAA was given, they were enrolled on a AAA surveillance programme, and the patient's GP was informed by letter. Patients with AAAs ≥5cm were referred to a consultant within the vascular unit on an elective or emergent basis depending on the presence or absence of symptoms.

2.1.6. Psychological assessment

The psychological consequences of the study were assessed with the HAD scale at the time of self-examination, immediately after abdominal ultrasound scanning and 1 month after scanning.

Hospital anxiety and depression scale is a rapid 1-side of A4, 14 item psychological assessment questionnaire that takes about 3-5 minutes to complete. It is made up of questions from 2 separate subscales which provide independent anxiety and depression scores. Each of the 14 questions is assigned a mark from 0-3, allowing a result between 0-21 for each of the subscales. Results from 0-7 were considered normal, 8-10 mild, 11-14 moderate, 15-21 severe for each scale.

The HAD scale was designed to be simple and reliable, in response to the fact that many other scales were lengthy and required administration by trained staff (Zigmond and Snaith, 1983, Snaith, 2003). In order for it to be short, the scale was designed only to look at anxiety and depression, with depression focusing on anhedonia. It has been frequently validated for use by the elderly and also for use in other age groups, and has been reported to be suitable for screening (White et al., 1999). It does not have to be used in the context of an in-patient hospital stay, having been validated in the community and primary care setting (Bjelland et al., 2002).

HAD scales were read off the printed sheet straight into a spreadsheet (Microsoft Excel XP) with the appropriate calculator for the 2 independent subscale results (anxiety and depression). These 2 separate marks were recorded into our database. These results were recorded for the 3 time-points – at self-examination, post scan, and 1 month post scan. Analysis was carried out using a statistics package (SPSS v11.5) using paired t-test for comparison of 2 time periods at a time within a subscale. The proportion of patients with abnormal scores (>7) in each of the 2 subscales was also analysed, and compared at different time points.

2.1.7. Analysis of self-examination

Sensitivity, the proportion of patients correctly diagnosed by the test as having an AAA out of all the patients that actually had an AAA, was calculated by dividing true positives by true positives plus false negatives. Specificity, the proportion of patients correctly diagnosed by the test as not having an AAA, was calculated by dividing the true negatives by the true negatives plus false positives.

The positive predictive value (PPV), the proportion of patients with positive test results whom actually had an AAA, was calculated by dividing the true positives by the true positives plus the false positives. The negative predictive value (NPV), the proportion of patients with negative results who did not have an AAA, was calculated by dividing the true negatives by the true negatives plus the false negatives.

The positive likelihood ratio (PLR) indicates the value of the test for increasing certainty about a positive diagnosis and provides a direct estimate of how much a test result will change the odds of having a disease. It is calculated by dividing sensitivity by (1-specificity). The negative likelihood ratio (NLR) [(1-sensitivity) / specificity] expresses the decrease in the odds of having a disease when the finding is negative.

2.2. Results

2.2.1. Patients and demographics

We invited 10,591 men ≥65 years of age. 9,474 (89%) replied resulting in a non-response rate of 11% (1,117) (table 3, below).

Of the 9,474 men who replied, 2,006 (19%) declined to participate, whilst a further 580 (5%) did not participate for one reason or another (detailed below). Thus 6,888

(65%) men, median age 72 (IQR 68-77) years, agreed to participate, and took part in the study.

Of the 580 (5%) men who did not decline, but did not participate in the study, the reasons were; 1.7% having a known or previously repaired AAA, 0.7% being recently deceased (despite mechanisms in place to reduce this number), 1.3% wrong address, 0.4% recent scan, 0.5% unwell but willing to join later, 0.5% failed to attend the ultrasound appointment twice, 0.1% subsequently changed mind (did not now want to participate in study), 0.1% out of country, 0.1% moving or moved out of area.

5,881 replies (62% of all replies) were received within 2 weeks of the initial pack being sent, and a further 1,468 replies (15%) were received prior to the end of week 4. 3,262 reminders (31% of invited group) were sent out after 4 weeks, and 2,127 replies (22% of all replies) were received after this (table 4, below).

Demographic analysis showed an incidence of 65% of ex-smokers, 47% hypertension, 39% hypercholesterolaemia, 32% ischaemic heart disease, 15% diabetes, 11% current smokers, and 8% previous cerebrovascular event. 2.4% also reported a family history of AAA (table 5, below).

Table 3. AAA Screening: response

	no.	%
Total invited	10591	
Total replied	9474	89%
No reply	1117	11%
Agreed	6888	65%
Declined	2006	19%
See comments	580	5%
Reminder	3262	31%

Table 4. AAA Screening: time to response

	Total	yes	no	comments	blank	% of all replies	% of total yes
replied 2wks	5881	4612	933	336	0	62%	67%
replied 4wks	1468	1143	236	88	1	15%	17%
replied longer	2127	1133	837	156	1	22%	16%
Total	9476	6888	2006	580	2		

Table 5. Family history of AAA

	no.	% of total
Father	27	16.9%
Brother	59	36.9%
Son	25	15.6%
Uncle	5	3.1%
Nephew	6	3.8%
Mother	18	11.3%
Sister	8	5.0%
Daughter	1	0.6%
Aunt	3	1.9%
Niece	0	0.0%
Cousin	8	5.0%
Total	160	

2.2.2. Self, second person and tandem examinations

Self-examination

6035 patients carried out abdominal self-examination for AAA. This was 88% of patients who agreed to take part in the study, and 57% of the total number invited. 1262(21%) reported 'a pulse in their tummy,' whilst 4773(79%) could not.

Second person examination

4528 patients had abdominal examination carried out by a second person. This was 66% of patients who agreed to take part in the study, and 43% of the total number invited. 1125(25%) reported 'a pulse in the patients tummy, whilst 3403(75%) could not.

Tandem examination

6231 patients had either abdominal self-examination or second person abdominal examination or both. This was 90% of patients who agreed to take part in the study, and 59% of the total number invited. 1652(27%) reported detecting 'a pulse in the tummy' in at least one of the 2 tests, whilst 4579(73%) could not detect 'a pulse in the tummy' in both tests.

2.2.3. Results by practice

15 GP practices from a variety of situations were recruited. Roos, Hedon, Patrington and Preston are small villages on the outskirts of a large city (Hull), Beverley, Market Weighton and Driffield are small market towns, Withernsea and Hornsea are small seaside towns and Bridlington is a slightly bigger seaside town and holiday resort.

Reply rates varied from 85% to 94%. 2 week replies varied from 40% to 65%. Consent varied from 56% to 77%. Self-examination varied from 45% to 68% (table 6, below).

Table 6. AAA screening response rate by general practice

GP Practice	Location	Men	Replied %	Replied in 2wks %	Consent %	Self-exam %
Manor Rd Surgery	Beverley	937	90	56	64	57
Cranwell Rd Surgery	Driffield	862	91	56	64	58
Eastgate Surgery	Hornsea	1185	90	57	66	58
Patrington Surgery	Patrington	259	86	54	68	61
Withernsea Surgery	Withernsea	748	88	40	64	56
Roos Surgery	Roos	90	86	50	70	66
Morrill Street Surgery	Hull	796	87	53	60	50
Hedon Group Practice	Hedon	823	90	57	70	62
Church View Surgery	Preston	798	93	58	77	68
Market Weighton	Market Weighton	559	94	58	68	58
Bridlington Surgery	Bridlington	1032	89	52	62	55
Marfleet Group Practice	Hull	915	85	58	56	45
Practice 1 Bridlington	Bridlington	489	93	65	66	59
Faith House Surgery	Hull	443	87	55	67	60
Practice 2 Bridlington	Bridlington	655	90	63	65	57
	Total/Mean	10591	89	56	65	57

General practices were recruited in the above order, starting with Manor Road Surgery in Beverley.

2.2.4. Abdominal aortic aneurysms

216(3.1 % of consented patients) AAAs (diameter ≥3cm) were detected by abdominal ultrasonography, of which 45(0.7%) were ≥5 cm in diameter.

Self-examination

Clinically significant AAAs (≥5cm) had a self-examination sensitivity of 49%, specificity of 79%, PPV of 1.6% and NPV of 99.6%. The PLR was 2.35, and NLR was 0.65 (table 7, below).

Sensitivity for self-examination in the detection of all AAAs was 37%, with a specificity of 80%. PPV was 5.8% and NPV was 97%.

Second person examination

Second person examination for AAA ≥5cm had a sensitivity of 69%, specificity of 75%, PPV 1.8%, NPV 99.7%, PLR 2.81, and NLR 0.41.

Tandem examination

When analysis was carried out on tandem 2nd person examination (at least one positive result from either self-examination *or* 2nd person examination) for AAA ≥5cm, sensitivity was 68%, specificity 79%, PPV 2.2% and NPV 99.7%. PLR was 3.31, and NLR 0.4.

Variation with time

Sensitivity for self-examination in AAA ≥5cm was 83% after analysis of the first cohort of 1000 patients, this reduced to 67% after the first 2 cohorts and finally on completion of the study it was 49%. Specificity was 86%, 83% then 79% respectively at the same points in the study (table 8, below).

Table 7. Sensitivity and specificity of self, second person and tandem examinations

	Self Exam		2nd Person Exam		Tandem Exam	
	≥3cm	≥5cm	≥3cm	≥5cm		≥5cm
Sensitivity	37	49	48	69		68
Specificity	80	79	76	75		79
Positive predictive value	5.8	1.6	6	1.8		2.2
Negative predictive value	97.4	99.6	97.9	99.7		99.7
Positive likelihood ratio	1.84	2.35	1.98	2.81		3.31
Neg likelihood ratio	0.79	0.65	0.69	0.41		0.4

Table 8. Sensitivity and specificity of self-examination with study progression

Invited pts	Sensitivity	Specificity
1000	83	86
1984	67	83
10591	49	79

2.2.5. Psychological scores

Psychological impact as measured by scores from the HAD subscales of anxiety and depression decreased significantly between self-examination (mean scores anxiety 4.17, depression 3.42) and immediately post-scan (mean scores anxiety 3.80, depression 3.04) (anxiety $p<0.001$, depression $p<0.001$), and between self-examination and 1 month post scan (mean scores anxiety 3.89, depression 3.22) (anxiety $p<0.001$, depression $p=0.022$) (figure 11, below). No significant change was seen between immediate post scan and 1-month post scan anxiety scores ($p=0.678$), although there was an increase in depression score ($p=0.02$) between immediate post scan and 1

month post scan scores. The proportion of patients with abnormal scores on the anxiety subscale was 16.8% at self-examination, reducing to 12.8% after ultrasonography and 16.1% after 1 month.

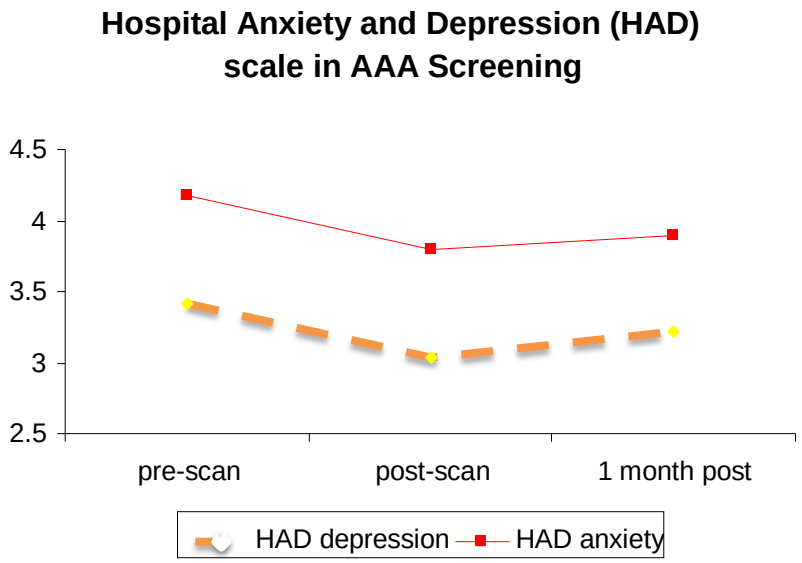


Figure 11. AAA screening: hospital anxiety and depression score

2.3. Discussion

This study was set up during the time when it was thought that AAA screening would be on the border for acceptability for the NHS in the UK, and thus the purpose of the study was to reduce costs in a way which was clinically reasonable. The idea of a screening test to filter patients prior to a diagnostic test is quite normal for screening programmes, however due to the diagnostic test for AAAs (ultrasonography) being relatively cheap, all the studies on screening have been done with ultrasonography. The principle however is valid, if the diagnostic test is too expensive, a screening test or filtering test should be done prior to the diagnostic test to bring the costs of a screening programme down to an acceptable level.

2.3.1. Participation

The response rate was 89%, with 65% agreeing to participate, but only 59% completed some form of abdominal examination. 19% of the cohort declined to participate. Through informal discussions with the participants, suggested reasons for not participating were; 'not wanting to know,' 'not wanting surgery,' 'too much trouble,' time constraints, perceived difficulty of self-examination, and having to attend for an ultrasound scan. It was also felt by some participants that the research nature of this programme could have adversely affect them, since this would suggest there was an experimental and voluntary side to the programme, and that this was not part of standard recommended medical care.

6% of the entire cohort failed to complete any kind of abdominal examination despite having already consented to participate. Direct questioning by the team during scanning sessions revealed that many had actually carried out self-examination but were afraid of getting the question 'wrong' and therefore just left the section blank. Others just failed to turn the page over (2 sided sheet), and the introduction of a simple "PTO" (please turn over) seemed to improve this. In addition, we did not restrict the upper age group as this was a study to look at the feasibility of self-examination rather than primarily to pick up AAAs, thus some may not have been able to complete the examination.

It was also interesting how the consent rates varied with area; villages - Preston 77%, Roos 70%, Hedon 70%, Patrington 68%; market / seaside towns – Market Weighton 68%, Hornsea 66%, Beverley 64%, Driffield 64%, Withernsea 64%; and bigger towns / cities (Bridlington / Hull) – 67%, 66%, 65%, 62%, 60%, 56%. This effect has been noted before (Smith et al., 1993), and seems applicable in this study.

2.3.2. Designed to detect AAAs >5cm

Data from our pilot study showed poor detection of small aneurysms, and that detection generally improved with AAA diameter. Therefore we aimed to detect the largest diameter AAA that would not affect normal AAA management. At 5cm we felt there would be enough time to invite the patient to a normal, routine out patient clinic appointment, and carry out all standard pre-AAA repair investigations. Choosing a large AAA diameter would also mean that fewer patients would be subject to the anxiety involved in a long term surveillance programme. This would also lower the costs associated with the surveillance. The ability to repeat the questionnaire would obviate the obvious risk of missing smaller AAAs.

2.3.3. Test effectiveness

Sensitivity or detection rate, is the ability of a test to find those with the condition in question. Our pilot study (164 patients -125 AAAs, 39 controls) showed that it was possible to obtain a sensitivity of 93% for self-examination in clinically significant AAAs (≥5cm) with an un-blinded group of AAA patients. However in this real-life community setting a sensitivity of 49% and specificity of 79% was obtained. This was rather disappointing, but the pilot study served to demonstrate the best possible sensitivity obtainable, a target sensitivity level.

Interestingly the sensitivity of self-examination for clinically significant AAAs (≥5cm) seemed to drop as the study progressed. When the first cohort of patients was completed (n=1,000), the sensitivity was 83.3%, and specificity was 85.5%. On completion of the second cohort (n=1,984) the sensitivity became 67%, and specificity 83%. At completion of the study the sensitivity was 49% and specificity 79%. It is

possible that these results stem from the fact that the later cohorts of patients came from more deprived inner city areas. However why should deprivation lead to a poorer sensitivity and specificity for self-examination? Uptake of tests could be expected to be poorer due to lack of interest or time or both, but here we are effectively only comparing groups that have 'administered' the test. We could not think of any reason that deprivation could explain this finding. This would not explain the initial fall either between cohort 1 and cohort 2. The instructions and diagrams for self and second person examination have not been altered, although the general leaflet design has changed to allow more efficient capture of information. Again we do not believe that this could account for the decrease in sensitivity and specificity.

Tandem testing involves carrying out 2 or more tests, with a positive test from either identifying disease – or in this case the possibility of disease. Analysis of the entire sample by this method (not only where a 2nd person actually carried out examination) increased sensitivity to 68%. The best sensitivity however comes from second person examination of clinically significant AAAs at 69%. This perhaps makes sense as it make be difficult to detect a pulsation if this is the same as your heart. Second person examination by doctors is also the method that AAAs have been detected for decades, albeit with varying degrees of success.

These sensitivities compare poorly to mammography for breast cancer screening 82-85%, and reasonably to conventional cervical cytology for cervical cancer screening 29-56%, prostate specific antigen (4ug/l cutoff) for prostate cancer screening 44%, and faecal occult blood testing for colorectal cancer in an average risk population 10.8%. The problem in AAA screening is that ultrasonography is so good. It can visualise the

aorta in over 99% of patients (Lindholt et al., 1999), with sensitivities estimated at 87-99%

Specificity is the ability of a test to correctly identify those without the condition. Non-specific tests ie low specificities, result in more worry for patients and unnecessary investigations for these false positives. Our specificity for self-examination of 79%, or tandem 2nd person examination specificity also of 79% compares unfavourably with mammography (90%), and PSA (4um/l) (94%), however both the latter situations would result in a biopsy whereas, our test requires a non-invasive, low risk, rapid examination (ultrasound) if a false positive is detected. Again ultrasonography is the gold standard with sensitivities of 99% (Lindholt et al., 1999).

These assessments of test effectiveness are thus not directly comparable (but are certainly of interest) as situations, interventions, and outcome differ depending on the condition involved.

To clinicians, positive and negative predictive values (PPV & NPV) are perhaps more important. This gives the chance that the patient has the condition after a positive result, and conversely the chance that the patient doesn't have the disease given a negative test result. This unfortunately is immensely affected by disease prevalence, and therefore primarily because the incidence of AAA in the population is low, the PPV is low and the NPV is high.

The likelihood ratio gives the increase in odds that the patient has the condition when the test is positive (positive likelihood ratio - PLR) or the decrease in odds that the patient does have the condition when the test is negative (negative likelihood ratio – NLR). This is not affected by the prevalence of the condition involved and therefore a

better clinical indicator of the usefulness of a test or otherwise. The positive likelihood ratio for self-examination in clinically significant AAAs is 2.35, suggesting that these patients are more than 2 times as likely as the average man aged 65 and above to have an AAA. The best result in PLR comes from tandem examination (3.31). This reflects the fact that PLR is proportional to sensitivity and inversely proportional to specificity, and although it is marginally superior to second person examination (≥5cm) in sensitivity, it is much better in specificity.

2.3.4. Psychological Assessment

HAD scores, at all time points were within normal population ranges (0-7), ie invitation for self-examination and ultrasound scanning did not produce pathological anxiety or depression. However, comparison of scores at self-examination to both immediately post-scan, and 1 month for both anxiety and depression were significantly reduced, indicating either a subtle elevation initially and subsequent reduction, or a general reduction in anxiety and depression due to "one less thing to worry about." No formal baseline was established prior to self-examination, so no comparison could be made to pre-screen levels. Examination of the percentage of patients with abnormal anxiety or depression levels also showed a similar trend, with levels decreasing significantly between self-examination and post-ultrasonography, but levels then rise significantly between post-ultrasonography and 1 month time points.

Other screening programmes and methods have also demonstrated the possible ill effects of screening, and in particular false positives. This needs to be a real consideration for any screening study, as the effects can be devastating to the individuals concerned.

2.4. Conclusion

Our results show that screening for AAA by self-examination is ineffective for clinically significant (≥ 5cm) AAAs, particularly in comparison to ultrasonography. Although psychological anxiety and depression were within normal range throughout the study, there was a statistically significant reduction on completion. Second person examination and self-examination *in tandem with 2nd person examination* can marginally improve sensitivity, and specificity, however these do not improve results enough to justify this method of screening for AAAs.

The cost-effectiveness, sensitivity, and acceptability of ultrasonography leaves little room for other AAA screening modalities. The funding for a national ultrasound screening programme for AAAs is finally here, so the final cost barrier to ultrasonographic screening has now been removed.

In future perhaps, this method can be used in other groups – women, smokers under 65 year of age, young and middle aged relations with a family history of AAA - that remains to be explored.

3. MMPs

3.1. Materials & Methods

3.1.1. Planning and ethics

Full local research ethics committee (LREC) approval was gained through application on the web-based COREC application system and attendance at the Hull LREC (COREC study number: 04/Q1104/42).

Institutional research and development department approval (Hull and East Yorkshire Hospitals NHS Trust (HEYT) and University of Hull (UoH)) was also gained (Hull and East Yorkshire NHS Trust Research and Development no: LG/EL/R0067 with a memorandum of understanding with the UoH).

All university staff involved had honorary contracts with HEYT.

3.1.2. Sample size calculation

Sample size calculation

The sample size calculation was carried out during the study design period, prior to submission to the regional ethics committee. Certain variable have therefore changed during the course of the study, but the original samples size calculations and assumptions are detailed.

A normal distribution was assumed and the "Paired T-Test" was used on calculations involving a null hypothesis. Correlation between each 2 paired samples was taken to be worse-case scenario and assumed at 0. Data was taken from a previously presented

study with details published in the conference proceedings (Wilson et al., 2004) The study examined MMP levels in ruptured AAA from arteriotomy and rupture sites.

The null hypothesis (H_0) for this study was – there is no difference in tissue MMP level between high and low stress sites in elective aneurysms.

Population means (of MMP level) taken to be;

- high stress: 30ng/mg (previous study ruptured site 44 ng/mg) (taken to be lower than rupture site as not yet ruptured, but higher than "normal" as we hypothesize that local MMP elevation contributes to aneurysm expansion and local change in geometry leading to high stress areas and eventual rupture).
- low stress: 15ng/mg (previous study arteriotomy site 17ng/mg) (taken to be slightly lower then arteriotomy site as this is the lowest wall tensile stress site and we hypothesize that this has "normal" levels of MMP).

Range of results (MMP level) taken to be;

- High stress areas 50ng/mg (previous study ruptures site 59ng/mg (20-79)) (assume slightly lower degree of variation due to lower mean value assumed and the observation that variance seems to be very much reduced in the arteriotomy site samples with their much lower mean level of MMP).
- Low stress areas: 7ng/mg (previous study arteriotomy site 8ng/mg (14-22) (assume variance continues to decrease with smaller size of mean).

Mean difference in tissue MMP between high and low stress sites = 15ng/mg.

Standard deviation of mean difference calculated as 12.6ng/mg with correlation between samples = 0.

Sample size calculation carried out with statistical software package "PASS 2002" from NCSS (Number Crunching Statistical Systems, Kaysville, Utah, US), using paired T-test. N=10 for 95% power at p=0.05 (N=8 for 81% power at p=0.05).

3.1.3. Patient recruitment & history

Patients listed for elective open AAA repair surgery with any of the 5 consultant surgeons in the academic vascular unit, Hull and East Yorkshire Hospitals NHS Trust were approached and full informed consent was obtained for inclusion in this trial. Information including relevant co-morbid and drug history was obtained, as was systolic blood pressure obtained at pre-assessment clinic, on admission for elective AAA repair or at most recent surgical clinic (in order of preference).

Exclusions

Patients undergoing emergency AAA repair and elective EVAR were not included.

3.1.4. CT scan and calculation of peak wall stress

Abdominal spiral CT scans were carried out as part of normal care (Mx8000 quad slice and Mx8000 dual slice, Philips) with scan initiation at peak of contrast uptake (100ml Ultravist 300, Schering AG Germany, product licence 0053/0174, administered with bolus pro software). Nominal slice thickness was 3.2mm with 50% overlap and a helical pitch of 0.875. The scanners were set to 120kV and 250mA with a standard algorithm. Abdomen and pelvis was imaged to visualise the infra-diaphragmatic aorta down to common iliac artery.

3.1.4.1. AAA image extraction / FEA analysis

Our AAA image extraction technique has been published (Heng et al., 2008), and is as follows.

CT images from renal arteries to aortic bifurcation were selected and imported to image processing software (Scion Image v4.0, Scion corporation, Maryland, USA), and each individual slice was analysed manually to obtain the location of the external aortic wall by a number (30-50) of mouse clicks, recording x,y coordinates (figure 12, below). The z coordinate was subsequently added by inputting the slice thickness. This data formed a cloud of points (figure 13, below) which was imported into 3D image rendering software (Rhinoceros v2, Seattle, USA), creating a 3D image of the external surface of the aneurysm. This surface data was exported in a RAW-triangle format into a finite element analysis programme (Ansys 7.0, ASN Systems Ltd, Cannonsburg, USA). Other information was also input including material properties (Raghavan et al., 2000), constraints and loading conditions (which includes systolic blood pressure). A von-Mises stress diagram was produced (figure 14, below) which showed the aneurysm in a state of equilibrium with stress values represented by different colours. It also specifically indicated with maximum stress site (MX) and value for each aneurysm, and also the minimum stress site (MN). This data was conveyed to the vascular surgeons in the form of a simplified diagram indicating the peak stress site (see tissue biopsy section below), and this information was also given as clock face position, and position with relation to aortic bifurcation and renal arteries (ie 3 o'clock, 3/5 of way up from aortic bifurcation to renal arteries).

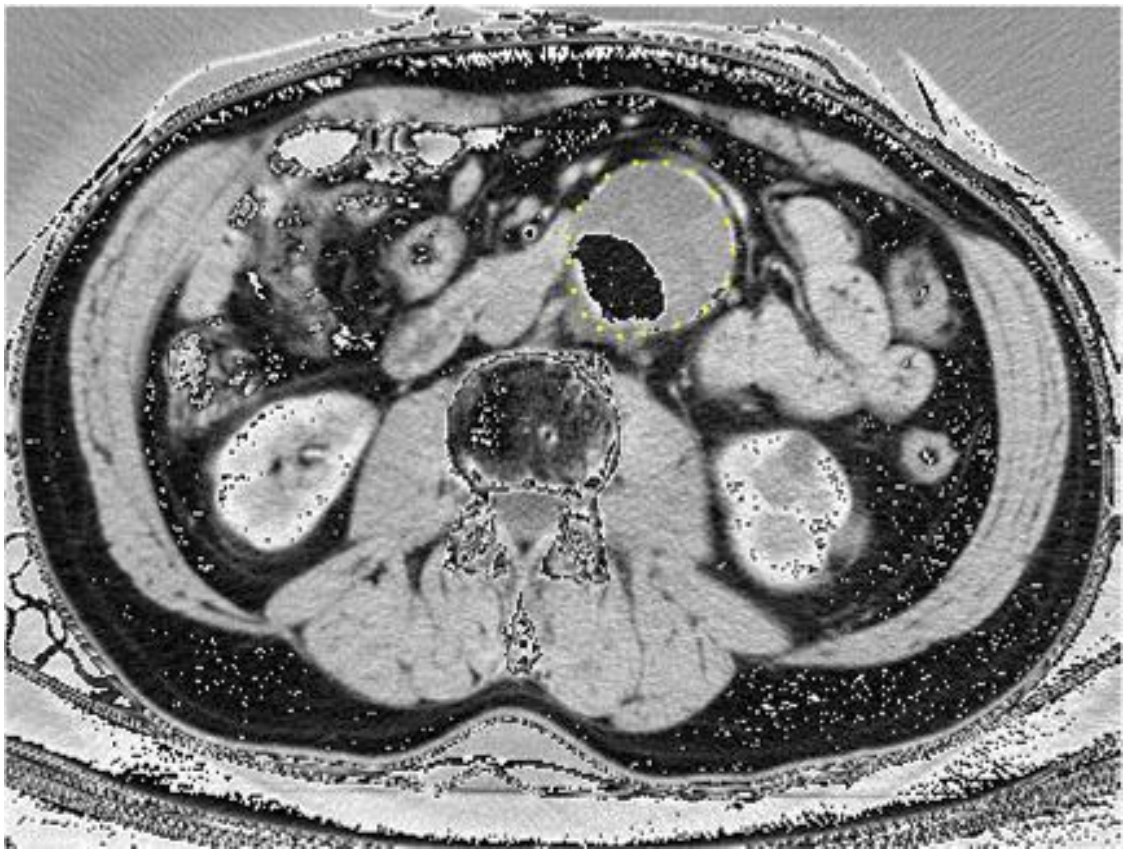


Figure 12. CT image with manually outlined edge of AAA

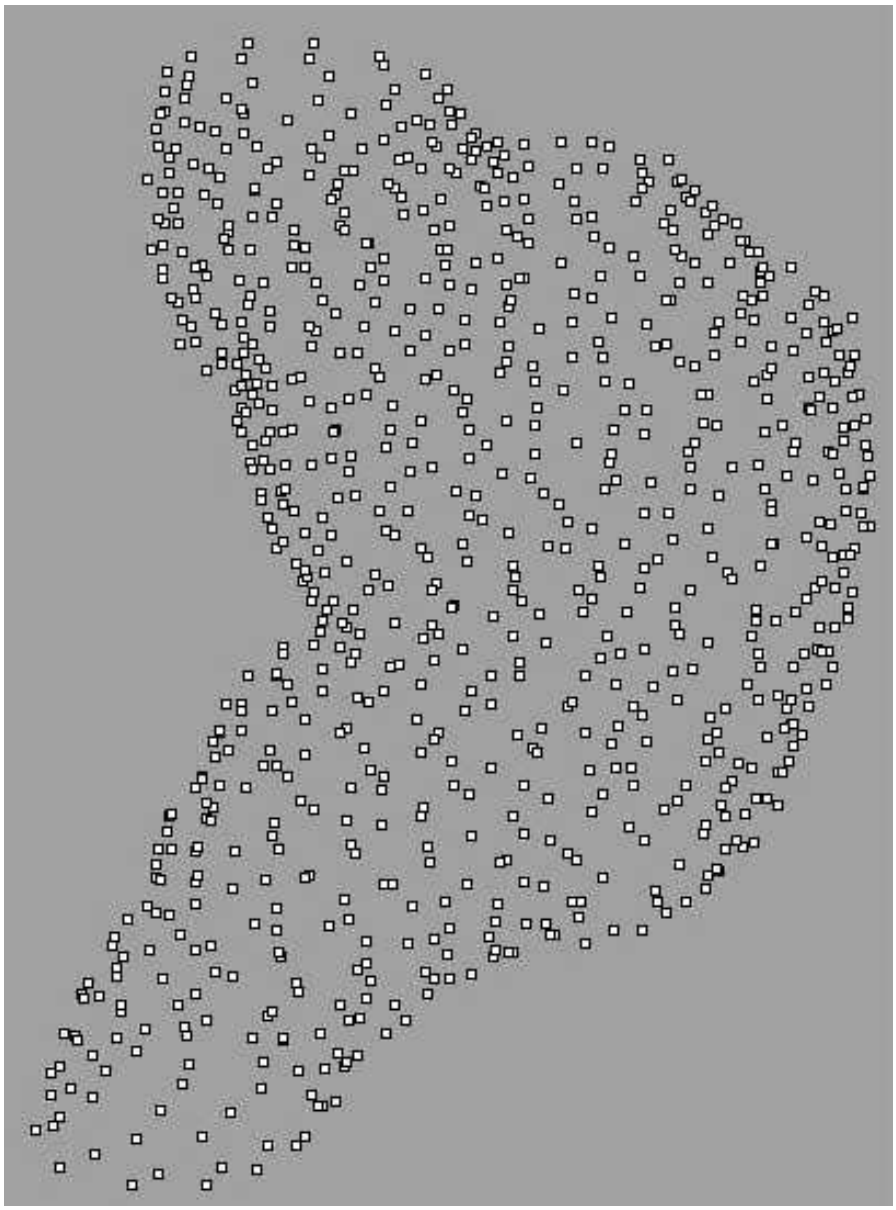


Figure 13. Cloud of points generated from manually extracted CT AAA edge data

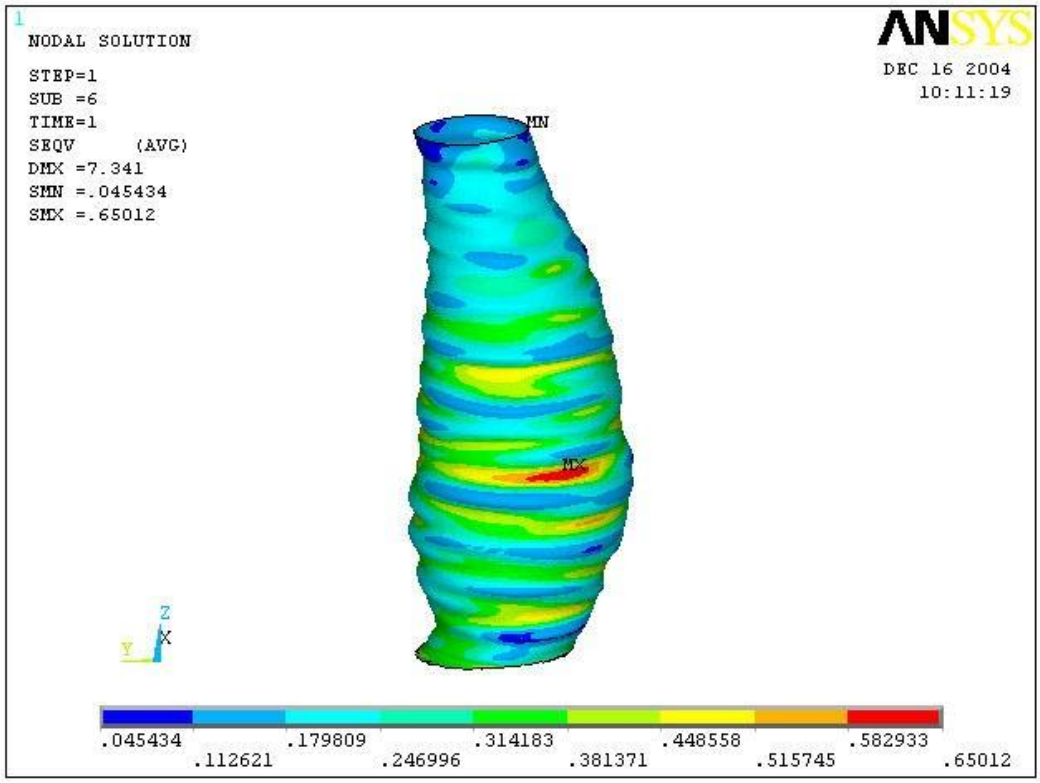


Figure 14. Von Mises stress diagram demonstrating stress patterns within the AAA, and the peak stress site (MX - peak wall stress site; MN – minimum stress site)

3.1.5. Blood samples

Both venous plasma and serum samples were obtained with consent as part of the protocol from patients involved in the study, on the day of admission, (1 day prior to AAA repair). The serum blood samples were collected into a gold top SST BD vacutainer bottles containing spray-coated silica and a polymer gel for serum separation. The plasma samples were collected into dark green top BD vacutainer bottles containing spray coated lithium heparin, with no gel separator. These samples

were centrifuged within 30 minutes of venepuncture, the liquid fraction pipetted into sarstedt tubes, labelled and slow frozen to -80°C.

3.1.6. Tissue Biopsy

A clinical vascular research fellow would be in theatre at the time of the aneurysm repair, to liaise with the surgeon, receive the tissue biopsy and prepare it for snap freezing in liquid nitrogen.

A surgeon's stress map was produced for each patient with the area of peak stress marked (figure 15, below), together with positional information as detailed in the AAA image extraction section above. The low stress areas could also be marked on the stress map, but this wasn't used since the second biopsy sample was an arteriotomy sample. 2 biopsies were obtained from each patient, one from the peak wall stress site and one from the arteriotomy site. The biopsies were requested to be 15 x 5mm minimum in dimension, but sometimes due to technical difficulties, smaller samples were obtained. The sample preparation was carried out with gloves and separate instruments for each biopsy site, and consisted; washing the sample in sterile sodium chloride, dividing the sample into 3 (minimum size 5 x 5mm) for MMP testing, weighing these resultant samples and wrapping the samples in aluminium foil with appropriate labelling prior to insertion into the liquid nitrogen for snap freezing, to ensure preservation of the enzymes involved. Where smaller samples were obtained, less dividing of samples was carried out as each specific sample had a minimum size. The maximum time between harvesting of sample and snap freezing was 10 minutes. The liquid nitrogen canister was transferred to the Biological Sciences Laboratory, University of Hull, once a week for transfer of samples and storage in -80°C freezers.

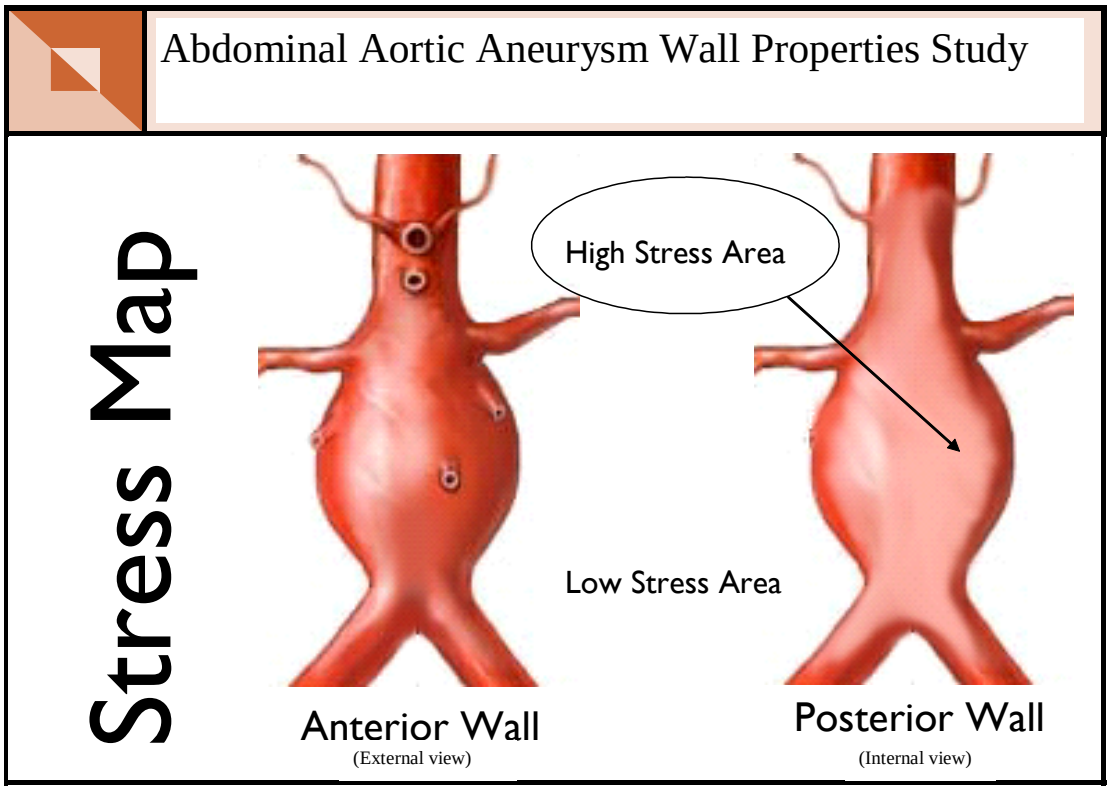


Figure 15. Surgeon's stress map

3.1.7. Tissue storage

Tissue was stored at -80°C freezers, in a secure university biomedical laboratory, with remote high temperature alarms.

3.1.8. Tissue extraction and purification of MMPs / TIMPs

Tissue extraction was carried out following a validated technique, previously used in several published papers (Vine and Powell, 1991, Wilson et al., 2006).

3.1.8.1. Tissue homogenisation

Tissue homogenisation is a procedure enabling substances within cells and the extracellular matrix to be analysed, and consists of mechanical and chemical disruption

of the substance to destroy cell membranes and release proteases from chemical bonds. Tissue homogenisation was carried out with approximately 1ml/100mg wet tissue weight cold homogenising buffer (distilled water, Brij 35, Urea, Tris-hydrochloric acid, EDTA, pH 7.6 – details below). The average sample size was 300mg requiring 3ml of buffer. Due to running ELISA tests in duplicate, and analysing active and total fractions of MMPs, a set of ELISA plates (1 ELISA plate each to test for MMP2, MMP8, MMP9, TIMP1, TIMP2) required 20 samples, and thus 60ml (3ml x 20) of buffer was required, but 100ml was prepared to allow for wastage. The mechanical homogenisation process was carried out manually with 2 scalpels, and the sample on ice. Physical disruption of the cell membrane and extracellular matrix is often carried out with a specialised tissue blender, but after consultation, this was considered unnecessary. More importance was associated with chemical homogenisation, and the details of previous studies regarding homogenising buffer was followed exactly.

Homogenising buffer

The homogenising buffer was prepared 1 to 2 days in advance of use, but pH was adjusted on the day of use and protease inhibitor was also added at that time. The buffer was stored in the fridge and used cold. As detailed above, distilled water is used as the main ingredient of the homogenising buffer. The ingredients of the homogenising buffer are detailed in the appendix.

3.1.8.2. Centrifugation

Centrifugation was at 15,000rpm for 59minutes at 2°C then the supernatant was aspirated leaving debris behind.

3.1.8.3. Dialysis

Samples were then dialysed overnight in dialysis tubing (pre-boiled visking tubing 12-14kDa molecular cut-off) with 75ml/1ml sample dialysis buffer (pH 8.5, distilled water, Brij35, calcium chloride, Tris-hydrochloric acid, details below), with both ends of each sample sealed with plastic clips. The samples were then labelled and slow frozen down to -80°C.

Visking tubing is a form of processed cellulose which has pores, and is considered a partially permeable membrane. It is available in a variety of pore sizes from 1350Da to 12-14kDa which is one of the bigger sizes of pore. This size is chosen as it is smaller than the smallest of substances with which this study is interested (TIMP2, 21kDa). Since the membrane is supplied dry, it is necessary to carefully re-hydrate it, and some cleaning is usually recommended. Boiling the visking tubing achieves both these ends, but it is necessary to ensure that the prepared tubing is kept fully immersed in distilled water until it is used, and that this is not for a prolonged period of time.

3.1.8.3.1. Dialysis Buffer

The dialysis buffer was prepared 1 to 2 days prior to dialysis, and the pH was adjusted on the day of use. Approximately 3 litres of dialysis buffer was used each time. The ingredients of the buffer are detailed in the appendix.

3.1.9. ELISA testing & protein concentration determination

ELISA testing carried out with Biotrak MMP activity assay kits, Biotrak TIMP assay kits, and protein concentrations were determined using the Bradford Assay, with Biorad protein concentration assay.

MMP, TIMP and protein levels were routinely analysed in duplicate or more as per standard lab practice for quality control purposes. Certain trials were carried out with individual samples where this was simply to gauge dilution levels prior to full plate testing. Certain plasma samples were also tested individually alongside duplicated samples (which would validate the plate and my technique) in an attempt to analyse more samples.

Duplicated levels that disagreed more than 35% - suggesting a problem with the pair of samples, were removed at an early stage before analysis. Values out of standard range were removed at an early stage, prior to analysis. Where several results were obtained for a given sample, the most recent result was used for final analysis (final results table). Where values were found that demonstrated a problem with the MMP detection of the sample ie total level of MMP2 found to be lower than active level of MMP2 in a given sample, the pair of results was removed from the final analysis table, and an older set of results used. If possible, the entire set of results (ie patient no. 4 active MMP2 high stress site, patient no. 4 total MMP2 high stress site, patient no. 4 active MMP2 arteriotomy site, and patient no. 4 total MMP2 arteriotomy site) was used from an earlier plate.

Final results for analysis were taken to be the latest result, as these plates would have the benefit of the earlier plates to sort out teething problems, and methodology. Where the standard and extended ranges for a plate was used, if no additional results were gained in the extended range compared to the standard range, then the extended range was disregarded.

Details of individual kits and procedures used as detailed in the appendix.

3.1.10. Data recording and statistical analysis

Lab data including plate identification tables, and dilutions were recorded in the lab on pre-prepared plate template sheets, and notes were taken of actual timings etc. These were then stuck into a lab book.

Electronic lab data including optical density plate readings results were recorded on Microsoft Excel format and stored as separately labelled files. The output tables were labelled, and this labelling was kept with the data in order to minimise the risk of the results tables being mixed up. These source files have been kept unaltered except for data transfer computer to computer and for data backup purposes.

These results tables were copied into analysis files where data was manipulated as demonstrated in the examples above to obtain results in ng/mg protein for tissue samples, and ng/ml for plasma samples.

Basic descriptive data was obtained from Microsoft Excel, and detailed descriptive together with statistical analysis were obtained using SPSS for windows version 11.5 to version 16.

3.1.11. Statistical analysis

Descriptives

The age of patients involved in the study was presented as range (median). Various co-morbid factors were presented as % (absolute number). AAA diameter and peak wall stress are also presented as range (median).

Correlations

Correlations for normally distributed data were reported with Pearson's correlation coefficient (r), and the appropriate significance level. Correlations for non-normally distributed data were reported with rank correlation coefficients - Spearman's ρ (r_s), and Kendall's τ (τ), with the appropriate significance.

Tests for normality

MMP tissue and plasma concentration data was tested for normal distribution, using visual histogram, Q-Q plots and the Kolmogorov-Smirnov test (non-significance indicating normal distribution, i.e. $p>0.05$). In general these data were non-normally distributed therefore non-parametric tests were used for analysis of these data.

MMP / TIMP concentrations

These were reported as median (interquartile range), and the null hypothesis was that there was no difference between concentrations of an individual MMP or TIMP at the peak stress site versus the arteriotomy site. This hypothesis was tested using the data and the Wilcoxon signed ranks test for testing non-parametric paired data. Significance was taken at $p\leq0.05$, and indicated rejection of the null hypothesis.

MMP ratios

MMP ratios (eg the ratio of active to total MMP2 at peak stress site, and the ratio of active to total MMP2 at arteriotomy site) were tested and reported as normally distributed. The difference in ratios were reported as means, 95% confidence interval, and tested with the paired t-test.

TIMP ratios

The ratio of individual TIMPs at arteriotomy site to peak stress site was simply reported. The ratios of certain MMPs against certain TIMPs at peak stress versus arteriotomy sites were also compared by non-paramatric paired tests (Wilcoxon signed ranks test).

Reliability of runs

This was compared using the samples from the same patients and analysing these samples using the same tests, but after a time interval. Correlations, and the significance of these were assessed, together with analysis to determine the mean absolute difference, mean percentage differences, and mean differences from the mean found between the test results.

3.2. Results

3.2.1. Patients and demographics

36 patients were recruited into the study with informed consent, of which 26 CT scans successfully retrieved, finite element analysis performed and peak wall stress levels calculated. 22 of these patients then went on to have tissue biopsied. There were various reasons that patients recruited did not have tissue biopsied including, digital CT scan images not obtained (not sufficient time to obtain scan, CT archive not accessible, scan carried out at separate facility -private), finite element analysis not performed (not sufficient time, lab computer not available), research fellow not available at time of AAA repair to direct and obtain tissue sample (on-call at separate hospital), request for tissue denied at time of AAA repair due to patient instability (n=1). There were no patient refusals. Of these 36 patients, the age range was 63 to

85(median 73), 83%(30) were male, 39%(14) were current smokers, 89%(32) smoked at some point in their life, the median number of pack years was 32. 56%(20) had hypertension, 25%(9) had a previous cerebrovascular accident or transient ischaemic attack, 25%(9) had a history of ischaemic heart disease, 22%(8) had a previous myocardial infarction and 8%(3) had a history of diabetes mellitus. In the drug history, 61%(22) were on aspirin, and 39%(14) were on statins (table 9, below).

Table 9. Demographics of recruited patients

Demographics n=36	
Age range (median)	63-85 (73)
Sex (male)	83% (30)
Co-morbid History	
Current smokers	39%(14)
History of smoking (at any point)	89%(32)
Pack years range (median)	5-104(32)
Hypertension	56%(20)
Range of systolic blood pressure (median)	105-194(155)
Previous CVA / TIA	25%(9)
Ischaemic heart disease	25%(9)
Myocardial infarction	22%(8)
Diabetes mellitus	8%(3)
Drug History	
Aspirin	61%(22)
Statin	39%(14)

3.2.2. AAA Diameter

AAA diameter ranged from 5cm to 8.5cm, with a median size of 5.8cm.

3.2.3. Peak wall stress

Peak wall stress varied from 0.46 to 1.33MPa (median 0.74MPa). There was no correlation between Peak Wall Stress and AAA diameter (r_s = 0.49, p=0.18, Spearman's ranked correlation coefficient) (figure 16, below).

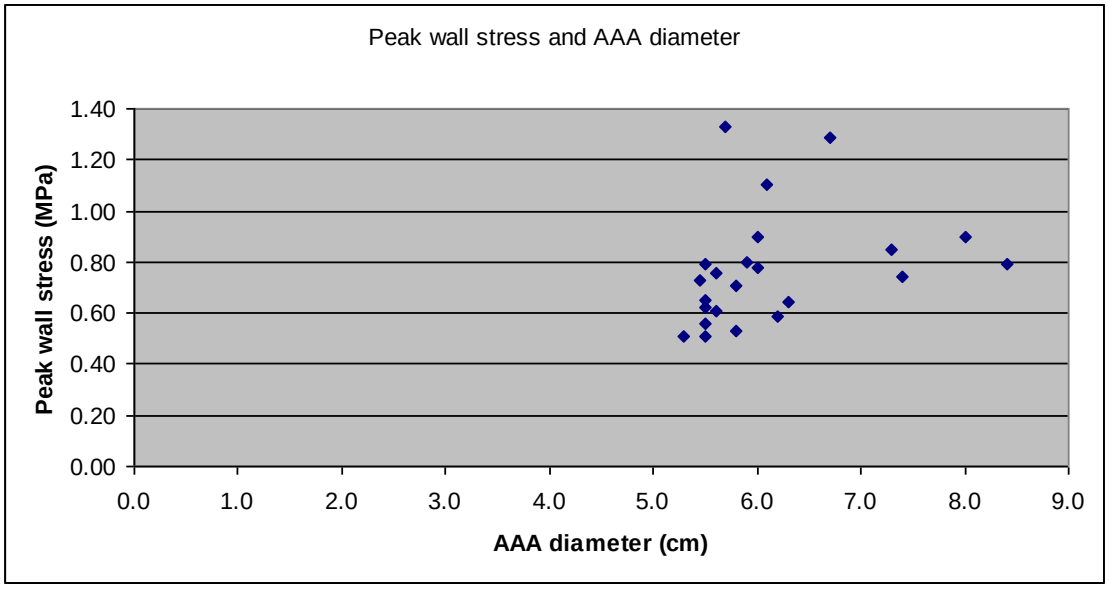


Figure 16. Scatter graph of AAA diameter and peak wall stress

3.2.4. Tissue MMP & TIMP

7 testing runs were carried out during the course of this project. A run consisted of the process from the removal of a snap frozen selection of tissue samples from -80°C storage, through tissue homogenisation, dialysis, and ELISA analysis of MMP and/or TIMP concentration, with protein determination of each selection of tissue samples. Tissue homogenates were frozen after dialysis, and thawed prior to ELISA analysis and protein concentration determination.

Run 1 was mainly to establish the range of concentrations on our samples (obtained initially from literature), and to fine tune dilutions and techniques. Run 2 was a straightforward run. Run 3 was a duplicate of run 2 in order to ascertain the reproducibility or reliability of the results obtained from run to run. Run 4 was a limited run involving only some tests as not all tests were available. Run 5 was carried out to complete the tests not carried out in run 4. Run 6 was again a complete run involving AAA tissue, and finally run 7 was for plasma MMP / TIMP concentration determination.

3.2.4.1. MMP2

The number of samples analysed for MMP2 was 22. An inadequate sample was obtained for subject 11 resulting in no total MMP2 concentration at the arteriotomy site being obtained. Analysis of active and total forms of MMP2 measured at peak stress and arteriotomy sites showed a non-normal distribution, except for active MMP2 at the arteriotomy site which may have a normal distribution (p=0.2, Kolmogorov-Smirnov test). Non-parametric tests were therefore used to compare these results. Raw results are found in a tabulated form in appendix 4.

Active MMP2

The median concentration of active MMP2 was 2.80ng/mg (interquartile range 1.49-4.89) at the peak stress site, and 2.45ng/mg (1.38-4.87) at the arteriotomy site (figure 17, below). There was no statistically significant difference between these sites (p=0.81, Wilcoxon signed ranks test).

No correlation was found between active MMP2 at peak wall stress and arteriotomy sites [$\tau = 0.117$ p=0.446 (Kendall's τ rank correlation coefficient), $r_s = 0.171$ p=0.446

(Spearman's ρ rank correlation coefficient)]. A scatter graph with regression line for the above data is shown below (figure 18, below).

Total MMP2

The median concentration of total MMP2 was 24.41ng/mg (12.87-36.45) at the peak stress site, and 21.87ng/mg (13.00-32.82) at the arteriotomy site (figure 17, below). Again there was no statistically significant difference between these sites (p=0.66, Wilcoxon signed ranks test).

Correlation was found between total MMP2 samples found in peak wall stress and arteriotomy sites ($\tau = 0.314$ p=0.046, $r_s = 0.440$ p=0.046). A scatter graph with regression line for the above data is shown below (figure 19, below).

MMP2 Ratios

The ratio of active to total MMP2 at peak wall stress sites, and arteriotomy sites (figure 20, below) were normally distributed (p=0.2, p=0.09 respectively, Kolmogorov-Smirnov test). There was no significant difference between the ratios of active MMP2 to total MMP2 at peak wall stress versus arteriotomy sites (Mean difference -0.024, 95% CI -0.062-0.015, p=0.212, paired t-test). The negative mean difference seen here suggests that the ratio of active to total MMP2 is actually higher at the arteriotomy site compared to the peak wall stress site, although the 95% confidence interval includes 0 and therefore shows no significant difference between the ratios.

There was also no difference found on comparing the ratio of MMP2 to total MMPs (MMP2+MMP8+MMP9) at the peak stress site to the arteriotomy site.

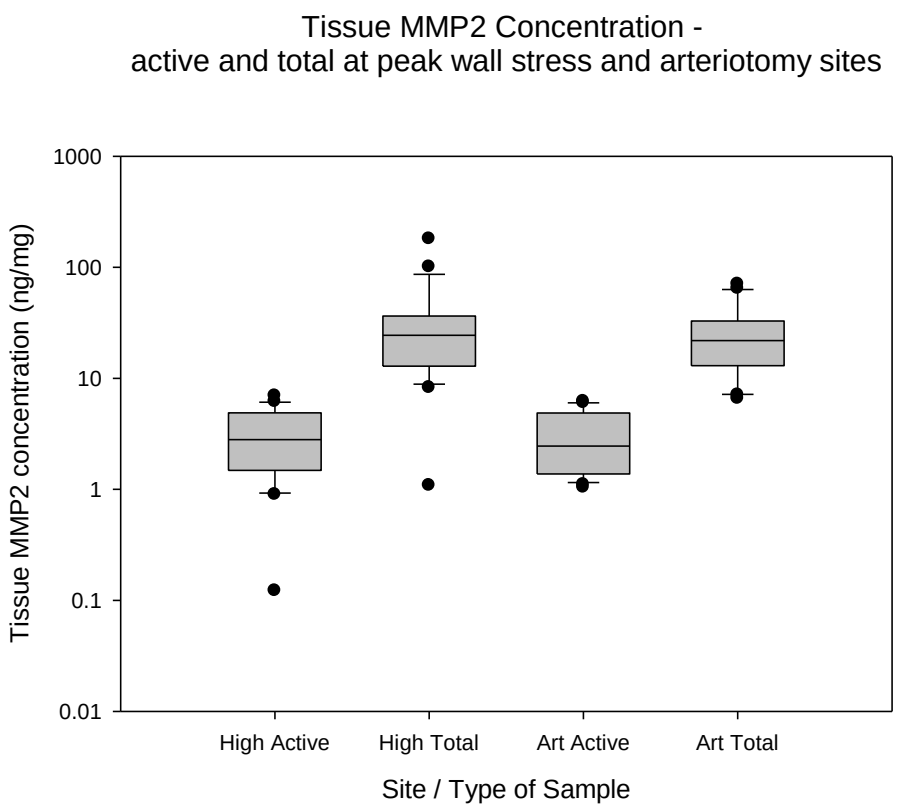


Figure 17. Tissue MMP2 concentration – active and total at peak wall stress and arteriotomy sites

High Active: Active MMP2 at peak wall stress site, High Total: Total MMP2 at peak wall stress site; Art Active: Active MMP2 at arteriotomy site; Art Total: Total MMP2 at arteriotomy site. Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

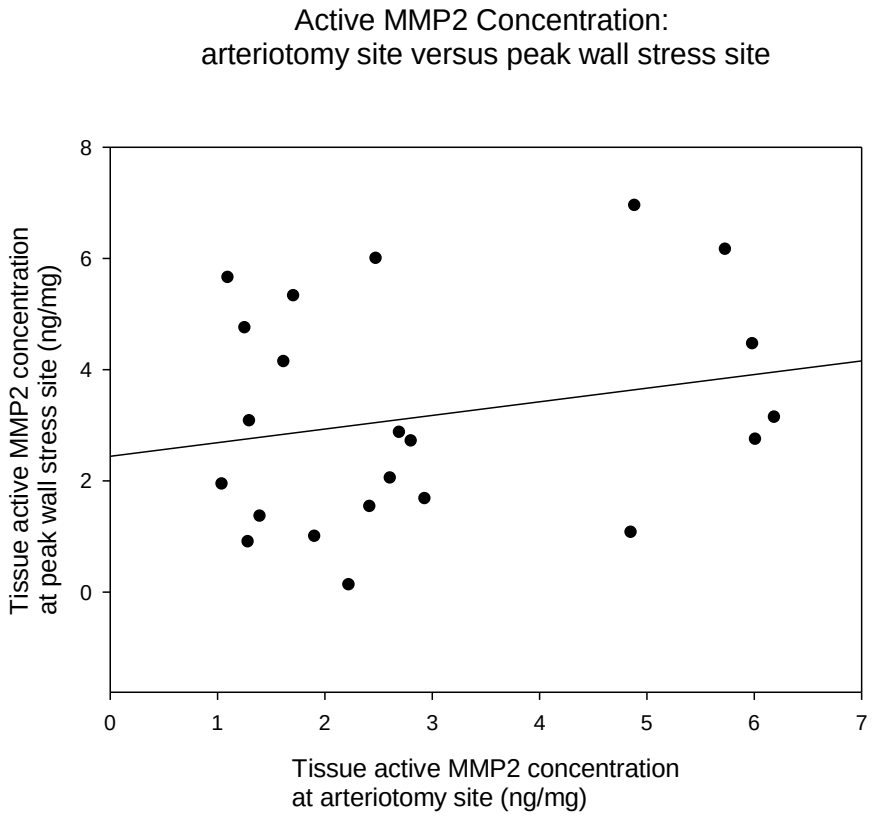


Figure 18. Active MMP2 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line.

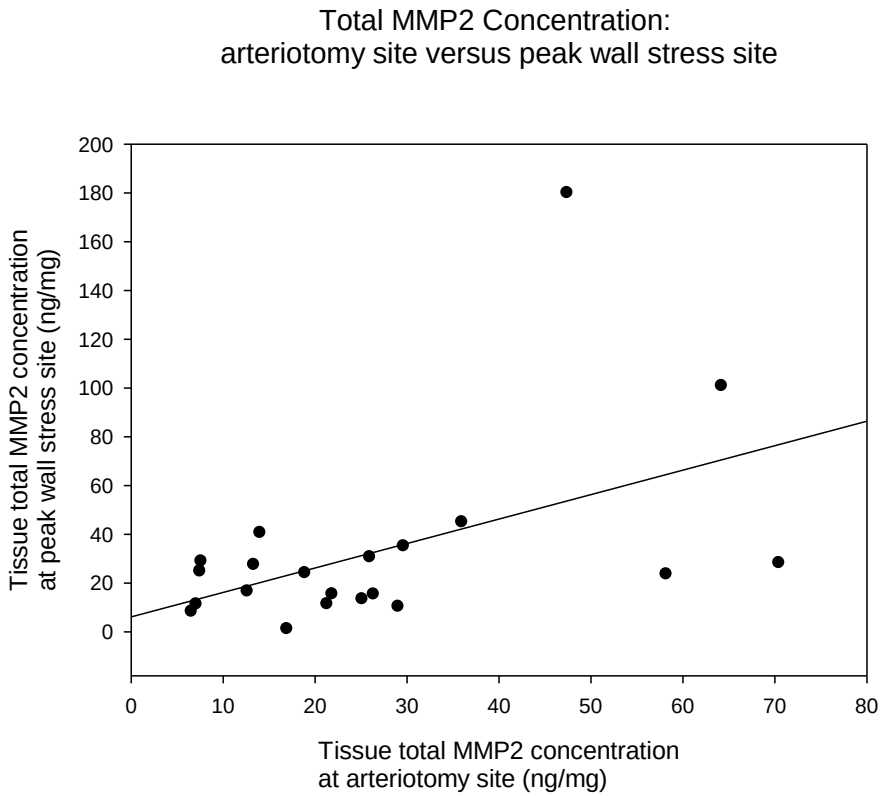


Figure 19. Total MMP2 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line.

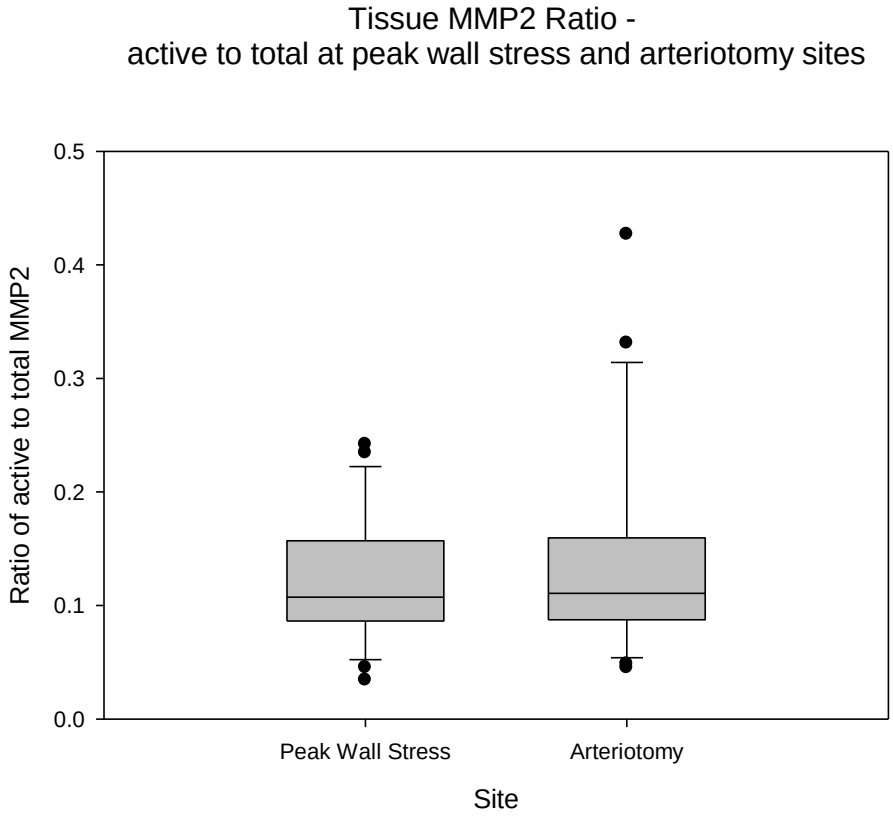


Figure 20. Tissue MMP2 Ratio – active to total at peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.2. MMP8

The number of samples analysed for MMP8 was 22. Subject 11 had no satisfactory result obtained for total MMP8 concentration either at the peak wall stress site or the arteriotomy site. Analysis of active and total forms of MMP8 measured at peak stress and arteriotomy sites showed possible normal distributions (active MMP8 peak stress site p=0.2, total MMP8 peak stress site p=0.12, active MMP8 arteriotomy site p=0.16, Kolmogorov-Smirnov test), except for total MMP8 at the arteriotomy site which has a

non-parametric distribution. As not all data had normal distributions, non-parametric tests were used to compare these results. Raw results are found in a tabulated form in appendix 4.

Active MMP8

The median concentration of active MMP8 was 5.20ng/mg (interquartile range 2.54-9.82) at the peak stress site, and 6.99ng/mg (3.65-15.72) at the arteriotomy site (figure 21, below). There was no statistically significant difference between these sites (p=0.15, Wilcoxon signed ranks test).

A positive significant correlation was found between active MMP8 samples found in peak wall stress and arteriotomy sites ($\tau = 0.385$ p=0.012, $r_s = 0.523$ p=0.012). A scatter graph with regression line for the above data is shown below (figure 22, below).

Total MMP8

The median concentration of total MMP8 was 18.80ng/mg (9.82-31.85) at the peak stress site, and 16.44ng/mg (10.81-31.98) at the arteriotomy site (figure 21, below). There was no statistically significant difference between these sites (p=0.52, Wilcoxon signed ranks test).

A positive significant correlation was found between total MMP8 samples found in peak wall stress and arteriotomy sites ($\tau = 0.381$ p=0.016, $r_s = 0.508$ p=0.019). A scatter graph with regression line for the above data is shown below (figure 23, below).

Ratios

The ratio of active to total MMP8 at peak wall stress sites, and arteriotomy sites (figure 24, below) were normally distributed (p=0.13, p=0.2 respectively, Kolmogorov-

Smirnov test). There was a trend towards significance in the difference between the ratios of active MMP8 to total MMP8 at peak wall stress versus arteriotomy sites (Mean -0.14, 95% CI -0.29-0.01, p=0.064, paired t-test), with higher ratios being seen in 14 of the 21 samples at the arteriotomy sites compared to the peak wall stress site.

There was also no difference found on comparing the ratio of MMP8 to total MMPs (MMP2+MMP8+MMP9) at the peak stress site to the arteriotomy site.

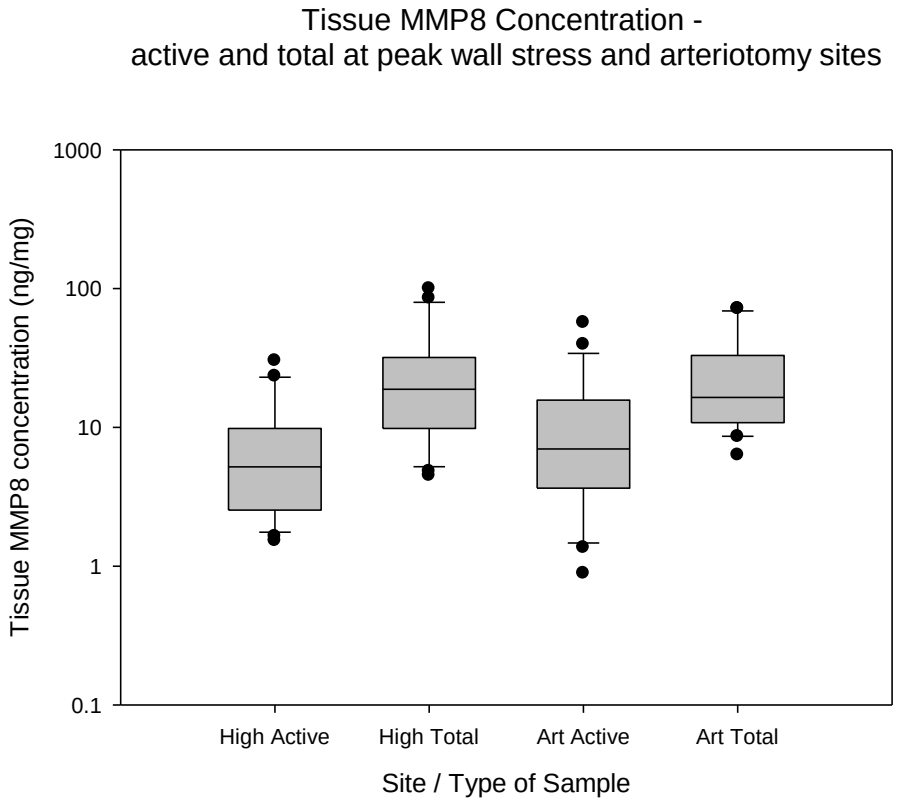


Figure 21. Tissue MMP8 Concentration – active and total at peak wall stress and arteriotomy sites

High Active: Active MMP8 at peak wall stress site, High Total: Total MMP8 at peak wall stress site; Art Act: Active MMP8 at arteriotomy site; Art Total: Total MMP8 at arteriotomy site. Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

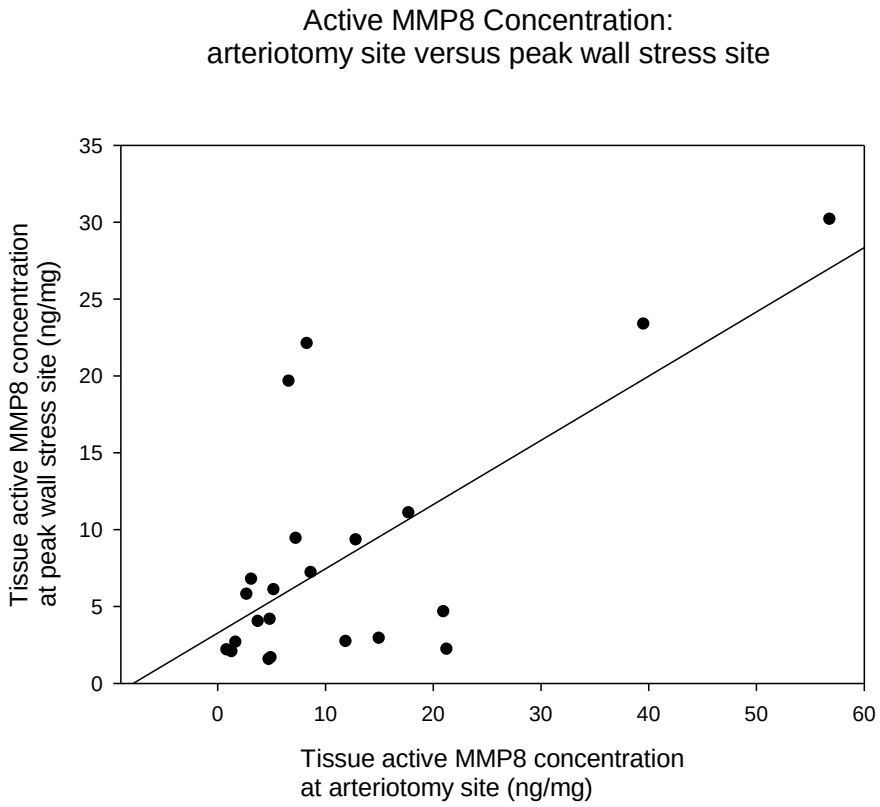


Figure 22. Active MMP8 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line

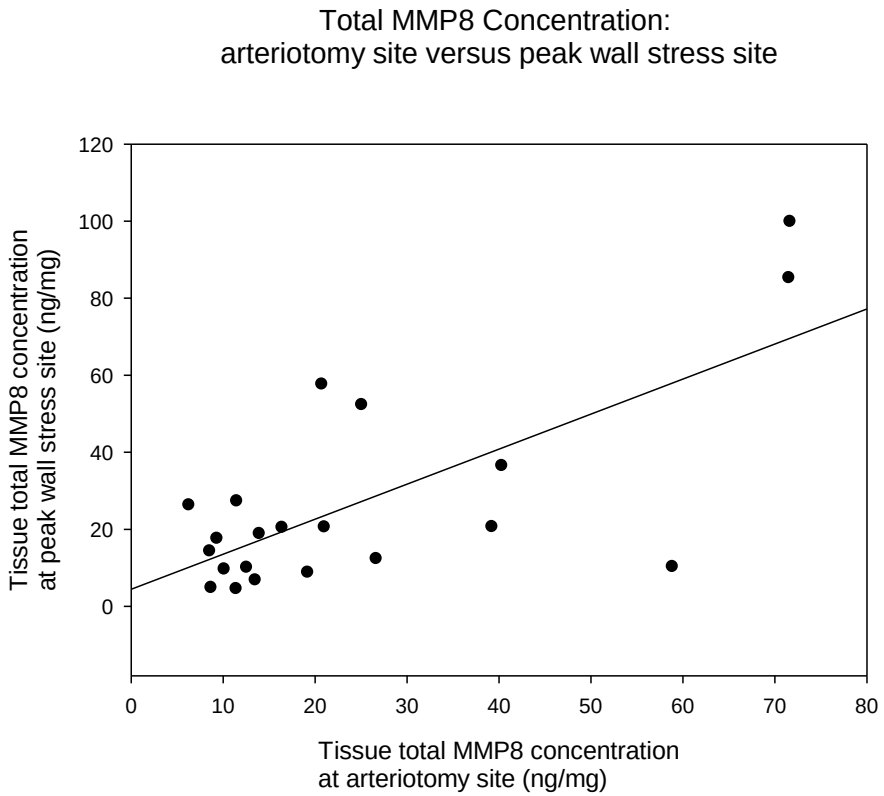


Figure 23. Total MMP8 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line

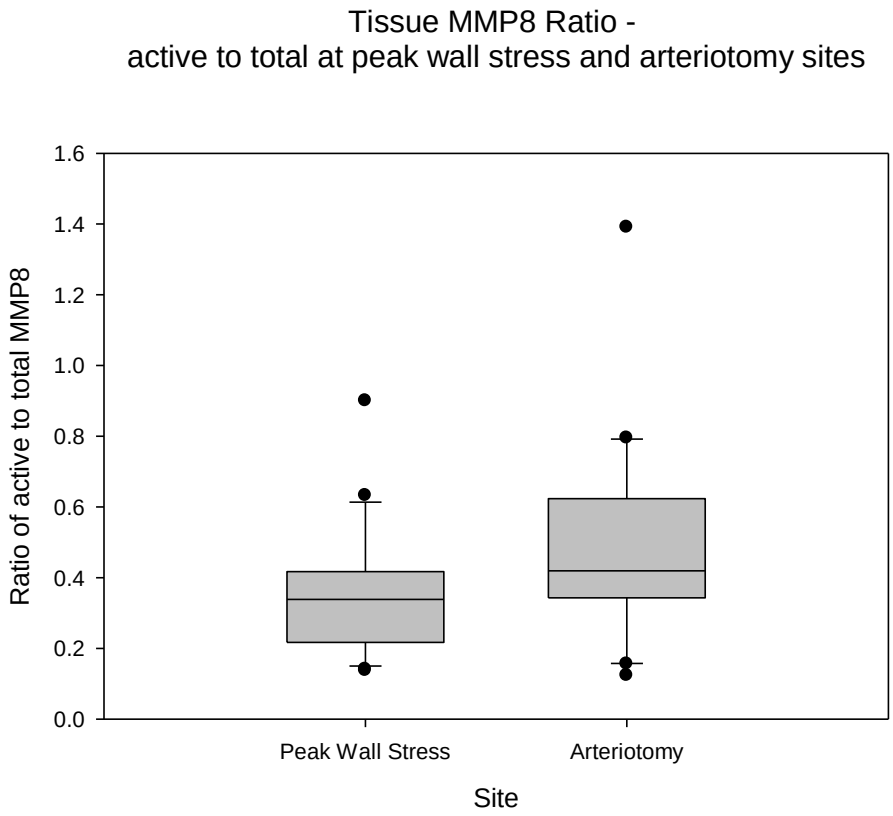


Figure 24. Tissue MMP8 Ratio – active to total at peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.3. MMP9

The number of samples analysed for MMP9 was 22. Subject 11 had no satisfactory result obtained for total MMP9 concentration either at the peak wall stress site or the arteriotomy site. Analysis of active and total forms of MMP9 measured at peak stress and arteriotomy sites showed a non-normal distribution, except for total MMP9 at the peak stress site (p=0.2, Kolmogorov-Smirnov test), and total MMP9 at the arteriotomy site which may have a normal distribution (p=0.14). Since not all data was normally

distributed, non-parametric tests were used to compare these results. Raw results are found in a tabulated form in appendix 4.

Active MMP9

The median concentration of active MMP9 was 0.58ng/mg (interquartile range 0.38-0.85) at the peak stress site, and 0.54ng/mg (0.32-0.97) at the arteriotomy site (figure 25, below). There was no statistically significant difference between these sites (p=0.78, Wilcoxon signed ranks test).

No correlation was found between active MMP9 at peak wall stress and arteriotomy sites ($\tau = 0.083$ p=0.592, $r_s = 0.154$ p=0.493). A scatter graph with regression line for the above data is shown below (figure 26, below).

Total MMP9

The median concentration of total MMP9 was 9.70ng/mg (3.95-24.47) at the peak stress site, and 11.34ng/mg (8.55-20.49) at the arteriotomy site (figure 25, below). There was no statistically significant difference between these sites (p=0.85, Wilcoxon signed ranks test).

A positive significant correlation was found between total MMP9 samples found in peak wall stress and arteriotomy sites ($\tau = 0.457$ p=0.004, $r_s = 0.629$ p=0.002). A scatter graph with regression line for the above data is shown below (figure 27, below)

Ratios

The ratio of active to total MMP9 at peak wall stress sites, and arteriotomy sites (figure 28, below) were normally distributed (p=0.12, p=0.20 respectively, Kolmogorov-Smirnov test). There was no significant difference between the ratios of active MMP9

to total MMP9 at peak wall stress versus arteriotomy sites (Mean 0.0054, 95% CI -0.0070-0.018, p=0.38, paired t-test).

There was also no difference found on comparing the ratio of MMP9 to total MMPs (MMP2+MMP8+MMP9) at the peak stress site to the arteriotomy site.

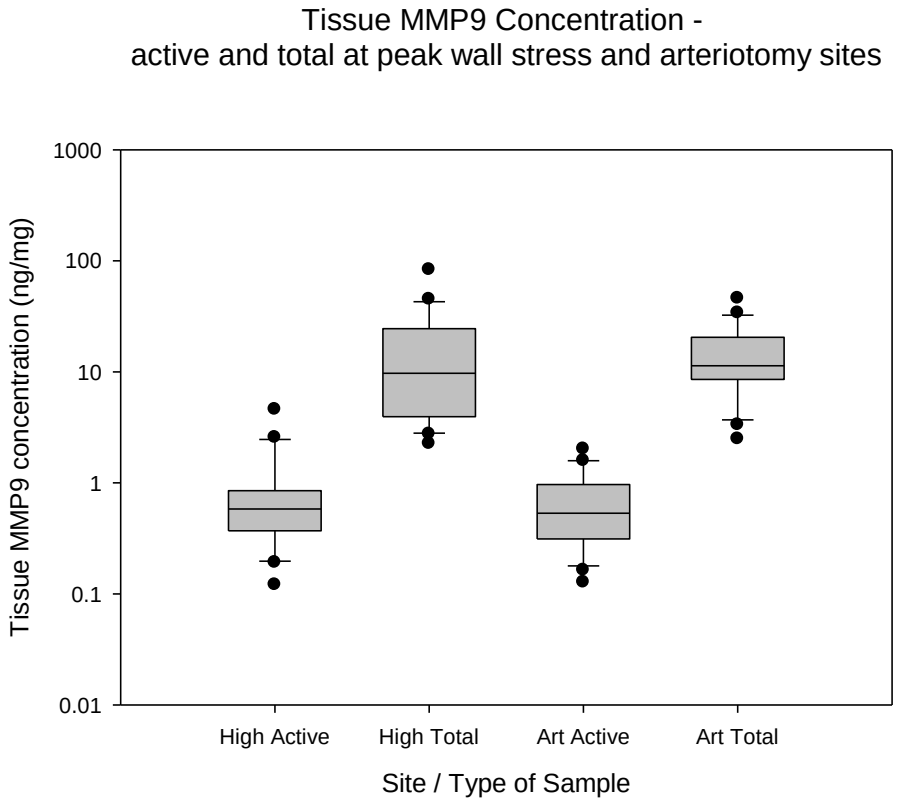


Figure 25. Tissue MMP9 Concentration – active and total at peak wall stress and arteriotomy sites

High Active: Active MMP9 at peak wall stress site, High Total: Total MMP9 at peak wall stress site; Art Active: Active MMP9 at arteriotomy site; Art Total: Total MMP9 at arteriotomy site. Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

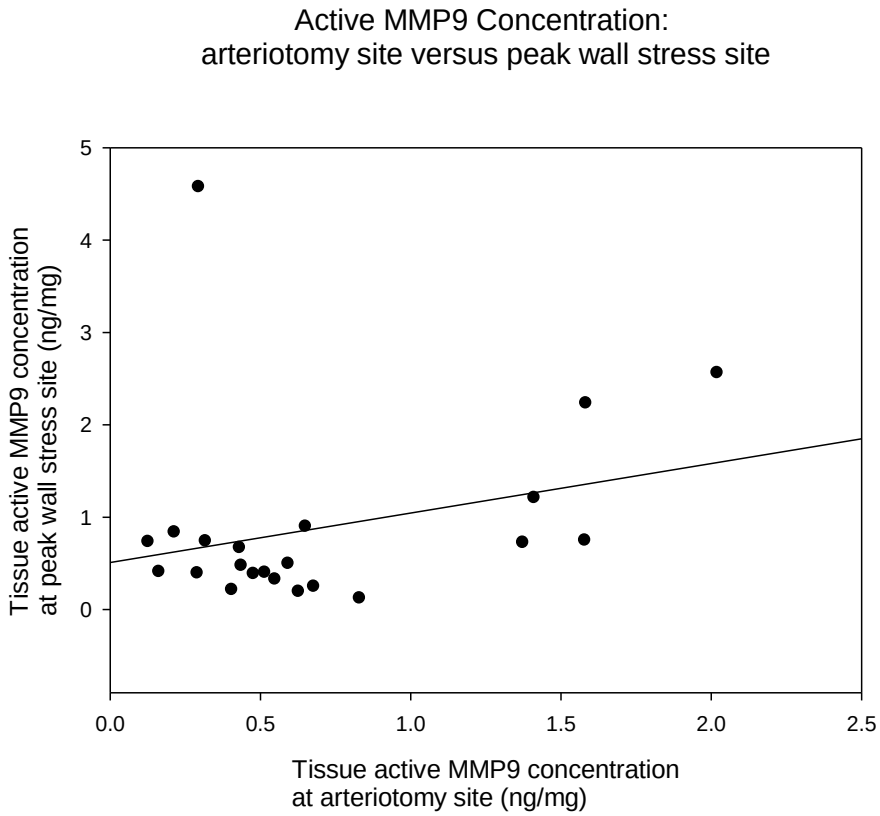


Figure 26. Active MMP9 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line

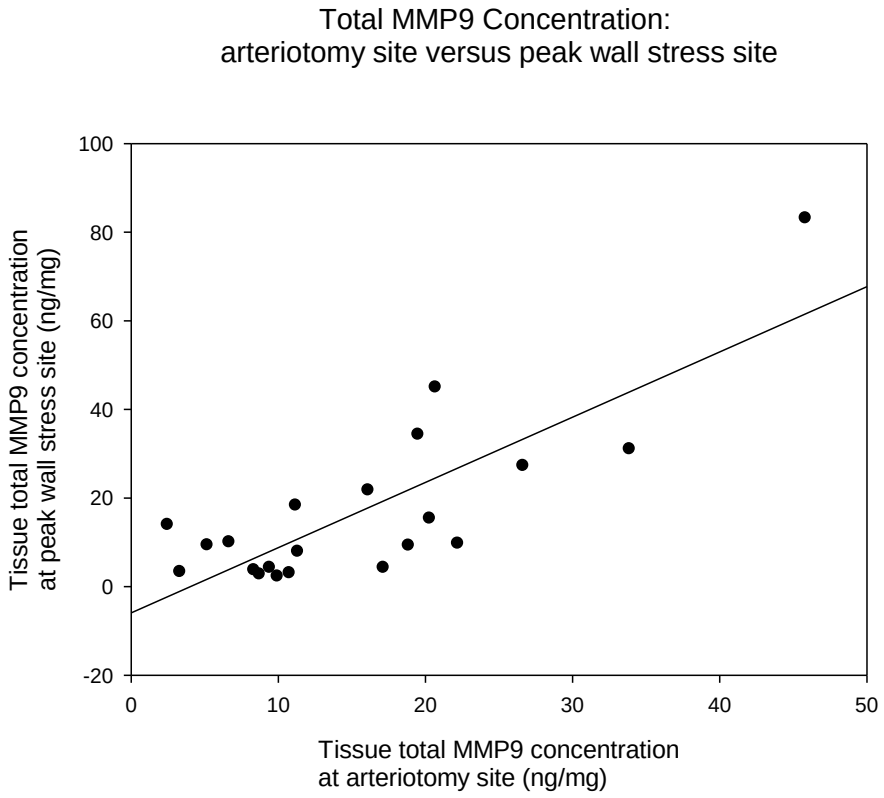


Figure 27. Total MMP9 Concentration: arteriotomy versus peak wall stress sites

Scatter plot with regression line

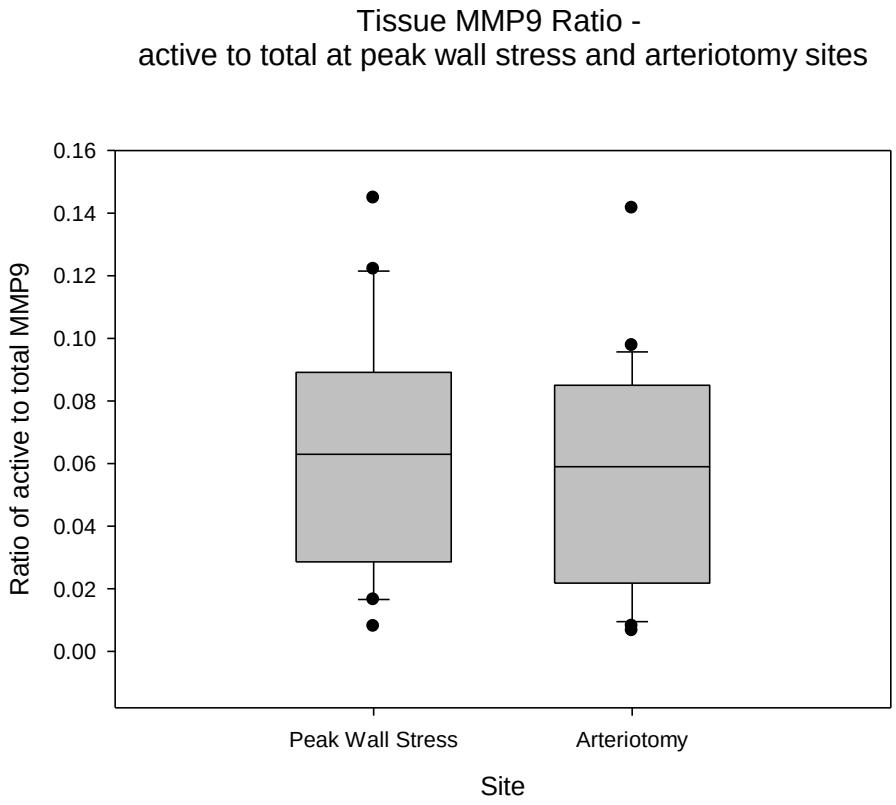


Figure 28. Tissue MMP9 Ratio – active to total at peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.4. TIMP1

The number of samples analysed for TIMP1 was 22. Analysis of TIMP1 measured at peak stress sites showed a non-normal distribution, but TIMP1 at the arteriotomy site may have a normal distribution (p=0.2, Kolmogorov-Smirnov test). Non-parametric tests were therefore used, since not all the data had normal distributions. Raw results are found in a tabulated form in appendix 4.

TIMP1 concentration

The median concentration of TIMP1 was 364.84ng/mg (Interquartile range 210.49-523.26) at the peak stress site, and 229.84ng/mg (86.80-370.95) at the arteriotomy site (figure 29, below). No statistically significant difference was seen between the sites (p=0.2, Wilcoxon-signed ranks test).

There was no significant correlation between TIMP1 at the arteriotomy site and the peak wall stress site (see tables in appendix 4),

TIMP1 Ratio

The mean ratio of TIMP1 at arteriotomy to peak wall stress sites was 0.93 (range 0.07-3.10).

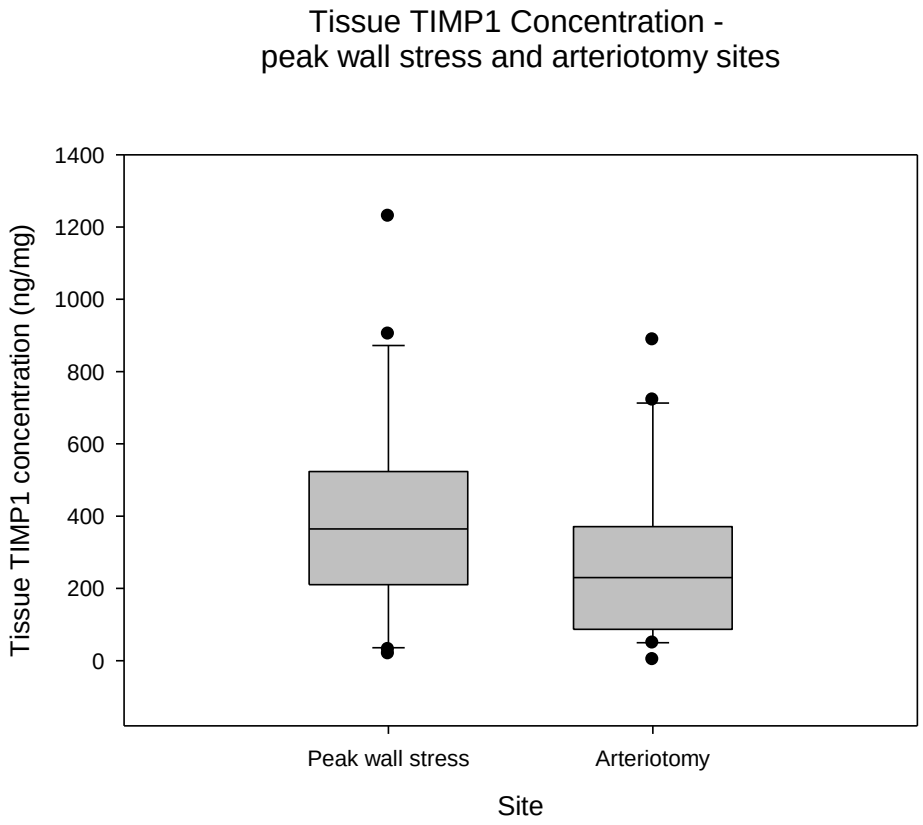


Figure 29. Tissue TIMP1 Concentration – peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.5. TIMP2

The number of samples analysed for TIMP2 was 22. Analysis of TIMP2 measured at peak stress (p=0.14, Kolmogorov-Smirnov test) and arteriotomy sites (p=0.14) showed a possibly normal distribution, however since the rest of the data was tested using non-parametric tests, these were also used here. Raw results are found in a tabulated form in appendix 4.

TIMP2 concentration

The median concentration of TIMP2 was 17.10ng/mg (Interquartile range 8.55-29.08) at the peak stress site, and 14.64ng/mg (8.56-29.93) at the arteriotomy site (figure 30, below). No statistically significant difference was seen between the 2 sites (p=0.76, Wilcoxon-signed ranks test).

There was no significant correlation between TIMP2 at the arteriotomy site and the peak wall stress site (see tables in appendix 4)

TIMP2 Ratio

The mean ratio of TIMP2 at arteriotomy to peak wall stress sites was 2.24 (range 0.15-14.32).

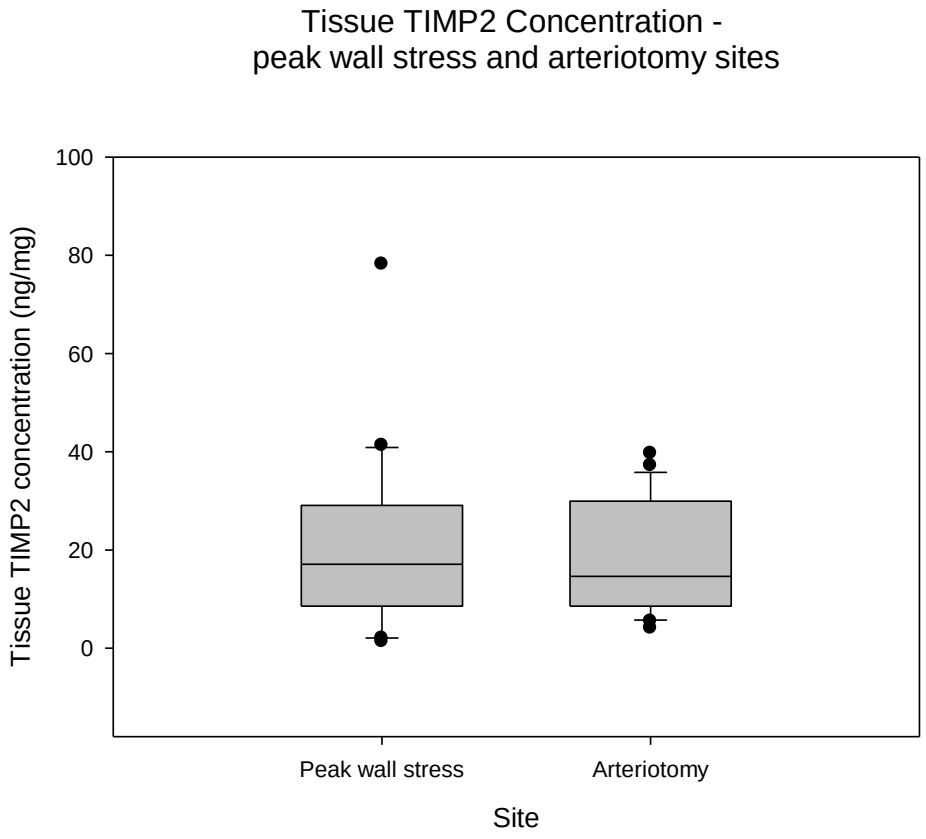


Figure 30. Tissue TIMP2 Concentration – peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.6. Protein concentration

The number of samples analysed for protein concentration was 22. Analysis of protein concentrations measured at peak stress (p=0.2, Kolmogorov-Smirnov test) and arteriotomy sites (p=0.2) showed a possibly normal distribution, however since the rest of the data was tested using non-parametric tests, these were also used here. Raw results are found in a tabulated form in appendix 4.

Protein concentration

The median concentration of Protein was 2.27mg/ml (Interquartile range 1.55-3.28) at the peak stress site, and 2.23mg/ml (1.16-3.77) at the arteriotomy site (figure 31, below). No statistically significant difference was seen between the 2 sites (p=0.96, Wilcoxon-signed ranks test).

Ratio of protein concentration at arteriotomy to peak stress sites

The mean ratio of protein concentration at arteriotomy to peak wall stress sites was 1.22 (range 0.07-5.12).

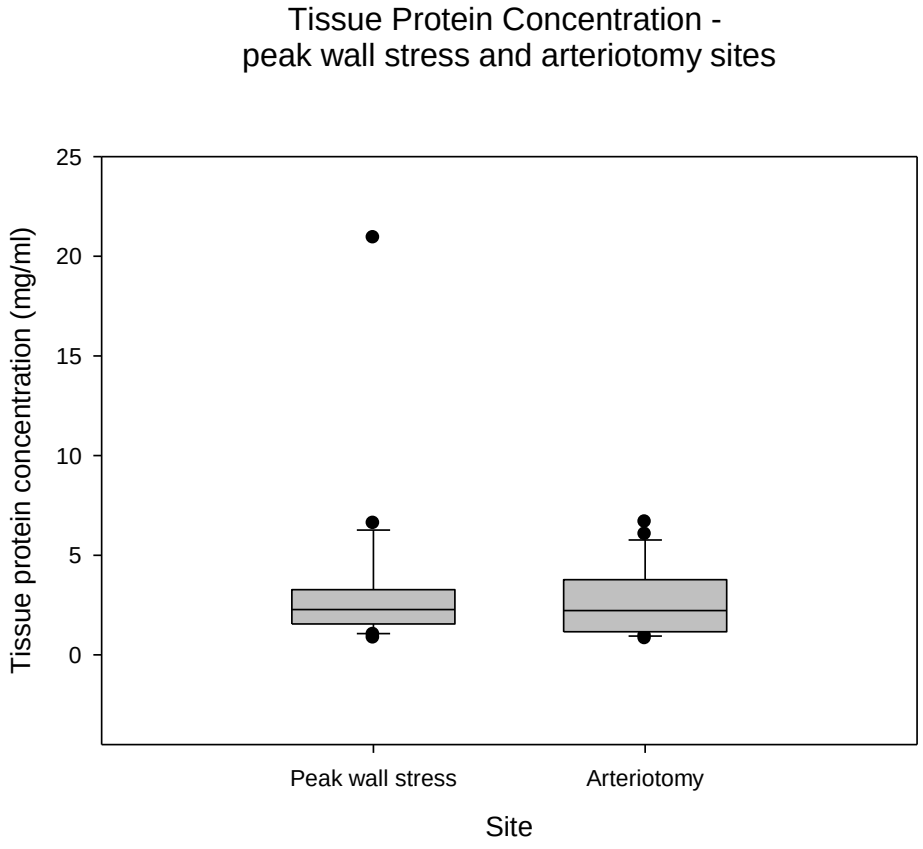


Figure 31. Tissue Protein Concentration – peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

3.2.4.7. Special MMP/TIMP ratios

MMP2/TIMP ratios

No significant difference was found in MMP2/TIMP1 or MMP2/TIMP2 ratios between peak stress and arteriotomy sites.

MMP8/TIMP ratios

Analysis of MMP8/TIMP ratios revealed all had non-normal distributions, therefore analysis was carried out with the Wilcoxon signed ranks test.

Active MMP8 / TIMP1 ratio at peak wall stress sites had a median value of 0.0161 (IQR 0.0064-0.0386). Active MMP8 / TIMP1 ratio at arteriotomy site had a median value of 0.0341 (IQR 0.0159-0.1004)(figure 32, below). A statistically significant difference was found between active MMP8 / TIMP1 at peak stress sites versus arteriotomy sites ($p=0.039$, Wilcoxon signed ranks test)(see table 10 below). Thus the ratio of active MMP8 / TIMP1 was significantly lower at peak wall stress sites compared to arteriotomy sites.

Due to the number of statistical tests carried out in this study, the Bonferroni method (Bland, 2000) should be applied to the above finding. If we assume that we have 5 independent populations (corresponding to each of the MMPs and TIMPs – even though they are not fully independent), then we should only accept significance at the α/κ (=0.05/5) 0.01 level. Thus we cannot assume that the above result is statistically significant.

No other significant differences were found.

MMP9/TIMP ratios

No significant difference was found in MMP9/TIMP1 or MMP9/TIMP2 ratios between peak stress and arteriotomy sites.

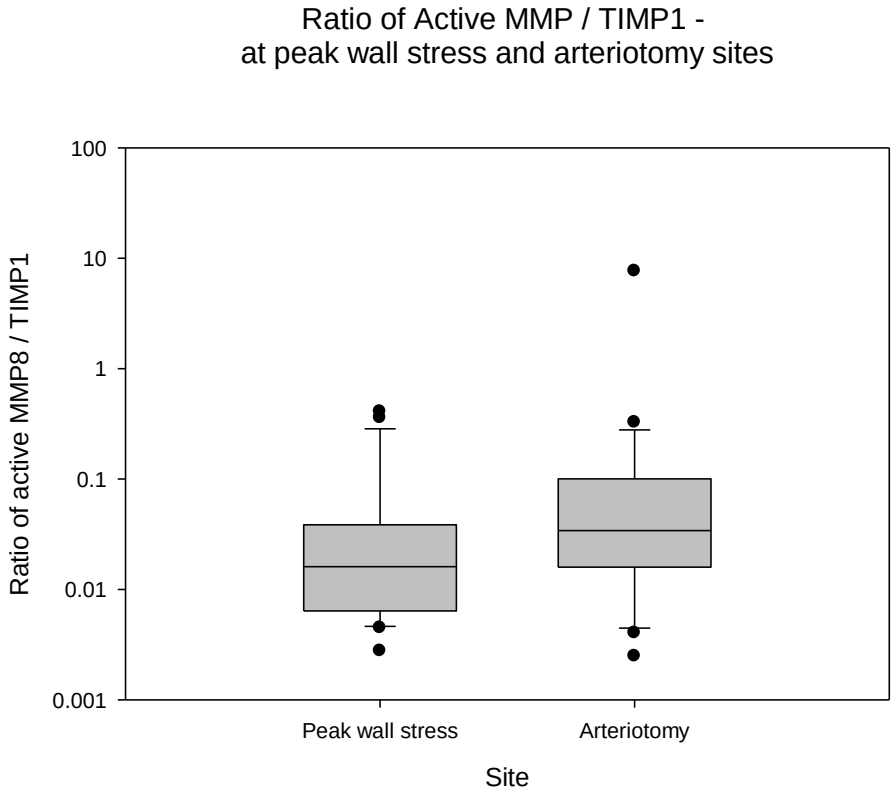


Figure 32. Ratio of Active MMP8 / TIMP1 – at peak wall stress and arteriotomy sites

Box and whisker plots show median, 25th, 75th centiles with whiskers extending to 10th and 90th centiles and outliers beyond this indicated.

Table 10. Comparison of MMP8/TIMP ratios at peak stress versus arteriotomy sites

Test Statistics[c]				
	MMP8 art active / TIMP1 art - MMP8 high active / TIMP1 high	MMP8 art total / TIMP1 art - MMP8 high total / TIMP1 high	MMP8 art active / TIMP2 art - MMP8 high active / TIMP2 high	MMP8 art total / TIMP2 art - MMP8 high total / TIMP2 high
Z	-2.094[a]	-1.373[a]	-.666[a]	-1.338[b]
Asymp. Sig. (2-tailed)	.036	.170	.506	.181

a. Based on negative ranks.

b. Based on positive ranks.

c. Wilcoxon Signed Ranks Test

3.2.4.8. Reliability of runs

The degree of reproducibility of results from run to run was tested between run 2 and run 3 (table 11, below). This repeated the entire process from frozen tissue sample, through MMP/TIMP extraction from tissue, freeze/thaw cycle, ELISA testing for MMP and TIMP concentrations and Bradford analysis for protein concentration in order to compare the final MMP/TIMP concentrations for these particular subjects. The samples although from the same subjects, had been separated immediately after harvesting. The runs were carried out 4 months apart.

Table 11. Reliability of results (comparison of run2 and run3)

Reliability of results (comparison of run2 & run3)						
	MMP2	MMP8	MMP9	TIMP1	TIMP2	Protein
Spearman's correlation coefficient	0.80	0.61	0.80	0.64	0.66	0.49
Significance	0.00	0.00	0.00	0.00	0.00	0.04
Mean absolute difference (sd)	23.27 (58)	17.6 (88)	13.78 (26)	600.55 (1804)	38.74 (89)	1.93(2.6)
Median absolute difference	5.40	5.88	5.00	196.71	13.33	0.77
Median sample value	5.16	9.58	2.90	224.05	31.80	2.20
Mean % absolute difference	86	80	99	105	72	64
Mean % difference from mean	43	40	49	53	36	32
% positive (run2-run3)	22	28	47	8	34	72

Correlation

Although there was good significance of correlation between the runs for the various plates (mostly significant at the 0.01 level), the actual degree of correlation was surprisingly low, with Spearman's correlation coefficient varying from 0.49 (protein concentration) to 0.80 (MMP2 and MMP9).

Differences

There was quite a marked difference between the results in run 2 and run 3, with percentage differences varying from 64% (protein concentration) to 105% (TIMP1). However often reporting of spread often looks at difference from the mean (see mean % difference from mean) from which variances and standard deviations are calculated, and is effectively half the value of the absolute difference.

The 'percentage positive' results show that generally higher results were obtained in the later test (run 3), except for protein concentration.

3.2.5. Plasma

Blood plasma levels of MMP and TIMP are reported in weight per unit volume (ng/ml) of plasma therefore results are also reported in these units here. For comparison to tissue MMP however, it is necessary to analyse concentrations in comparative units to which tissue MMP was reported (weight per unit of protein weight - ng/mg). For this purpose therefore the concentration of plasma protein was also measured, and this provided plasma MMP concentration in ng/mg protein – similar units to the reporting of tissue MMP although the protein divisor in the case of tissue MMP is obviously tissue protein.

3.2.5.1. Plasma MMP2

The number of samples analysed for MMP2 was 20. Analysis of active and total forms of plasma MMP2 demonstrated a possibly normal distribution for both (p=0.08, p=0.2, Kolmogorov-Smirnov test).

Plasma active MMP2

The median concentration of active MMP2 was 23.71ng/ml (interquartile range (IQR) 20.99-35.64).

Plasma total MMP2

The median concentration of total MMP2 was 655.64ng/ml (IQR 549-813).

Plasma active to total MMP2 ratio

The ratio of active to total plasma MMP2 was normally distributed (p=0.2, Kolmogorov-Smirnov test). The mean ratio was 0.04 (IQR 0.29-0.06).

Tissue and plasma MMP2

Concentrations compared were tissue MMP2 in ng/mg tissue protein and plasma MMP2 in ng/mg plasma protein.

A significant correlation was found between plasma active MMP2 and tissue active MMP2 from peak wall stress site [τ = -0.345 (Kendall's τ rank correlation coefficient)(p=0.039), r_s = -0.498 (p=0.03)(Spearman's ρ rank correlation coefficient)]. A scatter graph with regression line for the above data is shown below (figure 33, below).

There were no other significant correlations between tissue and plasma MMP2 either active or total forms (see tables in appendix 4).

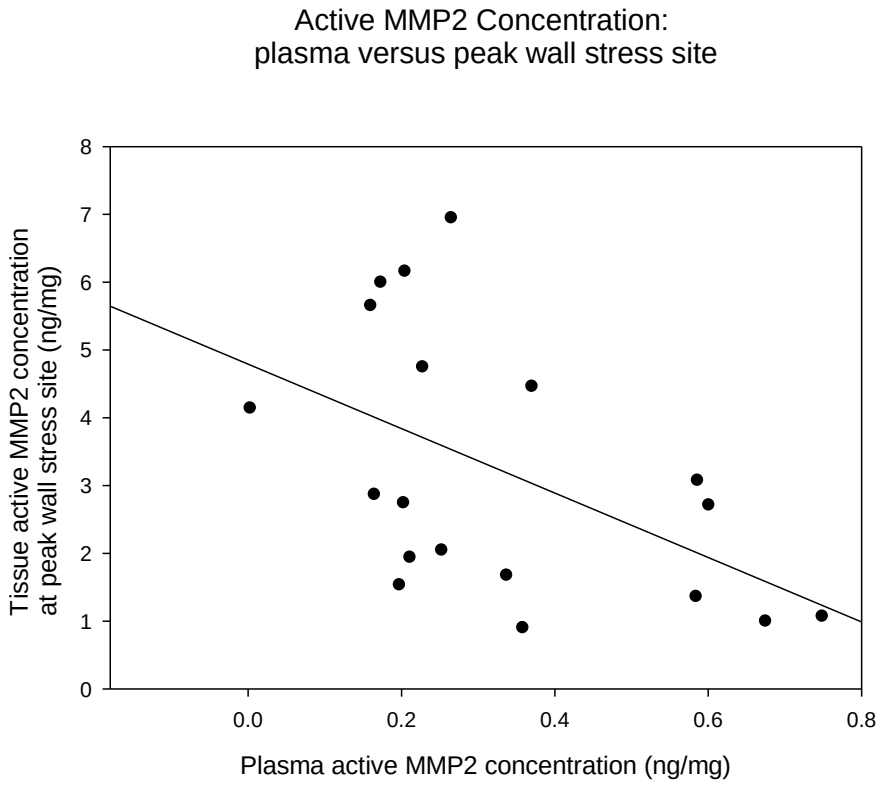


Figure 33. Active MMP2 Concentration: plasma versus peak wall stress site

Scatter plot with regression line.

3.2.5.2. Plasma MMP8

Unfortunately 50% of plasma levels for active MMP8 were too low for analysis at the plate concentration. This was despite earlier trial analysis on tissue MMP8 plates to determine rough concentrations needed for the plasma MMP8 plate. Active MMP8 was analysed at 8 times the concentration of total MMP8, but still to no avail. 11 results were however obtained. Plasma total MMP8 was unaffected, although less plasma active to total ratios were obtained.

The number of samples analysed for plasma active MMP8 was 10, and for plasma total MMP8, 20. Analysis of active and total forms of plasma MMP8 demonstrated a possibly normal distribution for both (p=0.2, p=0.2, Kolmogorov-Smirnov test).

Plasma active MMP8

The median concentration of active MMP8 was 0.51ng/ml (interquartile range (IQR) 0.36-0.70).

Plasma total MMP8

The median concentration of total MMP8 was 3.10ng/ml (IQR 1.73-5.59).

Plasma active to total MMP8 ratio

The ratio of active to total plasma MMP8 was normally distributed (p=0.07, Kolmogorov-Smirnov test). The mean ratio was 0.21 (IQR 0.070-0.372).

Tissue and plasma MMP8

Concentrations compared were tissue MMP8 in ng/mg tissue protein and plasma MMP8 in ng/mg plasma protein.

A significant correlation was found between plasma total MMP8 and tissue total MMP8 from arteriotomy site ($\tau = 0.340$ p=0.042, $r_s = 0.475$ p=0.040). A scatter graph with regression line for the above data is shown below (figure 34, below).

There were no other significant correlations between tissue and plasma MMP8 either active or total forms (see tables in appendix 4).

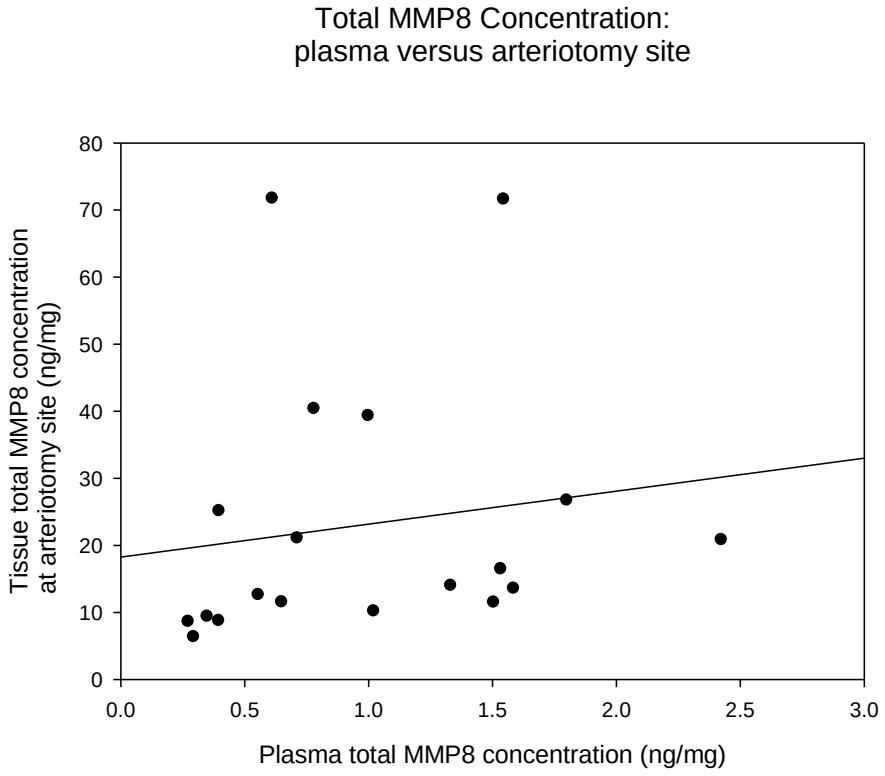


Figure 34. Total MMP8 Concentration: plasma versus arteriotomy site

Scatter plot with regression line

3.2.5.3. Plasma MMP9

The number of samples analysed for MMP9 was 20. Analysis of active and total forms of plasma MMP9 demonstrated a possibly normal distribution for total MMP9

(p=0.2, Kolmogorov-Smirnov test), but non-normal distribution for active MMP9 (p=0.023).

Plasma active MMP9

The median concentration of active MMP9 was 4.26ng/ml (interquartile range (IQR) 4.00-6.66).

Plasma total MMP9

The median concentration of total MMP9 was 55.51ng/ml (IQR 38.98-84.04).

Plasma active to total MMP9 ratio

The ratio of active to total plasma MMP9 was normally distributed (p=0.057, Kolmogorov-Smirnov test). The mean ratio was 0.064 (IQR 0.048-0.185).

Tissue and plasma MMP9

Concentrations compared were tissue MMP9 in ng/mg tissue protein and plasma MMP9 in ng/mg plasma protein.

There were no significant correlations between tissue and plasma MMP9 either active or total forms (see tables in appendix 4).

3.2.5.4. Plasma TIMP1

The number of samples analysed for TIMP1 was 20. Analysis of plasma TIMP1 demonstrated a possibly normal distribution (p=0.2, Kolmogorov-Smirnov test).

Plasma TIMP1

The median concentration of TIMP1 was 201.5ng/ml (interquartile range (IQR) 158.9-217.1).

Tissue and plasma TIMP1

Concentrations compared were tissue TIMP1 in ng/mg tissue protein and plasma TIMP1 in ng/mg plasma protein.

There were no significant correlations between tissue and plasma TIMP1 (see tables in appendix 4).

3.2.5.5. Plasma TIMP2

The number of samples analysed for TIMP2 was 20. Analysis of plasma TIMP2 demonstrated a possibly normal distribution (p=0.2, Kolmogorov-Smirnov test).

Plasma TIMP2

The median concentration of TIMP2 was 141.8ng/ml (interquartile range (IQR) 118.7-147.4).

Tissue and plasma TIMP2

Concentrations compared were tissue TIMP2 in ng/mg tissue protein and plasma TIMP2 in ng/mg plasma protein.

There were no significant correlations between tissue and plasma TIMP2 (see tables in appendix 4).

3.2.5.6. Degradation of plasma MMP samples

A large percentage (50%) of the active MMP8 plasma samples produced unrecordably low results when ELISA tested for concentration. This was due to having diluted down the samples too much despite several earlier trial runs with 2 or 3 wells of an ELISA plate alongside tissue samples for MMP8. These earlier tests are done to

determine an appropriate dilution for the ELISA plate, and these dilutions seemed to change. In fact the plasma sample of active MMP8 needed to be less and less dilute to keep in within the detection range of the ELISA kits as time went on. The first dilution was 5mcl sample to 95mcl buffer (first plasma run – ELISA run 3 (10 Jan 2006) – 2/2 samples in range), through 10mcl sample to 90mcl buffer (second plasma run – ELISA run 5 (2 March 2006) – 0/2 samples in range), and 40mcl sample to 60mcl buffer (Third plasma run – ELISA run 7 (13 March 2006) - 9/24 samples in range). So in fact despite a sample which was 8 times less concentrated in run 7, the early spot-on dilution became too dilute. The samples had almost lost 90% of their activity within that 3 month timeframe. A similar effect also seemed to have taken place on the plasma total MMP8 samples. The samples were all kept together, so in fact this must have affected the MMP8 in all the samples. Other MMPs or TIMPs did not have such noticeable problems with their plasma dilutions occurring.

3.3. Discussion

3.3.1. Results

3.3.1.1. Peak wall stress levels

The AAA peak wall stress levels found in this study varied from 0.46 to 1.33MPa. This is in line with AAA peak stress levels of 29 – 45 N/cm^2 (0.29-0.45MPa) found by Raghavan and Vorp in their seminal paper in 2000 (Raghavan et al., 2000). Interestingly, in that paper they suggested that the typical failure strength of AAAs was about 65N/ cm^2)(0.65MPa), referring to work done earlier (Vorp et al., 1996b). This would indicate that these patients are being repaired at the right time, and that potential

rupture is not far off. Indeed 15 out of 26 patients had peak wall stress values over 0.65MPa. Three patients had values over 1MPa, with AAA diameter 5.7, 6.1, and 6.7cm, and systolic blood pressure over 150mmHg. Of note, all 3 were women. Venkatasubramaniam of this unit obtained mean values for peak wall stress of 0.62MPa in non-ruptured AAAs and 1.02MPa in ruptured AAAs (Venkatasubramaniam et al., 2004a), and Doyle found a mean value of peak wall stress in non-ruptured AAAs of 0.45MPa (range 0.32-0.90MPa)(Doyle et al., 2009).

A correlation coefficient of 0.56 was obtained by Raghavan between peak wall stress and AAA diameter in his above paper, but unfortunately there was no mention of whether this was statistically significant. This study found a non-significant (p=0.18) correlation coefficient of 0.49.

3.3.1.2. MMP concentrations at peak wall vs arteriotomy sites

Levels of tissue MMP and TIMP

Levels of MMP and TIMP are broadly similar to those obtained by Wilson et al (Wilson et al., 2006)(table 12, below), however there are significant individual differences. Comparing arteriotomy site samples in this study to anterior abdominal wall site samples from their study, there seems to be a large difference in the level of active MMP9, and a smaller difference in total MMP2. The median level of active MMP9 in this study is 11 - 16 times less than quoted by Wilson et al, and does not fall between their 25^{th} and 75^{th} centiles. Total MMP2 has a median in this study less than half quoted by Wilson et al, again with this median not falling between their 25^{th} and 75^{th} centiles.

The ‘peak wall stress site’ used in this study is less directly comparable to the ‘site of rupture’ used by Wilson as although we are hypothesizing that the peak wall stress site has a high chance of becoming the ‘site of rupture’, the process leading up to rupture at this site may only be beginning, and the actual event of rupture may also contribute to the enzymes in this area. Comparison of these sites reveals several enzyme groups in which the median concentration found in this study does not fall within the 25^{th} to 75^{th} centiles as quoted by Wilson, namely; active and total MMP9, total MMP8, and TIMP1.

Table 12. Concentration of MMP / TIMP: comparison with Wilson et al

	Peak stress site	Site of rupture	Arteriotomy	AAA wall (n)	AAA wall (r)
MMP2 (a)	2.80 (1.49-4.89)	-	2.45 (1.38-4.87)	-	-
MMP2 (t)	24.41 (12.87-36.45)	65.9 (12.8-83.4)	21.87 (13.00-32.82)	54.1 (34.1-94.2)	63.8 (41.1-108)
MMP8 (a)	5.20 (2.54-9.82)	10.3 (3.98-16.0)	6.99 (3.65-15.72)	4.47 (1.41-10.3)	2.41 (1.19-5.31)
MMP8 (t)	18.80 (9.82-31.85)	43.6 (20.1-79.3)	16.44 (10.81-32.98)	17.3 (12.1-24.6)	16.4 (6.17-30.9)
MMP9 (a)	0.58 (0.38-0.85)	12.2 (7.77-16.2)	0.54 (0.32-0.97)	9.04 (5.25-13.8)	6.29 (4.68-14.6)
MMP9 (t)	9.70 (3.95-24.47)	87.1 (56.3-113)	11.34 (8.55-20.49)	33.2 (12.1-87.7)	28.5 (8.02-97.0)
TIMP1	364.84 (210.49)	180 (82.3-280)	229.84 (86.80-370.95)	151 (77.6-260)	148 (70.8-333)
TIMP2	17.10 (8.55-29.08)	8.04 (5.24-20.0)	14.64 (8.56-29.93)	8.07 (4.13-16.8)	7.20 (4.10-12.9)
Prot conc	2.27 (1.55-3.28)	-	2.23 (1.16-3.77)	-	-

Concentration in ng/mg protein, (n) anterior wall of non-ruptured AAA, (r) anterior wall of ruptured AAA, (a) active MMP, (t) total MMP, protein concentration, median (interquartile range), ‘site of rupture’, ‘AAA wall (n)’ and ‘AAA wall (r)’ columns originate from Wilson et al (Wilson et al., 2006).

Loss of MMP activity

A major concern when measuring and analysing MMP and TIMP concentrations is the degradation attributable to storage and freeze-thaw cycles. It is therefore worth

considering this factor in the above comparison of the results of this study with the Wilson study.

A substantial problem with degradation would produce a wide-scale lowering of the MMP or TIMP concentrations in one of the studies compared to the other. This is not seen. Rather there is a more subtle difference with the gelatinases (MMP2 and 9) reduced, and the collagenase (MMP8) preserved compared to the Wilson study. TIMPs on the other hand seem to be reduced in the Wilson study.

The TIMPs have been suggested to be more susceptible to freeze-thaw cycles (Alby et al., 2002), but plasma MMP9 has been shown to degrade even during -80°C storage (Rouy et al., 2005), with TIMP1 and MMP2 stable over time in these storage conditions. Auto-degradation of MMP9 is possible as MMP9 can self-activate, but this is also possible with MMP2 (Bergmann et al., 1995), which does not seem to degrade with time. The effect of incomplete inhibition by TIMP1 on MMP9 allows auto-activation to occur whilst TIMP2 more successfully inhibits MMP2 from auto-activation (Fridman et al., 1993) by forming complexes with MMP2. In addition these MMP2/TIMP2 complexes themselves are more susceptible to inhibition (Denhardt et al., 1993). Contrary to this mechanism would be the fact that no active MMP9 was detected in the samples without activation by APMA. Regardless of these points however, Rouy points out that if samples did undergo degradation as described above – whatever the cause – in most situations all the samples would be subject to similar degradation and therefore comparative findings would still be valid. The situation would be different if recruitment is markedly skewed for example, elective AAA patients recruited in the first 3 months of a study, but acute AAA patients recruited throughout a 1 year period, with all samples analysed at the end of this time.

Tissue samples (as opposed to the plasma samples discussed above) should be automatically protected from the above degradation due to various inhibitors which are normally administered during the various initial MMP extraction processes. Some of these processes include dialysis, which may remove inhibitors. However since a number of these inhibitors cause irreversible inhibition, the function of these proteases is not recovered. The problem comes from inhibitors that are lost during dialysis which only reversibly inhibit. In addition, if the problem really stems from auto-activation or MMP activating each other, then –as inhibitors to MMPs are not used since they affect functional assays (assays that test the function of MMPs such as zymograms, or ELISA-style activity assays)- this could be a real problem. Reliance is normally on the -80°C storage to prevent MMP activity prior to MMP activity testing. The way forward could possibly be reversible inhibitors of MMPs that can be removed or deactivated prior to MMP activity testing.

Although the problem of protein loss due to protease activity and MMP loss due to possible self-activation may be a problem in our study, the important results come from paired samples from the same patient, harvested, stored and processed under the same conditions. The loss would therefore presumably be the same in each sample and enable valid comparisons to be made between these samples.

The above process could account for the difference in MMP9 from the study of Wilson et al, but the difference in MMP2 or TIMP1 is still not satisfactorily accounted for.

Thoracic aorta aneurysmal data

Data has also been obtained by Schmoker et al looking at thoracic aortic aneurysms, and quantifying MMP and TIMP concentrations via methods including ELISA activity

kits (Schmoker et al., 2007). Interestingly they found that active and total MMP2 was greater in their control group compared to atherosclerotic or non-atherosclerotic thoracic aortic aneurysms, and this was also the case with active MMP9, TIMP1 and TIMP2 (table 13, below). However total MMP9 was found to be higher in atherosclerotic thoracic aortic aneurysms than control or non-atherosclerotic thoracic aortic aneurysms. These results were surprising until the identity of their control group was revealed as patients undergoing elective coronary artery bypass grafting, when possible explanations come to mind, such as areas of relative ischaemia within the vessel wall leading to areas of remodelling and proteolysis. Useful data in the way of MMP and TIMP concentrations in atherosclerotic thoracic aortic aneurysms – ascending and descending portions - can be used to compare to the results obtained in this study. The low gelatinase concentrations as compared to the results of Wilson et al, now do not seem so low in the light of Schmoker's results. Granted the tissue is not exactly the same, but there is no reason to suppose that there should be a marked difference between the descending aorta data and the arteriotomy data from this study. In the absence of other aortic MMP / TIMP concentration data in aneurysmal patients, this seems a reasonable comparison. Much of the previous data uses more traditional methods including mRNA data or zymographic data.

Table 13. Concentration of MMP / TIMP: comparison with Schmoker et al

	Arteriotomy	Ascending	Descending
MMP2 (a)	2.45 (1.38-4.87)	0.56 (0.58)	0.72 (0.49)
MMP2 (t)	21.87 (13.00-32.82)	71 (59)	26 (20)
MMP8 (a)	6.99 (3.65-15.72)	-	-
MMP8 (t)	16.44 (10.81-32.98)	-	-
MMP9 (a)	0.54 (0.32-0.97)	0.22 (0.42)	0.29 (0.45)
MMP9 (t)	11.34 (8.55-20.49)	8.5 (8.4)	14.8 (20.5)
TIMP1	229.84 (86.80-370.95)	274 (224)	173 (240)
TIMP2	14.64 (8.56-29.93)	14.5 (10.0)	11.9 (15.7)
Prot conc	2.23 (1.16-3.77)		

Concentration in ng/mg protein, ascending aorta, anterior aspect descending aorta taken at most dilated part, (a) active, (t) total, protein concentration, median (IRQ) or mean (sd), 'ascending' and 'descending' columns from Schmoker et al (Schmoker et al., 2007).

Peak wall stress vs arteriotomy site: MMPs

Statistical analysis was carried out on the study results and suggested no significant difference in active or total MMPs or TIMPs at peak wall stress site compared to arteriotomy site.

Wilson et al (Wilson et al., 2006) found significant differences in MMP8 and 9 levels (both total and active) in anterior aneurysm wall versus site of rupture in patients with ruptured AAA, using 12 patients in non-parametric matched pairs analysis (Wilcoxon paired test). These levels were all higher at the site of rupture compared to anterior aneurysm wall. No significant difference was found in MMP2, TIMP1 or TIMP2. MMP2 was slightly higher at anterior aneurysm wall site, and TIMP1 and 2 was non-significantly elevated at rupture site. Although the tissue involved (ruptured AAA tissue) is not the same as in this study, Wilson also demonstrated no significant

difference in MMP or TIMP levels from anterior aneurysm wall in 55 patients undergoing elective AAA repair compared to 21 patients undergoing emergency AAA repair. Granted this is not the same as proving there is no difference between the enzymes levels at these 2 sites, but it does go some way in validating a reasonable comparison between anterior aneurysm wall MMPs and TIMPs in ruptured aneurysms and arteriotomy samples in this study.

The site of aneurysm rupture is certainly different to the peak wall stress site, however the underlying hypothesis in this study is that the peak wall stress site eventually becomes the site of rupture due to progressively increasing stresses at this site due to extensive and excessive remodelling at this site. The remodelling process would be directed by MMPs, initially elastases, and later collagenases as the histological make up of the aortic changes. On the basis of this hypothesis, it is therefore reasonable to assume an elevated level of MMPs – either elastase or collagenase depending perhaps on degree of aneurysm progression – higher than in non-peak wall stress tissue. This level may be at sub-rupture site levels, or perhaps even at supra-rupture site levels.

The results of this study however do not seem to support this hypothesis, as no significant difference was found between MMP levels at peak wall stress sites compared to arteriotomy sites. Furthermore median levels of active MMP8 (collagenase) are higher at arteriotomy sites compared to peak wall stress sites. This could however - as hypothesised above - be due to stage of aneurysm, with potential collagenase rises to be seen later (perhaps immediately prior to rupture) since this was the most significant difference seen in MMPs by Wilson et al in ruptured AAA tissue. The median level of elastases however, could support the hypothesis since both active forms (MMP2 and 9) are higher at peak wall stress sites compared to arteriotomy sites

(although these are non-significant differences in this study). There are only minute differences between the median levels between these sites, and the ranges are also quite similar. These suggest that if there is a difference a much larger study sample would be necessary to identify it. If there is a true difference, why is the observed difference so small? Possibilities include; 1) because this is the true difference, 2) because only a very tiny localised area has a very large difference (and we are therefore diluting down the MMP by harvesting a relatively large area, or 3) a large area has a moderately high level of MMP, but poor localisation leads us to only obtain part of this area together with lots of 'normal' aneurysm wall.

Peak wall stress vs arteriotomy site: TIMPs

Levels of TIMP1 and 2 are also non-significantly elevated in peak wall stress sites compared to arteriotomy sites. Elevated levels of TIMP1 and 2 have been observed alongside elevated MMP levels(Oberg et al., 2000, Susskind et al., 2003), presumably trying (but unable) to compensate for MMP levels(Zucker et al., 1999).

Type II statistical error

It is possible that this study incorrectly fails to reject the null hypothesis (that there is no difference between MMP and TIMP levels between peak wall stress and arteriotomy sites), as since the median levels seen are similar between sites, and there is a substantial degree of variance, a larger number of subjects would be necessary to test conclusively for a difference between the sites

This study assumed some similarity between MMP and TIMP levels seen at calculated peak wall stress sites and the rupture site in the Wilson study (Wilson et al., 2006). It was entirely reasonable therefore that we expected to be able to draw a valid

conclusion about whether there is a difference in MMP and TIMP levels between peak wall stress and arteriotomy sites.

What this study suggests is that peak wall stress site tissue is further from ruptures site tissue than was thought by our team. This difference is biochemical, but in terms of time and location, there is still no reason to believe that these may not be quite close. This biochemical difference may narrow in days as the tissue spirals towards rupture. The location may indeed be correct as anecdotal and observational data points towards this, and theoretically the peak wall stress site would be subject to a vicious cycle of remodelling, with gradually increasing stresses until rupture.

It is difficult to be certain that our negative findings in this study was not due to a type II statistical error, but we believe the study was designed appropriately with the information that was available at the time of study inception.

Tissue MMP ratios

Higher levels of activated MMP9 and MMP2 have been identified in AAA compared to normal tissue using traditional zymographic analysis (Sakalihasan, 1996). It was hoped therefore to demonstrate significantly different ratios of activated to total MMP9 and MMP2 in peak stress areas compared to arteriotomy areas. This was not seen.

Also demonstrated have been higher proportions of MMP9 compared to total MMPs in AAA, and higher proportions of MMP2 in normal tissue. In this study there was no significant difference found on comparing the ratios of the various MMPs to total MMPs (MMP2 + MMP8 + MMP9) at the peak stress site compared to the arteriotomy

site. A reason could be that not all the MMPs were accounted for in this study, and no test was done that measured total MMP activity, activated or not.

3.3.1.3. Plasma MMP / TIMPs

Plasma MMP concentration

Plasma MMP2 concentrations from this study was roughly in line with results from other contemporary studies, although again MMP and TIMP concentrations seem substantially lower in this study than in Wilson et al, and also McMillan (table14, below).

Table 14. Table comparing plasma MMP / TIMP concentrations

	Heng et al	Wilson et al	McMillan et al
MMP2	23.71 (20.99-35.64)	42.14 (28.71-68.69)	-
	655.64 (549-813)	-	-
MMP8	0.51 (0.36-0.70)	-	-
	3.10 (1.73-5.59)	-	-
MMP9	4.26 (4.00-6.66)	17.54 (10.30-34.24)	-
	55.51 (38.98-84.04)	-	85.66 [11.64]
TIMP1	201.5 (158.9-217.1)	460.80 (199.80-611.00)	-
TIMP2	141.8 (118.7-147.4)	-	-

ng/ml, Median (interquartile range), mean [standard error], Wilson (Wilson et al., 2008) McMillan(McMillan and Pearce, 1999)

Degradation of plasma active /total MMP8

It is interesting that when to date MMP9 has been noted to be the most sensitive to degradation in storage and freeze-thaw cycles (Rouy et al., 2005), this study found significant problems with plasma MMP8. Rouy et al studied only MMP9 so this perhaps just has not been reported in the past. It would be worthwhile exploring the

degradation of MMP8, to ascertain just how sensitive it is to storage conditions and freeze-thaw cycles. It is also interesting that although it is not possible to compare plasma MMP8 levels obtained here to levels obtained in another study, MMP2 and 9, and TIMP1 levels are reduced in this study compared to others. In contrast, *tissue* MMP8 concentrations in this study are relatively spared compared to the other MMPs as demonstrated above.

Ratio of tissue to plasma MMPs

A negative correlation was found to be statistically significant between active MMP2 from peak wall stress sites and active plasma MMP2 [rank correlation coefficient -0.345 (p=0.039)(Kendall's τ), rank correlation coefficient -0.498 (p=0.03)(Spearman's ρ]. Since only active plasma MMP2 was normally distributed (active MMP2 from peak wall stress site was not normally distributed) it would not have been appropriate to use the Pearson correlation coefficient (Altman, 1991).

3.3.2. Study design

3.3.2.1. Active and total MMP

At an early stage in the study it was decided to analyse both active and total forms of MMP via ELISA tests, although our initial proposal was only to analyse active forms of MMP. This resulted in using double the number of kits as initially specified in the proposal for a given number of patients.

3.3.2.2. Power calculation

The comparison of MMP and TIMP levels in peak wall stress and low wall stress sites was powered at 80% and significance 0.05 level. Sample calculation was based on the

best available data, and suggested n=8. For the reasons explained earlier, it was decided to take arteriotomy samples rather than low stress site samples.

Initial analysis was carried out and presented to the steering group at n=10, but statistical significance was not achieved. It was felt that this was due to a type II error due to the fact that the difference expected was now smaller due to the change to the secondary biopsy site - from low stress site to arteriotomy site - and as the power calculation was based on best available data, and not exact data, that it was valid to continue collecting data for repeat statistical analysis with double the sample.

3.3.3. Patient recruitment

Patient recruitment was generally straightforward although several patients were missed due to the research fellow not being available (1 in 4, 24hr on-call, off research site), however this did not pose a problem as regards recruiting enough patients for the study. There were no refusals from patients regarding recruitment in to the study.

Many patients did not complete the study again due to the research fellow not being available to guide localisation of AAA tissue biopsy, and also to process these prior to snap freezing at the time of elective AAA repair. On one occasion another research fellow was enlisted to direct AAA biopsy and process the tissue, and this was successful, however this was not continued as it is a time consuming process for the other research fellow, and the consistency about 1 research fellow processing all the tissue would be lost.

3.3.4. Effect of thrombus

Recent work has provided a constitutive model for intra-luminal thrombus (ILT) derived from biaxial testing but clearly the impact of ILT rupture risk is a complex one

and as stated by these authors, further work is required (Vande Geest et al., 2006b). We began our modelling before the effect of ILT on wall stress was apparent, thus elected not to incorporate ILT into our model. Subsequently, we wished to avoid major changes to the model mid study. It is now becoming clear that ILT probably has a role to play in rupture risk, although this role is not without ambiguity (Malkawi et al.).

3.3.5. Low stress versus arteriotomy samples

The study was initially set up to test peak wall stress samples against low wall stress samples, and both sites were identified from Von-Mises stress diagram, and conveyed to the surgeon. However due to some difficulties in theatre in harvesting AAA biopsies from 2 separate identified sites mainly to do with the fear of injuring adjacent structures and also of having to close these defects to reduce the risks of aorto-enteric fistulae, it was decided to take arteriotomy samples rather than low stress samples. This would reduce the risks high-lighted above. The peak wall stress sample was essential to the study, and this was harvested as initially planned. The change in biopsy site would affect the degree of difference likely to be seen between the sites, and would therefore increase the sample needed to avoid a type II statistical error.

3.3.6. Sample localisation

Sample localisation was inherently inaccurate due to problems relating to scale, and the difficulty in localising a position in a 3-dimensional uneven biological structure. The Surgeon's stress map, and oral information given regarding position of the peak stress site, gave a reasonable localisation however minute accuracy was absent.

This problem potentiates other factors such as wide variability in MMP reliability between runs as discussed below.

3.3.7. MMP / TIMP extraction & analysis

Tissue homogenisation

This was carried out manually with 2 scalpels on ice. Most other studies carried this out with a homogeniser, but after discussions with Dr Greenman, reader, division of cell and molecular medicine, medical research laboratory, University of Hull, and Dr Madden, post-doctoral fellow, medical research laboratory, University of Hull, it was decided that the homogenisation could reasonably be carried out manually. Although it is possible that due to this method of homogenisation, less MMP / TIMP was extracted, as the main object of this study was to compare matched samples, both obtained, and processed in the same way, this should not have been a major problem.

Buffers

EDTA at 10mmol/l concentration has been demonstrated to destroy MMP activity at pH3, reduce activity by 90% at higher pH levels, and to release active TIMP from TIMP/MMP complexes (Murphy et al., 1989). Although the concentration of EDTA used in the buffers is less than above - 0.1%(2.7mmol/l), there is still a chance that the EDTA may be inhibiting MMP activity. Vine and Powell experimented with removal of EDTA from the homogenizing buffer with no improvement in yield of MMPs (Vine and Powell, 1991). EDTA has been used in the preparation of plasma samples for MMP analysis, with no obvious decrease in expected activity (Jung, 2008).

PMSF (Phenylmethylsulfonyl Fluoride) was used at 0.1mM concentrations in both homogenising and dialysis buffers by Vine and Powell in their original paper (Vine and Powell, 1991). Furthermore it was also used in the work of Wilson et al in his modified version of their MMP tissue extraction protocol (personal communication)

although it wasn't specifically detailed in their paper (Wilson et al., 2006). It was decided to use a cocktail of protease inhibitors (Calbiochem protease inhibitor cocktail set III) which included a serine inhibitor (AEBSF, 4-(2-Aminoethyl) benzenesulfonylfluride, HCl), related to PMSF but more stable and non-toxic, and also recommended as a replacement for PMSF (Calbiochem website). This cocktail of inhibitors had no particular recorded activity against MMPs. Since AEBSF irreversibly inhibits the same range of proteases as PMSF, it was also thought that further dosing in the dialysis buffer was unnecessary.

Ammonium sulphate fractionation

The method of extracting MMPs used in this study originated with Vine and Powell (Vine and Powell, 1991). This included ammonium sulphate fractionating, where the proteins precipitating between 35 and 65% ammonium sulphate saturation were collected. Various changes to the original protocol were apparently successfully carried out by Wilson et al (Wilson et al., 2006), including dropping the use of ammonium sulphate fractionation during the extraction process. The fractionation process was carried out after centrifugation of the homogenate, and prior to dialysis which presumably was mainly to remove ammonium sulphate. Since the fractionation was after centrifugation - where the remainder solid was discarded - this process could not have extracted any more MMP from the tissue, but it would have 'cleaned up' the supernatant and reduced interference from other substances. Since the ELISA test used in this study relies on active MMP activating an enzyme, it is very specific, and there should not be much to gain by removing other substances. Wilson et al also used ELISA tests, but Vine and Powell used zymography and were thus much more affected

by other substances, especially if these substances had similar molecular weight to the MMPs in question.

Dialysis

Dialysis was carried out to remove the 2M urea and EDTA involved in homogenisation process. The urea used is at a much lower concentration than is used in many other extraction processes (often 10M), but could still interfere with later substance quantification and therefore had to be removed. The EDTA could inhibit MMP function at high concentrations and therefore some removal is advised. Dialysis will remove some EDTA, but will often leave a low concentration of EDTA.

Dialysis may also however remove various protease inhibitors and may thus result in the recovery of function in the proteases that were not irreversible inhibited.

ELISA plate design

Samples to be compared together were attempted to be kept within the same ELISA plate in order to minimise potential inter-plate, and inter-run reliability issues. Thus active MMP2 from peak wall and arteriotomy sites for subject 17 were always analysed in the same ELISA plates, and in general other samples that may also be tested against these (eg total MMP2 from peak stress and arteriotomy sites) were also analysed on the same plate if possible.

ELISA kit characteristics

Interestingly although the cross-sensitivity of the MMP2 kit was 100% for pro-MMP2, it was reported as only 77% for activated MMP2. As commented on the kit insert, this could have simply been due to their methods (pre-activation not allowed time enough to achieve full activation of sample), or somehow a loss of MMP2 (eg through rapid

degradation), but it is possible that the ELISA tests simply does not detect 33% of activated MMP2. This is an established kit, used in many previous papers, so comparison with these papers is valid, but there may be some discrepancy with other papers that did not use this test.

The ELISA kits used in this study had significant inter-assay variability with coefficient of variability reported as from 5.9% (TIMP2) to 21.7% (MMP9) (kit inserts as supplied by Amersham Biosciences).

Protein concentration determination

The Bradford assay for determination of protein concentrations has a limited range of linear response, therefore samples often have to be diluted to be within the appropriate range. The Coomassie Plus kit from Pierce seems to extend the range, but the microplate protocol required minute quantities (10ul) of standard, and as sample dilution is often still necessary, this leads to the use of even smaller quantities (3ul). Even with appropriate training, inexperienced laboratory staff are at risk of substantial titration errors at these quantities. For this reason from run 3 onwards, protein quantification was carried out in triplicate, in order that obviously skew results could be identified, removed, and still allow protein concentration determination in order to work with the suite of tests carried out for that sample (MMP and TIMP tests) in that run.

3.3.8. Reliability of runs

There was generally poorer than expected correlation of results between the 2 compared runs. However when considering the myriad of processes contributing to an individual run, the variability may not be as out of place as initially thought. Consider

that each sample is taken from a different area within the initially harvested peak stress or arteriotomy sample. Consider then the variability in the amount of MMP/TIMP that is successfully extracted from tissue. Finally there are variabilities from the actual ELISA test, and protein concentration detection.

More consistent results could be obtained by homogenising the entire tissue biopsy from the AAA in a single batch, which may be wise if the local MMP rise is extremely focused. This would help to overcome the real problems in localisation this peak stress area.

The coefficient of variation (CV) (ratio of standard deviation to mean) of ELISA kits range from 2.5% (TIMP2) to 21.7% (MMP9), and this contributes significantly to the overall poor consistency.

It was not appropriate to calculate the CV for our data comparing results from these 2 runs as a) the data was not normally distributed (failed when tested), and b) it is not appropriate for comparison of between subject variability (Swinscow, 1997).

The protein concentration was higher in run 2 - 72% of times this is higher than expected but raises particular suspicions due to the fact that all other substances were lower to some extent in run 2 than in run 3. The protein concentration is particularly important since all the other results are expressed in relation to unit weight of protein. So for example if protein concentration were to fall due to proteolysis during the intervening period between runs, the later run would have a lower concentration of overall protein, and if the MMPs have been preserved, these would appear to have a concentration in these later samples as the same quantity of MMP would be divided by a smaller quantity of protein. This however would be surprising since a general

protease inhibitor cocktail was included in the homogenising buffer. It is possible however to also include a protease inhibitor in the dialysis buffer which was done in certain previous papers, and would be again recommended.

One of the advantages of the ELISA activity kits is that activity can be quantified in a more comparable format. The results can be compared to other runs done at a late stage and date. This is through comparable standards and clearly quantifiable results. Other methods of assessing MMP or general protease activity for example Zymography relay on substrate digestion and the comparative quantification is fine intraplate, difficult inter-plate, and almost impossible between studies even with the use of a densitometer.

3.4. Conclusion

In this study there was no demonstrated statistical difference between concentrations of MMP2, 8, 9 or TIMP1, or 2 in peak wall stress sites compared to areteriotomy sites in the aneurysm walls of patients with abdominal aortic aneurysm undergoing elective repair.

Although there is a general level of agreement between the MMP and TIMP concentrations detected in this study and other published studies, there was enough discrepancy to wonder whether there was some degradation of MMP due to auto-activation or otherwise. This discrepancy could of course just be due to some slight variation in the extraction process.

In the plasma samples, there seemed to be a relatively fast loss in MMP8 concentration – again presumably due to some sort of degradation. In tissue samples MMP8 seemed

best preserved, perhaps due to proteases used to process the tissue. This should be studied in more depth.

There was a trend towards increased gelatinases (MMP2 & 9) in peak stress sites compared to arteriotomy sites, and a decrease in collagenase (MMP8) in peak stress sites compared to arteriotomy sites. If this difference is true, then it may suggest that our hypothesis about the peak wall site being the eventual rupture site is biochemically further away than we thought, since in line with general agreement we would expect elastolysis earlier, followed by later collagenolysis at the presupposed site of rupture.

Work continues to be carried out on patient specific biomechanical profiling in AAA, with improved and more rapid techniques (Malkawi et al.). This together with a more detailed understanding of the background biochemical changes may result in more accurate determination of AAA risk with better patient selection at an appropriate time to achieved maximum procedural benefit.

4. Appendix 1: Correspondence for AAA Screening Study

The following pages display correspondence and forms from the AAA screening study

- Invitation letter

  Invitation letter from GP (sent on their behalf) to practice patients inviting them to join the AAA screening study.

- Patient information sheet (on back of invitation letter)

- Reminder letter to patient (sent at 1 month)

- HAD scale

  This anxiety and depression scale was completed by a cohort of patients from 1 practice only. They completed identical forms; a) when initially carrying out self-examination for AAA, b) just after the ultrasound scan for AAA (at which time they were informed of their ultrasound result), and c) 1 month after their ultrasound scan (the form was mailed to them).

- HAD scale score sheet

- Letter to GP practice AAA 3-5cm

- Letter to GP practice AAA ≥5cm

Abdominal Aortic Aneurysm Screening Study – invitation letter

Date

Title First name Surname
Address 1
Address 2
Address 3
Post Code

Dear Title Surname

Abdominal Aortic Aneurysm Screening Study

There is a condition that affects men in your age group, which is completely hidden until it may be too late to treat: it is called an "abdominal aortic aneurysm", and is when the main artery in the abdomen becomes enlarged, like a bulge, which occasionally and unexpectedly can burst.

Specialists at Hull Royal Infirmary are looking for better ways to discover this condition, and are asking a simple question: can this be detected by feeling your own stomach? Early signs are that it can! We now need to test this out on a large number of men, and that is why I am inviting you to take part in a research study.

If you agree to take part, could you please complete the form, and return it in the enclosed stamped addressed envelope. You will then be invited to attend Alfred Bean Hospital Driffield for a simple ultrasound test within the next 6 months. This is similar to the scans done routinely on pregnant women, and is completely safe and totally painless! The test will either confirm for certain that you do not have the condition, or will show if there is a problem for which treatment will be offered. The specialists who carry out the scan will be able to answer your questions about the result.

In order for this research to be effective, we need to enrol nine thousand men! So I do hope you will agree to take part: it could save your life! There is more detailed information on the research study on the back of this letter, but **if you have any further questions, you can phone me on 01482 675523 or Jennie Bryce on 01482 674703 or Carole Tennison on 04182 675784**. Please do not ring the Cranwell Road Surgery, as they will not be able to answer your queries.

If you do not want to participate please tick here ☐ and return this letter in the stamped addressed envelope to prevent us contacting you again.

Thank you for your help. Yours sincerely,

Michael Heng

Mr Michael Heng MB ChB MRCS
Clinical Research Fellow
Cranwell Road Surgery & the Academic Vascular Unit, Hull Royal Infirmary.
Address: Vascular Laboratory, Alderson House, Hull Royal Infirmary, Anlaby Road, Hull HU3 2JZ

Patient information sheet: detailed information

Full Study Title: A prospective trial on a novel approach for screening clinically significant (>5cm) Abdominal Aortic Aneurysms
ELSY no: 2599
LREC no: 12/01/253

The Academic Vascular Unit at Hull Royal Infirmary in collaboration with Cranwell Road Surgery, are initiating a study to detect, a condition called Abdominal Aortic Aneurysm (AAA). This is a condition that mainly affects men over the age of 65, with dilation (expansion) of the main artery (the aorta) in the abdomen, and the progressive thinning of the arterial wall leading in some cases to the artery rupturing (bursting). Most people who have a rupture of their aneurysm (AAA) will die (88%), with 50% of people not even getting to hospital alive. When an aneurysm is found specialists decide what risk it has of rupturing, and will offer surgery in certain cases. Abdominal aortic aneurysms often cause no symptoms until they rupture, which means that people often do not know they have this condition until it is too late. The aim of this study is to detect these aneurysms at a stage when we can repair them. Once an aneurysm is repaired people have no further risk from it.

A pilot project has been done involving more than 1,000 patients and the results are promising with 7 large aneurysms discovered. We are continuing the project and will need 8,000 more patients for the project to be conclusive. You have been selected by Cranwell Road Surgery from a list of men over the age of 65.

You can choose whether or not you wish to participate in this study, and if you do decide to take part, you are still free to withdraw at any time without giving a reason. This will not affect any other care you receive.

Your participation involves answering a few questions and you feeling your own tummy in the manner described by us. You may also ask your wife/relative/friend to feel your tummy as described by us. Once completed you can return the questionnaire in the self addressed envelope provided. This will be followed by an ultrasound scan of your tummy at a later date (usually within 6 months). Ultrasound scanning of the tummy is a painless outpatient test that only takes a few minutes to do. It is used to decide whether an aneurysm is present and to measure its exact size.

If an aneurysm is detected you will be referred to the vascular surgical unit at Hull Royal Infirmary, and they will treat you according to their normal protocol (regular ultrasound scans if aneurysm <5.5cm, detailed discussion with vascular consultant if aneurysm >5.5cm and possible surgery).

All information that is collected about you during the course of the research will be kept strictly confidential. The overall results will be published in leading scientific and medical journals in approximately 3 years. Your confidentiality will be preserved in all published articles. We would be happy to supply you with a copy of the results on request.

The ethics behind this study have been reviewed and supported by the Hull and East Riding Local Research Ethics Committee.

Thank you for taking the time to read this information sheet. If you have any further queries or questions please contact;

Mr Michael Heng, Clinical Research Fellow, Cranwell Road Surgery and the Academic Vascular Unit, Hull Royal Infirmary. Telephone no: 01482 675523 or Jennie Bryce on 01482 674703 or Carole Tennison on 01482 675784

IS 4.3 12/07/2005

Abdominal Aortic Aneurysm Screening Study - Reminder

Date

Title / First name / Surname
Address1
Address2
Address3
Post code

Dear Title Surname

Thank you for taking the time to read this letter.

We wrote to you a month ago regarding the above study, but have not received a response. It is vitally important that you realise this study *could* save your life, and *will* help towards our understanding of this silent time-bomb that often kills without warning.

We realise that some people will not want to take part. If you do not want to participate please tick here ☐ and return this letter in the stamped addressed envelope to prevent us contacting you again. If you know that you have or have had an abdominal aortic aneurysm then please tick here ☐ as you will have had treatment or be on surveillance and will therefore not need to take part in this study.

We enclose another participation pack, and would be grateful if you could find the time to participate in this study. Your involvement in this study will involve only 3 things;

You checking your own abdomen

A simple questionnaire

A 5 minute ultrasound scan at Eastgate Medical Group Surgery

If you have any further questions, you can phone Jennie Bryce on 01482 674703 or Carole Tennison on 01482 5784 or me on 01482 675523. Please do not ring the Eastgate Medical Group, as they will not be able to answer your queries.

Thank you for your help. Yours sincerely,

Michael Heng
Mr Michael Heng MB ChB MRCS
Clinical Research Fellow, Eastgate Medical Group Surgery & the Academic Vascular Unit, Hull Royal Infirmary.
Postal Address: Vascular Laboratory, Alderson House, Hull Royal Infirmary, Anlaby Road, Hull, HU3 2JZ.

HAD (Hospital Anxiety and Depression) Scale

1 Month

Instructions: Doctors are aware that emotions play an important part in most illnesses. If your doctor knows about these feelings he or she will be able to help you more. This questionnaire is designed to help your doctor know how you feel. Read each item and place a firm tick in the box opposite the reply which comes closest to how you have been feeling in the past week. Don't take too long over your replies: your immediate reaction to each item will probably be more accurate than a long thought out response.

Please tick only 1 box in each section

I feel tense or 'wound up':		**I feel as if I am slowed down:**	
Most of the time		Nearly all of the time	
A lot of the time		Very often	
Time to time, occasionally		Sometimes	
Not at all		Not at all	
I still enjoy the things I used to enjoy:		**I get a sort of frightened feeling like 'butterflies in the stomach':**	
Definitely as much		Not at all	
Not quite so much		Occasionally	
Only a little		Quite often	
Not at all		Very often	
I get a sort of frightened feeling like something awful is about to happen:		**I have lost interest in my appearance:**	
Very definitely and quite badly		Definitely	
Yes, but not too badly		I don't take as much care as I should	
A little, but it doesn't worry me		I may not take quite as much care	
Not at all		I take just as much care as ever	
I can laugh and see the funny side of things:		**I feel restless as if I have to be on the move:**	
As much as I always could		Very much indeed	
Not quite so much now		Quite a lot	
Definitely not so much now		Not very much	
Not al all		Not at all	
Worrying thoughts go through my mind:		**I look forward with enjoyment to things:**	
A great deal of the time		A much as I ever did	
A lot of the time		Rather less than I used to	
From time to time but not too often		Definitely less than I used to	
Only occasionally		Hardly at all	
I feel cheerful:		**I get sudden feelings of panic:**	
Not at all		Very often indeed	
Not often		Quite often	
Sometimes		Not very often	
Most of the time		Not at all	
I can sit at ease and feel relaxed:		**I can enjoy a good book or radio or TV programme:**	
Definitely		Often	
Usually		Sometimes	
Not often		Not often	
Not at all		Very seldom	

Name: *mailmerge* **Date sent:** *mailmerge*

Date completed by patient (please complete):

Thank you for your help in this study, your participation is now complete. Now please send this form back to the vascular lab in the stamped address envelope enclosed.

2010412HM1.0 15/12/2004

Hospital Anxiety and Depression Scale (HADS) Score

Doctors often need a quick, reliable method of finding out whether a patient is suffering from depression, or generalised anxiety disorders, and how severe their suffering may be.

The Hospital Anxiety and Depression Scale (HADS) is an easy-to-use self-reporting questionnaire, that gives a measure of a person's present state of mind. It has been extensively researched and validated, and has a good reputation amongst psychiatrists and doctors.

Choose one response for each of the questions below, clicking on the button to register your answer. Try not to think too long about an answer and give an immediate response.

Questions relating to anxiety are marked "Anxiety:" and those relating to depression are marked "Depression:". The scores for both will be calculated separately at the bottom, along with guidelines to the level of any disorder.

Anxiety: **I feel tense or 'wound up'...**	Most of the time	3
	A lot of the time	2
	From time to time, occasionally	1
	Not at all	0
Depression: **I still enjoy the things I used to enjoy...**	Definitely as much	0
	Not quite so much	1
	Only a little	2
	Hardly at all	3
Anxiety: **I get sort of frightened feeling as if something awful is about to happen...**	Very definitely and quite badly	3
	Yes, but not too badly	2
	A little, but it doesn't worry me	1
	Not at all	0

Depression: **I can laugh and see the funny side of things...**	As much as I always could	0
	Not quite so much now	1
	Definitely not so much now	2
	Not at all	3
Anxiety: **Worrying thoughts go through my mind...**	A great deal of the time	3
	A lot of the time	2
	From time to time, but not too often	1
	Only occasionally	0
Depression: **I feel cheerful...**	Not at all	3
	Not often	2
	Sometimes	1
	Most of the time	0
Anxiety: **I can sit at ease and feel relaxed...**	Definitely	0
	Usually	1
	Not often	2
	Not at all	3

Depression: I feel as if I am slowed down...	Nearly all the time	3
	Very often	2
	Sometimes	1
	Not at all	0
Anxiety: I get a sort of frightened feeling like 'butterflies' in the stomach...	Not at all	0
	Occasionally	1
	Quite Often	2
	Very Often	3
Depression: I have lost interest in my appearance...	Definitely	3
	I don't take as much care as I should	2
	I may not take quite as much care	1
	I take just as much care as ever	0
Anxiety: I feel restless as I have to be on the move...	Very much indeed	3
	Quite a lot	2
	Not very much	1
	Not at all	0

Depression: **I look forward with enjoyment to things...**	As much as I ever did	0
	Rather less than I used to	1
	Definitely less than I used to	2
	Hardly at all	3
Anxiety: **I get sudden feelings of panic…**	Very often indeed	3
	Quite often	2
	Not very often	1
	Not at all	0
Depression: **I can enjoy a good book or radio or TV program...**	Often	0
	Sometimes	1
	Not often	2
	Very seldom	3
Anxiety Scale:	**Depression Scale:**	

Score	Interpretation
0-7	Normal
8-10	Mild
11-14	Moderate
15-21	Severe

Reference

Zigmond and Snaith (1983)

GP Name
Practice address 1
Practice address 2
Practice address 3
Practice address 4

Academic Vascular Unit
Vascular Laboratory
Alderson House
Hull Royal Infirmary
Anlaby Road
Hull HU3 2JZ

Date

Corec ethics study number: LREC/12/01/253
HEY Hospitals NHS Trust R & D number: ELSY 2599

A Prospective trial on a novel approach for screening clinically significant (>5cm) Abdominal Aortic Aneurysms

Dear GP Name

Re: Patient name (forename & surname)

Patient address 1
Patient address 2
Patient address 3

Patient dob

Patient hosp no.

During the above patient's involvement in the above study, an abdominal aortic aneurysm (AAA) was detected by abdominal ultrasonography.

The diameter of this patient's abdominal aortic aneurysm is: ☐

This patient has therefore been enrolled onto the AAA surveillance programme carried out by the Academic Vascular Unit, Hull and East Yorkshire Hospitals NHS Trust. This will entail 6 monthly to 2 yearly ultrasound scans in the vascular laboratory, Hull Royal Infirmary depending on the exact AAA diameter.

Please contact me should you require any further information.

Thank you for your help.

Yours sincerely

Mr Michael ST Heng MB ChB MRCS
Vascular Research Fellow
Academic Vascular Unit
University of Hull / Hull Royal Infirmary

Postal address:
Vascular Laboratory
Alderson House
Hull Royal Infirmary
Anlaby Road
Hull HU3 2JZ

Tel: 01482 675523
Mobile: 0775 9219050

Email: michael.heng@hey.nhs.uk

GP Name
Practice address 1
Practice address 2
Practice address 3
Practice address 4

Academic Vascular Unit
Vascular Laboratory
Alderson House
Hull Royal Infirmary
Anlaby Road

Date

Corec ethics study number: LREC/12/01/253
HEY Hospitals NHS Trust R & D number: ELSY 2599

A Prospective trial on a novel approach for screening clinically significant (>5cm) Abdominal Aortic Aneurysms

Dear GP Name

Re: Patient name (forename & surname)

Patient address 1
Patient address 2
Patient address 3

Patient dob
Patient hosp no.

During the above patient's involvement in the above study, a clinically significant abdominal aortic aneurysm (AAA) was detected by abdominal ultrasonography.

The diameter of this patient's abdominal aortic aneurysm is: ☐

This patient has therefore been referred to a consultant within the Academic Vascular unit of Hull and East Yorkshire Hospitals NHS Trust. They will write to you directly.

Please contact me should you require any further information.

Thank you for your help.

Yours sincerely

Mr Michael ST Heng MB ChB MRCS
Vascular Research Fellow
Academic Vascular Unit
University of Hull / Hull Royal Infirmary

Postal address:
Vascular Laboratory
Alderson House
Hull Royal Infirmary
Anlaby Road
Hull HU3 2JZ

Tel: 01482 675523
Mobile: 0775 9219050

Email: michael.heng@hey.nhs.uk

5. Appendix 2: Ingredients of homogenising and dialysis buffers

These are the ingredients of the tissue homogenising buffer.

Distilled water

Urea

Urea is often used in buffers to disrupt non-covalent bonds in proteins, and thus to increase protein solubility in the buffer. Here it is used in a 2M concentration (molecular weight 60, therefore 120g/l or 12g in 100ml).

Brij 35

Brij 35 is 30% Polyoxyethyleneglycol dodecyl ether in water with preservative and surfactant (also known as lauryl alcohol ethylene oxide, ethoxylated lauryl alcohol, and polyoxyethylene lauryl ether). It is a non-ionic detergent, commonly used in buffers, aiding the removal of unreacted reagents, and useful in the isolation of functional membrane complexes. It also helps with the disruption of cell membranes, and structures and may also assist in mobilisation of MMPs from the extracellular matrix. It is difficult to remove from solution by dialysis, but can be removed by Extracti-Gel D detergent removal gel. It is used in this buffer at a concentration of 0.1% (1g/l or 100mg in 100ml).

Tris-hydrochloric acid

Tris-hydrochloric acid acts as an acid-base buffer stabilising the overall homogenising buffer for pH. It is used here in a concentration of 50mM (molecular weight 157.6, therefore 7.88g/l or 0.79g in 100ml)

EDTA

EDTA (Ethylenediaminetetraacetic acid) (figure 35, below) is a synthetic amino acid, which functions as a chelating agent, widely used to sequester di- and tri- valent metal ions. It binds using 4 carboxylate and 2 amine groups. EDTA also helps to disrupt cell membranes, and structures, thus it may assist in mobilising MMPs from the extra-cellular matrix. It may also be that EDTA is used here to aid extraction of MMPs from the tissue during homogenization through temporary chelation of the core zinc ion of MMP, although there is no current evidence to back this up.

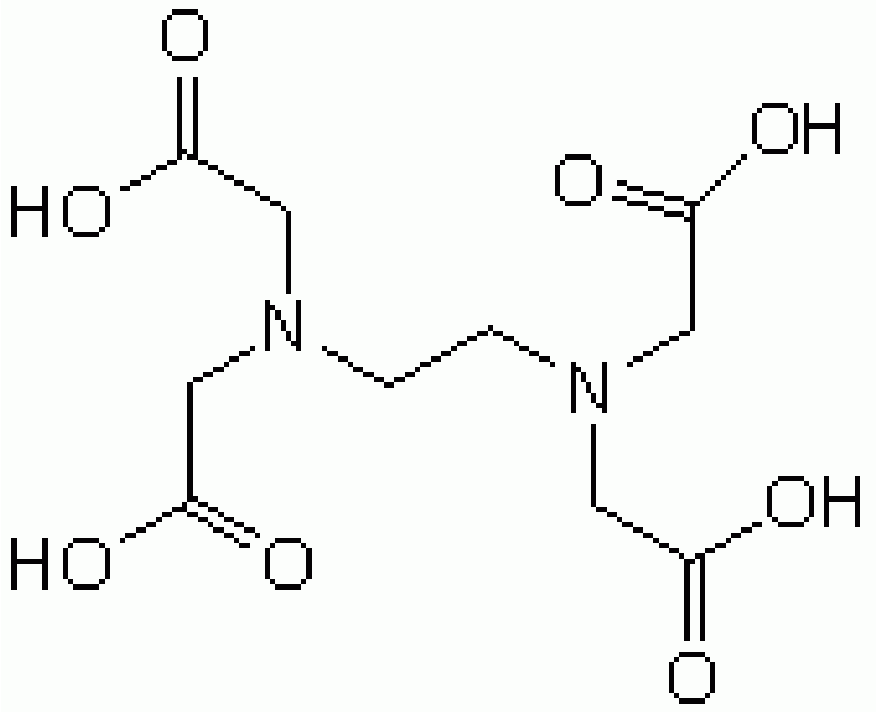


Figure 35. EDTA (www.merck-chemicals.com)

pH 7.6

Since low pH levels may lead to EDTA affecting MMP activity adversely, a higher pH was chosen.

Protease inhibitor

Calbiochem protease inhibitor cocktail set III was used in this buffer (table 15, below). It was added on the day of buffer use.

Table 15. Components of Calbiochem protease inhibitor cocktail set III (www.merckbiosciences.co.uk)

Product	Cat. No.	Mol. Wt.	Concentration in the Vial	Target Protease
AEBSF, Hydrochloride	101500	239.5	100 mM	Serine Proteases
Aprotinin, Bovine Lung, Crystalline	616370	6512	80 µM	Broad Spectrum, Serine Proteases
Bestatin	200484	308.4	5 mM	Aminopeptidase B and Leucine Aminopeptidase
E-64, Protease Inhibitor	324890	357.4	1.5 mM	Cysteine Proteases
Leupeptin, Hemisulfate	108975	475.6	2 mM	Cysteine Proteases and Trypsin-like Proteases
Pepstatin A	516482	685.9	1 mM	Aspartic Proteases

These are the ingredients of the dialysis buffer.

Distilled water

Brij 35

Brij 35 was added up to a concentration of 0.1%

Tris-hydrochloric acid

Tris-hydrochloric acid was added to a concentration of 25mmol/l

Calcium Chloride

It has been shown that calcium stabilises MMP in the presence of EDTA even at low pH levels. This is added to a concentration of 10mmol/l.

pH 8.5

The pH level was adjusted to 8.5 with sodium hydroxide.

6. Appendix 3: details of MMP kits and other laboratory equipment

6.1. Matrix metalloproteinase 2

The Matrix metalloproteinase-2 (MMP2) Biotrak activity assay system kit was used (Amersham code: RPN2631), produced by Amersham Biosciences. This was a 2-day test including an overnight incubation period. This kit system is specific for MMP2, and will measure active MMP2 directly, or total MMP2 by activation of pro-MMP2 by p-aminophenylmercuric acetate (APMA), in order that all MMP2 contained in the sample is activated and subsequently measured. A colorimetric assay is used and has been validated (Capper et al., 1999). Active MMP2 in the range 0.75 – 12ng/ml, with a sensitivity of 0.5ng/ml is measured. An increased sensitivity is available through an altered protocol to 190pg/ml with a range of 0.19 – 3ng/ml. 42 samples can be analysed in each microplate (in duplicate), after allowing for the recommended number of standard wells for this kit.

Mechanism of detection

A pro detection enzyme of modified urokinase is used, which is activated by active MMP2, and transformed into an active detection enzyme through a single proteolytic event. This active enzyme then catalyses a chromogenic peptide substrate allowing calculation of the level of active enzyme, through measurement of optical density at 405nm, using a spectrophotometric plate reader (see figure 36, below). Through comparison with known standards, run through the same microplate, and construction of a standard curve, the level of MMP2 in each of the samples can be interpolated.

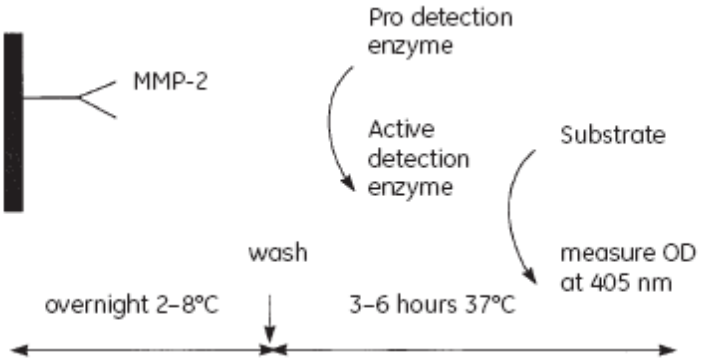


Figure 36. Protocol for MMP2 activity assay measuring endogenous active MMP2 (Amersham Biosciences)

Assay summary

The microplate is pre-coated with anti-MMP2 antibody. Control samples with known levels of human pro-MMP2, as well as unknown samples requiring quantification of MMP2 (active or total) are added to the microplate, with all samples being tested in duplicate (figure 37). Overnight incubation at 2-8°C allows MMP2 to be captured and bound to the microplate walls, with other components of the sample removed by repeated washing and aspiration. APMA is then added to wells where the total level of MMP2 is required. This activates pro-MMP2, but does not affect already active MMP2. In wells where only the level of active MMP2 is required, assay buffer is added to ensure comparable volume, and allow both sets of tests to use the same controls. The detection reagent is then added, and the microplate incubated again for a number of hours depending on the sensitivity required.

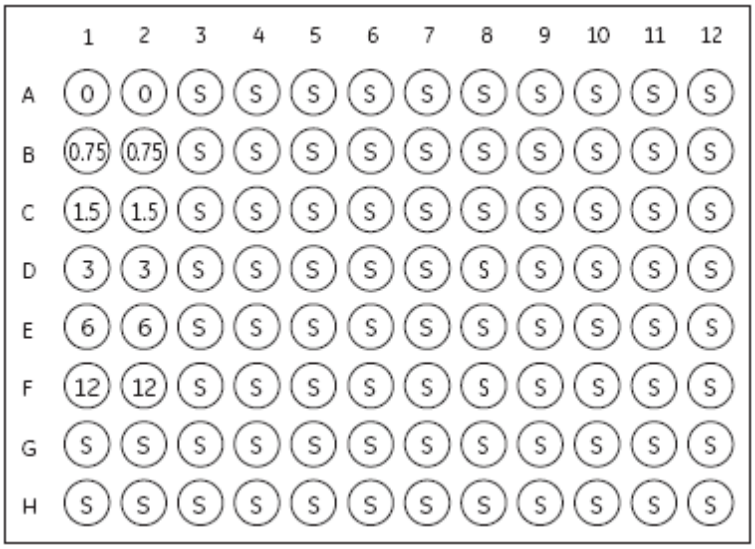


Figure 37. Recommended positioning of standard and sample wells (Amersham Biosciences)

Kit contents

This kit contained;

- 12 x 8 well microplate, pre-coated with anti-MMP2.
- Assay buffer containing 10ml Tris-HCl buffer concentrate. Made up with 100ml distilled water to give 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% by volume (v/v) BRIJ 35 and 0.01% by weight (w/v) gelatin.
- Standard contains 24ng of lyophilised (dried by freezing in a high vacuum) human pro MMP2. On reconstitution with 1ml of buffer, this gives a concentration of 24ng/ml pro MMP2 in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35 and 0.01% (w/v) gelatin.

- p-Aminophenylmercuric acetate (APMA). Bottle contains 352mg of APMA in powder form, made up with 1ml dimethylsulphoxide (DMSO) to 1M concentrated APMA solution. This is further diluted using 5µl of concentrated APMA (1M) to 10ml assay buffer to form a 0.5mM solution for use in the assay.
- Detection enzyme. Powdered concentrate reconstituted with assay buffer to 100µl of concentrated solution of modified urokinase in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1µM zinc chloride, 0.01% (v/v) BRIJ 35 and 0.01% (w/v) gelatin.
- Substrate. Bottle containing lyophilised S-2444 peptide substrate , reconstituted with 5.1ml of assay buffer to form a ready to use solution of S-2444 substrate in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1µM zinc chloride, 0.01% (v/v) BRIJ 35 and 0.01% (w/v) gelatin.
- Wash buffer. Bottle contains 12.5ml of phosphate buffer concentrate which when made up to 500ml with distilled water gives a 0.01M sodium phosphate buffer pH7.0 containing 0.05% Tween 20. Tween 20 (also known as polysorbate 20) is a stable relatively non-toxic polysorbate surfactant used here as a detergent.

The kit was stored between –15 and –30°C until needed. Kit instructions were fully followed as detailed including critical parameters. Kits were equilibrated at room temperature, particularly the microplates, as condensation affects the performance of the antibody with which the plates are coated.

MMP2 detection procedure protocol

The assay protocol for the assay range 0.75 – 12ng/ml of MMP2 was followed (higher endogenous levels). The extended detection range (for lower endogenous levels – 0.19-3ng/ml) involved the preparation of lower concentration standards, and a longer incubation time.

Day 1 reagent preparation

Assay buffer concentrate, anti-MMP2 coated microplate, and wash buffer concentrate were allowed to equilibrate to room temperature before use. Other components were removed from storage just before use. Assay buffer and wash buffer were prepared according to kit protocol, using distilled water. Standard was prepared using assay buffer. Both buffers were stored in a closed vessel at room temperature, and standard was stored on ice until needed.

Day 1 procedure

5 standards of known concentration of human pro-MMP2 (0.75, 1.5, 3, 6 and 12ng/ml) were freshly prepared by successive dilution with assay buffer and vortex mixing. 1 sample of assay buffer only was also prepared ('blank' - 0ng/ml MMP2 content). 100µl of blank, all standard concentrations and unknown samples were put in wells on the microplate in positions as suggested (figure above), in duplicate. The microplate was covered and incubated at 2-8°C overnight.

Day 2 reagent preparation

APMA, detection enzyme, substrate, detection reagent was prepared according to kit protocol. APMA was prepared as instructed using fresh dimethylsulphoxide (DMSO)(in a fume cupboard, with skin and eye protection), and then diluted again to

form the ready-to-use dilution. Prior to use, detection enzyme was thawed and stored on ice. Substrate was also stored on ice prior to use. Detection reagent was 100µl of detection enzyme added to the reconstituted substrate. This was prepared immediately prior to addition to wells and vortex mixed.

Day 2 procedure

All wells were washed with wash buffer and aspirated 4 times using a microplate washer ensuring each well was fully filled and emptied each time. 50µl of ready-to-use APMA was pipetted into all standard wells, and wells needing the quantification of total MMP2. 50µl of assay buffer was added to wells for quantification of active MMP2 only. All wells then had 50µl of detection reagent added. The microplate was shaken in a microplate shaker for 20 seconds and the initial (T_0) spectrophotometer reading at 405nm was taken. The microplate was then covered and incubated at 37°C for 3 hours, shaken and a subsequent spectrophotometer reading at 405nm taken (T_3).

Calculation of MMP2 activity levels

MMP2 activity is directly proportional to the cleavage of S-2444 (substrate), so it can be represented by the rate of change of optical absorbance at 405nm. The absorbance change is linear with respect to the square of the duration of incubation (figure 38).

$\delta Abs_{405} / h^2$

Figure 38. MMP2 activity proportional to rate of change of absorbance at 405nm

Multiplication by 1000 allows whole numbers to be plotted on the graft thus (figure 39).

$(\delta Abs_{405} / h^2) * 1000$

Figure 39. MMP2 activity

6.2. Matrix metalloproteinase 8

The Matrix metalloproteinase-8 (MMP8) Biotrak activity assay system kit was used (Amersham code: RPN2635), produced by Amersham Biosciences. This was a 2-day test including an overnight incubation period. This kit system is specific for MMP8, and will measure active MMP8 directly, or total MMP8 by activation of pro-MMP8 by APMA. Active MMP8 in the range 0.09 – 24ng/ml, with a sensitivity of 1.2ng/ml is measured for a 2 hour incubation. 41 samples can be analysed in each microplate (in duplicate).

Details of this activity assay are similar to the activity assay for MMP2, but differences will be pointed out and full details of the kit will be included as an appendix.

This assay uses a microplate that is pre-coated with goat, anti-mouse antibody, and subsequent exposure to mouse anti-MMP8, allows immobilisation of MMP8 within the microplate wells.

Mechanism of detection

The mechanism of detection is as for the above-described Amersham Biosciences MMP2 activity assay system and therefore does not need further description.

MMP8 activity assay kit contents

The kit contents were identical to the MMP2 assay except for;

- The microplate was pre-coated with $F(ab')_2$ goat anti-mouse antibody(rather than anti-MMP2).
- This kit contained lyophilised mouse anti-MMP8, which on reconstitution with 11ml of assay buffer gave mouse anti-MMP8 in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.
- The standard contained 48ng of lyophilised human pro MMP8 (rather than 24ng of lyophilised human pro-MMP2), which on reconstitution gave a concentration of 48ng/ml pro MMP8.
- The ready to use APMA solution was made up to 1.5mM (note concentration of APMA in MMP2 activity assay was 0.5mM).

MMP8 detection procedure protocol

The assay protocol for the assay range 0.75 – 24ng/ml of MMP8 was followed (higher endogenous levels). The extended detection range (for lower endogenous levels – 0.09-6ng/ml) was occasionally used and involved more standards, and standards of a lower concentration than in the normal protocol.

The assay protocol was identical to the MMP2 activity assay protocol except for the following details.

Microplate treatment

The microplate was pre-coated with goat anti-mouse antibody, and this was incubated at 37°C for 2 hours with 100µl reconstituted lyophilised mouse anti-MMP8. The microplate was then aspirated and washed 4 times with wash buffer using an automatic microplate washer. In contrast the microplate supplied with the MMP2 activity assay kit was pre-coated with anti-MMP2, and did not require this step (figure 40).

Standards

1 extra standard of known concentration human pro-MMP8 was used compared to the MMP2 activity assay (to make 6 in total) (0.75, 1.5, 3, 6, 12 and 24ng/ml). This only allowed 41 samples to be analysed (compared to the 42 samples in the MMP2 activity assay).

Day 2 – APMA activation of pro-MMP8

After addition of ready-to-use APMA, the microplate was incubated at 37°C for 1 hour prior to addition of detection reagent (In the MMP2 protocol, there was no interim incubation between addition of APMA and detection reagent).

Day 2 – final incubation period

Final incubation was at 37°C for 2 hours (as opposed to 3 hours – MMP2), after which normal microplate shaking and spectrophotometry at 405nm was carried out.

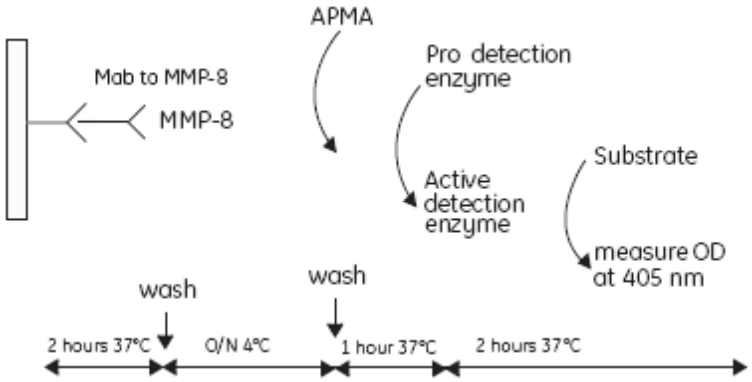


Figure 40. Protocol for MMP8 activity assay measuring endogenous active MMP8 (Amersham Biosciences)

Calculation of MMP8 activity levels

The mechanism of calculation for MMP8 activity levels is as for the above-described Amersham Biosciences MMP2 activity assay system and therefore does not need further description.

6.3. Matrix metalloproteinase 9

The Matrix metalloproteinase-9 (MMP9) Biotrak activity assay system kit was used (Amersham code: RPN2634), produced by Amersham Biosciences. This was a 2-day test including an overnight incubation period. This kit system is specific for MMP9, and will measure active MMP9 directly, or total MMP9 by activation of pro-MMP9 by APMA. Active MMP9 in the range 0.5 – 16ng/ml, with a sensitivity of 0.5ng/ml is measured. A prolonged incubation period protocol is available for lower endogenous MMP9 levels with an assay detection range of 0.125 – 4 ng/ml. 41 samples can be analysed in each microplate (in duplicate).

Details of this activity assay are similar to the activity assay for MMP2, but differences will be pointed out and full details of the kit will be included as an appendix.

Mechanism of detection

The mechanism of detection is as for the above-described Amersham Biosciences MMP2 activity assay system and therefore does not need further description.

Kit contents

The kit contents were identical to the MMP2 assay except for;

- 12 x 8 well microplate pre-coated with anti-MMP9.
- The standard contained 32ng of lyophilised human pro MMP9 (rather than 24ng of pro MMP2). On reconstitution with 1ml of buffer, this gave a concentration of 32ng/ml pro MMP9.
- Ready to use APMA solution was made up to 1mM (note 0.5mM for MMP2 assay, and 1.5mM for MMP8 assay).

MMP9 detection procedure protocol

The assay protocol for the assay range 0.5 – 16ng/ml of MMP9 was followed (normal endogenous levels). The extended detection range (for lower endogenous levels – 0.125-4ng/ml) was occasionally used and involved more standards, and standards of a lower concentration than in the normal protocol.

The assay protocol was identical to the MMP2 activity assay protocol except for the following details.

Standards

1 extra standard was used compared to the MMP2 assay protocol (5 standards) to make a total of 6 standards (0.5, 1, 2, 4, 8 and 16ng/ml).

APMA

After addition of ready-to-use APMA (or buffer), an interim incubation at 37°C for 1.5 hours was allowed prior to the addition of the detection reagent (no interim incubation for MMP2 assay).

Detection reagent

Incubation after addition of the detection reagent was at 37°C for 1 hour (MMP2 assay incubation 3 hours), followed by normal plate shaking and a subsequent spectrophotometry at 405nm.

Calculation of MMP9 activity levels

The mechanism of calculation for MMP9 activity levels is as for the above-described Amersham Biosciences MMP2 activity assay system and therefore does not need further description

6.4. Tissue inhibitor of metalloproteinase 1

The tissue inhibitor of metalloproteinase-1 (TIMP1), human, Biotrak ELISA system kit was used (Amersham code: RPN2611), produced by Amersham Biosciences. This was a 1-day test. This kit system is specific for total human TIMP1, including TIMP1 complexed with MMPs, but does not cross react with TIMP2, or MMP1. This kit uses an ELISA sandwich format. TIMP1 in the range 3.13 – 50ng/ml, with a sensitivity of 1.25ng/ml is measured. 42 samples can be analysed in each microplate (in duplicate).

Mechanism of detection

A sandwich ELISA format is used where TIMP1 is immobilised to the microplate walls which are coated with anti-TIMP1, and after washing, peroxidase conjugate (antibody to TIMP1 coupled to horseradish peroxide (HRP), sandwiches TIMP1 on the microplate wall (figure 41). After washing, the amount of peroxidase bound is then determined by TMB substrate (3,3', 5,5'- tetramethylbenzidine (TMB)/hydrogen peroxide mix), and sulphuric acid added to stop the reaction after 30mins.

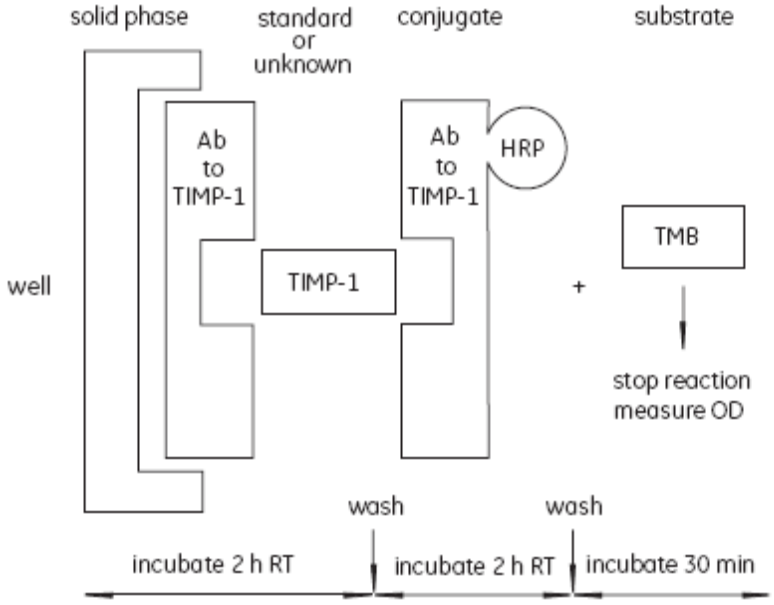


Figure 41. TIMP1 ELISA assay design

Kit contents

This kit contained;

- 12 x 8 well microplate, pre-coated with anti-TIMP1.
- Assay buffer containing 10ml phosphate buffer concentrate. Made up with 100ml distilled water to give 0.1M phosphate buffer pH7.5 containing 0.9% by

weight (w/v) sodium chloride, 0.1% (w/v) bovine serum albumin, and 0.1% Tween 20.

- Standard contains 100ng of lyophilised (dried by freezing in a high vacuum) TIMP1. On reconstitution, this gives a concentration of 100ng/ml TIMP1 in 0.1M phosphate buffer pH7.5 containing 0.9% by weight (w/v) sodium chloride, 0.1% (w/v) bovine serum albumin, and 0.1% Tween 20.
- Peroxidase conjugate. Lyophilised anti-TIMP1 horseradish peroxidase, which on reconstitution with 11ml of assay buffer gives anti-TIMP1 horseradish peroxidase in 0.1M phosphate buffer pH7.5 containing 0.9% by weight (w/v) sodium chloride, 0.1% (w/v) bovine serum albumin, and 0.1% Tween 20.
- Wash buffer. Bottle contains 12.5ml of phosphate buffer concentrate which when made up to 500ml with distilled water gives a 0.01M sodium phosphate buffer pH7.5 (note different pH to wash buffer for MMP activity kits) containing 0.05% Tween 20. Tween 20 (also known as polysorbate 20) is a stable relatively non-toxic polysorbate surfactant used here as a detergent.
- TMB Substrate. 3,3', 5,5'- tetramethylbenzidine (TMB)/hydrogen peroxide ready for use.

The kit was stored between –15 and –30°C unit needed. Kit instructions were fully followed as detailed including critical parameters. Certain kit components were equilibrated at room temperature before use as instructed.

TIMP1 detection procedure protocol

Reagent preparation

Assay buffer concentrate, anti-TIMP1 coated microplate, wash buffer concentrate, standard, peroxidase conjugate and TMB substrate were allowed to equilibrate to room temperature before use as was unknown sample. Assay buffer and wash buffer were prepared according to kit protocol, using distilled water. Both buffers were stored in a closed vessel at room temperature.

Procedure

5 standards of known concentration of TIMP1 (3.13, 6.25, 12.5, 25, 50ng/ml) were freshly prepared by successive dilution with assay buffer and vortex mixing. 1 sample of assay buffer only was also prepared ('blank' - 0ng/ml TIMP1 content). 100µl of blank, all standard concentrations and unknown samples were put in wells on the microplate in positions as suggested (figure 42 below), in duplicate. The microplate was covered and incubated at 20-25°C for exactly 2 hours.

All wells were washed with wash buffer and aspirated 4 times using a microplate washer ensuring each well was fully filled and emptied each time. 100µl of peroxidase conjugate was pipetted into all wells, and incubated at 20-25°C for exactly 2 hours.

All wells were washed and aspirated again 4 times, and 100µl room temperature equilibrated TMB substrate was dispensed into all wells. The plate was again covered and incubated at room temperature (15-30°C) for 30 minutes on a microplate shaker.

100µl of 1M sulphuric acid was then added to all wells, and the plate read at 450nm by a microplate spectrophotometer within 30minutes (note different wavelength of light to MMP activity kits).

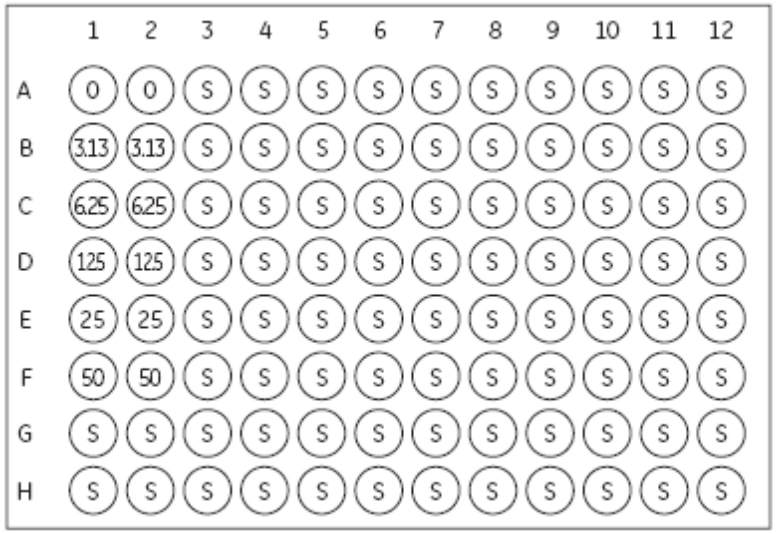


Figure 42. Recommended positioning of standard and sample wells (Amersham Biosciences)

Calculation of TIMP1 levels

TIMP1 is detected by peroxidase labelled antibody to TIMP1. The excess peroxidase is washed away, and levels of peroxidase are then detected by TMB ready to use substrate. 1M sulphuric acid is used to stop the reaction and the resulting colour indicating concentration of peroxidase can be measured at 450nm using a microplate spectrophotometer. The concentration of TIMP1 in the sample can be interpolated from the standard curve. We used a polynomial equation to the graph, and calculated TIMP1 concentrations straight from this.

6.5. Tissue inhibitor of metalloproteinase 2

The tissue inhibitor of metalloproteinase-2 (TIMP2), human, Biotrak ELISA system kit was used (Amersham code: RPN2618), produced by Amersham Biosciences. This was a 1-day test. This kit system recognises free TIMP2, and that complexed with active forms of MMPs, but not TIMP2 complexed with proMMP2. It does not cross react with TIMP1, or TIMP-3. This kit uses an ELISA sandwich format. TIMP2 in the range 8 – 128ng/ml, with a sensitivity of 3ng/ml is measured. 42 samples can be analysed in each microplate (in duplicate).

Details of this ELISA assay are similar to the above-described Biotrak TIMP1 ELISA assay, but differences will be pointed out and full details of the kit will be included as an appendix.

Mechanism of detection

A sandwich ELISA format is used (figure 43), as in the TIMP1 assay and therefore does not need further description.

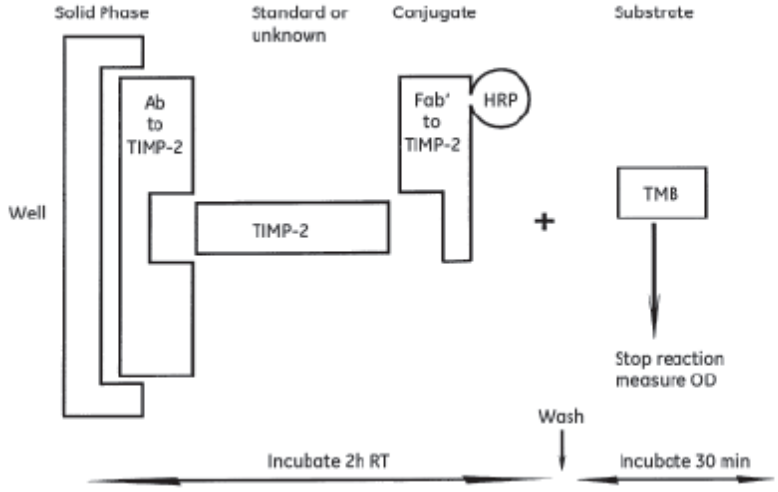


Figure 43. TIMP2 ELISA assay design

Kit contents

The kit contents were similar to the TIMP1 assay except for;

- 12 x 8 well microplate, pre-coated with anti-TIMP2.
- Assay buffer containing 10ml phosphate buffer concentrate. Made up with 100ml distilled water to give 0.03M phosphate buffer pH7.0 containing 0.1M sodium chloride, 0.3% (w/v) bovine serum albumin, and 0.01M EDTA (TIMP1 assay buffer - 0.1M phosphate buffer, with no EDTA).
- Standard contains 256ng of lyophilised TIMP2. On reconstitution with 1ml of assay buffer, this gives a concentration of 256ng/ml TIMP2 in 0.03M phosphate buffer pH7.0 containing 0.1M sodium chloride, 0.3% (w/v) bovine serum albumin, and 0.01M EDTA (TIMP1 standard contains 100ng of TIMP1, with different concentration of assay buffer).
- Peroxidase conjugate. Lyophilised anti-TIMP2 horseradish peroxidase, which on reconstitution with 12ml of assay buffer gives anti-TIMP2 horseradish peroxidase in 0.03M phosphate buffer pH7.0 containing 0.1M sodium chloride, 0.3% (w/v) bovine serum albumin, and 0.01M EDTA.

TIMP2 detection procedure protocol

The assay protocol was identical to the TIMP1 ELISA assay protocol except for the following details.

Standards

5 standards of known concentration of TIMP2 (8, 16, 32, 64, 128ng/ml) were freshly prepared by successive dilution with assay buffer and vortex mixing.

Sample volume and conjugate

50µl of blank, all standard concentrations and unknown samples were put in wells on the microplate, together with 50µl of conjugate to all wells and the microplate was covered and incubated at 20-27°C for exactly 2 hours (in the TIMP1 protocol 100µl of samples was added, incubated at 20-25°C for 2 hours, washed, then 100µl of peroxidase conjugate was added prior to further incubation at 20-25°C for 2 hours).

Calculation of TIMP2 levels

Calculation of TIMP2 concentration was carried out as for TIMP1 as detailed above.

6.6. Protein concentration

For the truly representative and comparative MMP concentration value in tissue, many studies have used a MMP concentration relative to total protein concentration of the tissue homogenate.

We used the modified Bradford assay to quantify the protein concentration using a test kit "Coomassie Plus – the better Bradford assay kit," (product number 23236, Pierce, Rockford, IL).

Mechanism of detection

The Bradford Assay was introduced in 1976 (Bradford, 1976) and is still in use today with some modifications, as a rapid and sensitive method of protein concentration determination. The technique is a colorimetric protein assay, and involves the binding of protein to dye which changes colour from red to blue (figure 44). An optical reading is taken at 595nm, and the increase in absorption at this level is proportional to the amount of bound protein. Known concentrations of protein are used as standards,

and this produces a standard curve from which protein concentration can be worked out for a given change in optical density (595nm), which in turn produces a reliable protein concentration for a particular solution.

Other protein determination methods were quite susceptible to interference from other substances which could be present in protein samples. This is less true of the Bradford Assay, however high concentrations of detergent which may be have been used in protein extraction, for example SDS (sodium dodecyl sulfate), can produce artificially low protein levels on testing due to detergent binding to protein, or conversely produce artificially high levels by binding to certain fractions of the dye, causing a change in equilibrium. Buffer can also cause an over-estimation in protein concentration due to the depletion of free protons (which is normally taken up by the protein).

It is often necessary to dilute protein samples in order that their concentrations lie within the linear section of the Bradford assay (2 – 120 µg/ml), but newer formulations have an extended range of linear response.

In our study we used a modified Bradford Assay, and 'Coomassie Plus – the better Bradford Assay kit' (Pierce, Rockford, IL), which included Coomassie Plus, and albumin standards (bovine serum albumin 2.0mg/ml in 0.9% saline and 0.05% sodium azide).

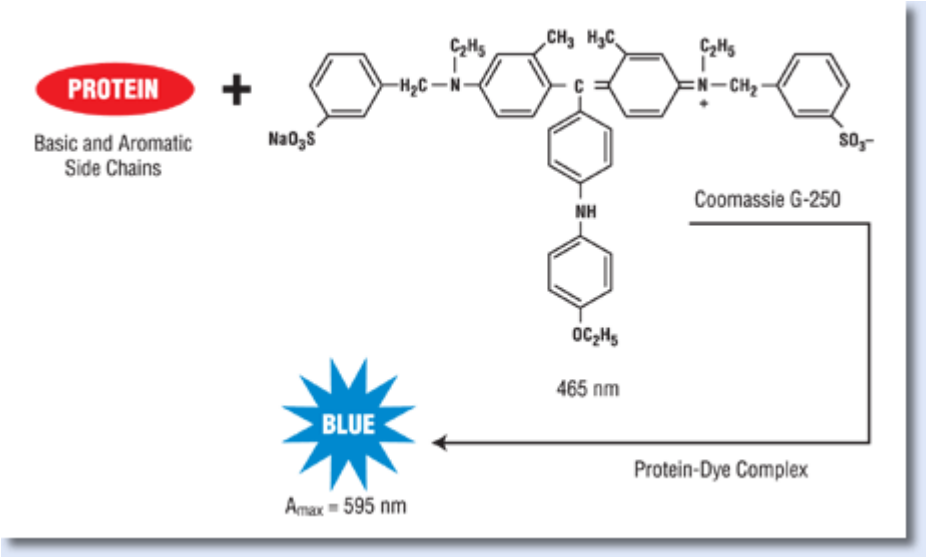


Figure 44. Reaction schematic for Coomassie Plus (Piercenet.com)

Assay protocol

Sterile, 96 well flat bottom microplate, with lids (Sarstedt tissue culture plate – ref: 83.1835) were used for protein concentration determination. Maximum volume of each well 380µl.

Protein standards were prepared according to the instructions provided with the kit for a working range of 100-1,500µg/ml (see table 16 below). Diluent used was assay buffer for MMP kits. The components of this buffer were fully compatible with the protein assay kit.

Table 16. Preparation of diluted albumin (BSA) standards (working range 100-1,500 µg/ml)

Vial	Volume of diluent	Volume/ source of BSA	Final BSA concentration
A	0	300µl of stock	2,000 µg/ml

B	125µl	375µl of stock	1,500 µg/ml
C	325µl	325µl of stock	1,000 µg/ml
D	175µl	175µl of vial B dilution	750 µg/ml
E	325µl	325µl of vial C dilution	500 µg/ml
F	325µl	325µl of vial E dilution	250 µg/ml
G	325µl	325µl of vial F dilution	125 µg/ml
H	400µl	100µl of vial G dilution	25 µg/ml
I	400µl	0	0 µg/ml = blank

Abstract from instructions "Coomassie plus – the better Bradford assay kit," Pierce product number 23236

Coomassie plus was allowed to equilibrate to room temperature before use, and was gently inverted several times to disperse any dye-dye aggregates that had formed.

10µl of each standard and each unknown was pipetted into each well of the microplate (all tests were carried out in at least duplicate – some triplicate – detailed in results section).

300µl of Coomassie plus was then dispensed into each well using a multi-channel pipette, and shaken on a plate shaker for 30 seconds. The plate was then left at room temperature for 10 minutes then optical density was read at 595nm with a plate reader.

Details were recorded on a Microsoft Excel spreadsheet.

Kit contents

- Coomassie plus assay reagent, 950ml, containing Coomassie G-250 dye, methanol, phosphoric acid and solubilizing agents in water.
- Albumin standard ampules 2mg/ml (10 x 1ml) with bovine serum albumin (BSA) at a concentration of 2.0mg/ml in a solution of 0.9% saline and 0.05% sodium azide.

Calculation of protein concentration

The average of the standard blank replicates optical density at 595nm were subtracted from all the other individual wells' optical densities.

A standard curve was produced using the known concentrations of protein in the standards' wells, and blank-corrected optical density at 595nm plotted against the known concentration of protein in µg/ml. A quadratic equation was fitted to the standard curve, and this was used to determine the protein concentration of each sample. If R^2 was <0.9 then the test was repeated and a pipetting error was assumed.

6.7. Other lab equipment

Pipettes

Single and multi-channel pipettes were used – all with disposable polypropylene tips.

Magnetic stirrer

Magnetic stirrers were used during the preparation of buffers.

Centrifuge

Centrifuge for 4 x 50ml screw-top polypropylene test tubes was used for early sample homogenate preparation.

A refrigerated centrifuge for flip-top Sarstedt tube was also used for sample homogenate preparation.

Microplate

96 well flat bottom microplate (Sarstedt tissue culture plate – ref: 83.1835) used for protein concentration determination. Other microplates supplied with kits as above.

Microplate shaker

A microplate shaker was used. Plates were covered during shaking.

Microplate washer

Thermo labsystems wellwash 4 Mk2 was used.

Microplate spectrophotometer

Anthos 2010 microplate spectrophotometer (ASYS Hitech GmbH, Austria) was used connected to a PC running Windows 2000 professional operating system (Microsoft, USA), and using Stingray ver1.1 (Dazdaq Ltd, Brighton) software.

For 405nm spectrophotometer readings, the above spectrophotometer did not have the appropriate filter and therefore a different machine was used. This was the Synergy HT (Bio-Tek, Vermont), using KC4 Kineticalc for windows version 3.3 revision 10.

7. Appendix 4: Raw MMP / TIMP results

The following are tables detailing results of MMP2, 8, 9, TIMP1, 2 and protein concentrations. In the main thesis they are presented summarised as box and whisker plots.

Table 17. Tissue MMP2 Concentration and Ratios - active and total at peak wall stress and arteriotomy sites

	MMP2 concentration (ng/mg protein)					
Subject	High Act	High Total	Art Act	Art Total	High Ratio	Art Ratio
6	4.14	44.91	1.62	35.99	0.09	0.05
7	6.15	179.92	5.74	47.43	0.03	0.12
8						
9	2.86	35.08	2.70	29.64	0.08	0.09
10	1.93	11.23	1.05	7.09	0.17	0.15
11	5.65	53.41	1.10		0.11	
12	1.53	15.41	2.42	21.87	0.10	0.11
13	4.74	30.60	1.26	25.98	0.15	0.05
14	3.14	13.38	6.19	25.14	0.23	0.25
15	3.07	28.85	1.30	7.64	0.11	0.17
16	1.36	16.53	1.40	12.65	0.08	0.11
17	2.71	24.03	2.81	18.92	0.11	0.15
18	6.94	100.76	4.89	64.23	0.07	0.08
19	4.46	40.55	5.99	14.03	0.11	0.43
20	0.89	8.26	1.29	6.58	0.11	0.20
21	2.04	15.29	2.61	26.37	0.13	0.10
22						
23	5.99	24.78	2.48	7.50	0.24	0.33
24						
25	1.06	23.56	4.86	58.21	0.05	0.08
26	5.32	27.41	1.71	13.35	0.19	0.13
27						
28						
29	2.74	28.19	6.02	70.46	0.10	0.09
30	0.99	11.33	1.91	21.31	0.09	0.09
31						
32						
33	1.67	10.24	2.94	29.06	0.16	0.10
34	0.12	1.08	2.23	16.96	0.11	0.13
35						
36						

Mean	3.16	33.85	2.93	26.69	0.12	0.14
Median	2.80	24.40	2.45	21.87	0.11	0.11
Min	0.12	1.08	1.05	6.58	0.03	0.05
Max	6.94	179.92	6.19	70.46	0.24	0.43
Sum	69.50	744.77	64.52	560.40	2.64	2.99
No	22	22	22	21	22	21

High Act: Active MMP2 at peak wall stress site, High Total: Total MMP2 at peak wall stress site; Art Act: Active MMP2 at arteriotomy site; Art Total: Total MMP2 at arteriotomy site; High Ratio: Ratio of active MMP2 to total MMP2 at peak wall stress site; Art Ratio: Ratio of active MMP2 to total MMP2 at arteriotomy site.

Table 18. Tissue MMP8 Concentration and Ratios - active and total at peak wall stress and arteriotomy sites

	MMP8 concentration (ng/mg protein)					
Subject	High Act	High Total	Art Act	Art Total	High Ratio	Art Ratio
6	22.07	57.58	8.34	20.78	0.38	0.40
7	30.15	85.20	56.86	71.56	0.35	0.79
8						
9	19.62	36.44	6.66	40.35	0.54	0.16
10	9.40	27.26	7.31	11.51	0.34	0.63
11	2.15		0.89			
12	4.13	10.04	4.91	12.58	0.41	0.39
13	6.74	17.55	3.18	9.36	0.38	0.34
14	2.90	8.74	15.03	19.22	0.33	0.78
15	1.52	4.50	4.80	11.45	0.34	0.42
16	1.64	6.77	4.99	13.54	0.24	0.37
17	5.76	26.24	2.75	6.32	0.22	0.43
18	7.17	52.25	8.70	25.10	0.14	0.35
19	2.69	14.25	11.94	8.58	0.19	1.39
20	11.06	12.29	17.77	26.68	0.90	0.67
21	6.06	9.59	5.26	10.16	0.63	0.52
22						
23	3.99	20.40	3.80	16.44	0.20	0.23
24						
25	2.65	18.80	1.72	13.97	0.14	0.12
26	9.30	20.50	12.88	21.04	0.45	0.61
27						
28						
29	4.63	20.58	21.01	39.27	0.22	0.54
30	2.03	4.81	1.36	8.72	0.42	0.16
31						
32						
33	23.34	99.80	39.57	71.69	0.23	0.55
34	2.19	10.21	21.31	58.89	0.21	0.36
35						
36						

Mean	8.24	26.85	11.86	24.63	0.35	0.49
Median	5.20	18.80	6.98	16.44	0.34	0.42
Min	1.52	4.50	0.89	6.32	0.14	0.12
Max	30.15	99.80	56.86	71.69	0.90	1.39
Sum	181.22	563.80	261.02	517.21	7.29	10.22
No	22	21	22	21	21	21

High Act: Active MMP8 at peak wall stress site, High Total: Total MMP8 at peak wall stress site; Art Act: Active MMP8 at arteriotomy site; Art Total: Total MMP8 at arteriotomy site; High Ratio: Ratio of active MMP8 to total MMP8 at peak wall stress site; Art Ratio: Ratio of active MMP8 to total MMP8 at arteriotomy site

Table 19. Tissue MMP9 Concentration and Ratios - active and total at peak wall stress and arteriotomy sites

	MMP9 concentration (ng/mg protein)					
Subject	High Act	High Total	Art Act	Art Total	High Ratio	Art Ratio
6	0.67	9.33	0.43	5.19	0.072	0.083
7	1.21	21.71	1.41	16.12	0.056	0.088
8						
9	0.49	7.86	0.59	11.34	0.063	0.052
10	0.74	44.93	0.32	20.69	0.016	0.015
11	4.57		0.30			
12	0.41	15.34	0.16	20.29	0.026	0.008
13	0.90	9.98	0.65	6.67	0.090	0.098
14	0.21	4.22	0.41	17.15	0.050	0.024
15	0.40	2.75	0.52	8.74	0.145	0.059
16	0.73	34.27	0.13	19.52	0.021	0.007
17	0.83	13.92	0.21	2.49	0.060	0.086
18	2.56	83.12	2.02	45.84	0.031	0.044
19	0.39	3.28	0.29	3.33	0.119	0.087
20	0.19	3.02	0.63	10.76	0.063	0.058
21	0.39	4.24	0.48	9.42	0.091	0.051
22						
23	2.23	18.28	1.58	11.19	0.122	0.142
24						
25	0.25	31.00	0.68	33.88	0.008	0.020
26	0.47	27.22	0.44	26.65	0.017	0.016
27						
28						
29	0.75	9.23	1.58	18.86	0.081	0.084
30	0.33	3.68	0.55	8.36	0.088	0.066
31						
32						
33	0.72	9.70	1.37	22.21	0.074	0.062
34	0.12	2.26	0.83	9.96	0.053	0.083
35						
36						

Mean	0.89	17.11	0.71	15.65	0.064	0.059
Median	0.58	9.70	0.53	11.34	0.063	0.059
Min	0.12	2.26	0.13	2.49	0.008	0.007
Max	4.57	83.12	2.02	45.84	0.145	0.142
Sum	19.55	359.32	15.58	328.66	1.348	1.233
No	22	21	22	21	21	21

High Act: Active MMP9 at peak wall stress site, High Total: Total MMP9 at peak wall stress site; Art Act: Active MMP9 at arteriotomy site; Art Total: Total MMP9 at arteriotomy site; High Ratio: Ratio of active MMP9 to total MMP9 at peak wall stress site; Art Ratio: Ratio of active MMP9 to total MMP9 at arteriotomy site

Table 20. Tissue TIMP1 concentrations at peak wall stress and arteriotomy sites

	TIMP1 concentration (ng/mg protein)		
Subject	High	Art	Art/H Ratio
6	903.56	288.56	0.32
7	574.36	629.36	1.10
8			
9	48.12	52.85	1.10
10	371.09	176.38	0.48
11	775.10	55.34	0.07
12	506.23	887.31	1.75
13	494.18	147.17	0.30
14	483.10	144.25	0.30
15	234.13	48.44	0.21
16	365.53	224.07	0.61
17	169.91	301.30	1.77
18	1229.42	467.25	0.38
19	145.68	158.90	1.09
20	30.87	54.55	1.77
21	253.89	338.85	1.33
22			
23	799.53	97.28	0.12
24			
25	224.02	693.51	3.10
26	364.14	313.52	0.86
27			
28			
29	420.12	721.16	1.72
30	298.13	338.19	1.13
31			
32			
33	319.94	235.60	0.74
34	19.33	2.79	0.14
35			
36			

Mean	410.47	289.85	0.93
Median	364.84	229.84	0.80
Min	19.33	2.79	0.07
Max	1229.42	887.31	3.10
Sum	9030.38	6376.62	20.38
No	22	22	22

High: TIMP1 at peak wall stress site, Art: TIMP1 at arteriotomy site; Art/H Ratio: Ratio of TIMP1 at arteriotomy site to TIMP1 at peak wall stress site

Table 21. Tissue TIMP2 concentration at peak wall stress and arteriotomy sites

	TIMP2concentration (ng/mg protein)		
Subject	High	Art	Art/H Ratio
6	4.67	6.33	1.35
7	8.56	12.79	1.49
8			
9	2.01	5.46	2.72
10	17.14	10.08	0.59
11	39.94	6.88	0.17
12	13.76	18.71	1.36
13	39.85	17.25	0.43
14	78.16	11.91	0.15
15	9.27	8.70	0.94
16	22.63	39.64	1.75
17	19.93	8.14	0.41
18	41.28	29.53	0.72
19	20.15	11.91	0.59
20	2.18	31.14	14.32
21	8.53	4.06	0.48
22			
23	29.01	14.07	0.48
24			
25	17.05	31.35	1.84
26	19.18	15.20	0.79
27			
28			
29	29.28	18.76	0.64
30	14.54	32.55	2.24
31			
32			
33	13.53	37.18	2.75
34	1.34	17.62	13.11
35			
36			

Mean	20.55	17.69	2.24
Median	17.09	14.63	0.87
Min	1.34	4.06	0.15
Max	78.16	39.64	14.32
Sum	452.01	389.27	49.32
No	22	22	22

High: TIMP2 at peak wall stress site, Art: TIMP2 at arteriotomy site; Art/H Ratio: Ratio of TIMP2 at arteriotomy site to TIMP2 at peak wall stress site

Table 22. Tissue protein concentration at peak wall stress and arteriotomy sites

	Protein concentration (mg/ml)		
Subject	High	Low	Art/H Ratio
6	2.09	2.83	1.35
7	1.01	0.93	0.92
8			
9	2.71	2.98	1.10
10	2.27	3.95	1.74
11	1.30	6.65	5.12
12	2.84	1.93	0.68
13	1.29	1.70	1.32
14	1.98	1.16	0.59
15	4.93	6.04	1.23
16	2.37	2.48	1.05
17	2.74	5.12	1.87
18	0.84	1.22	1.45
19	1.86	4.24	2.28
20	6.59	3.71	0.56
21	4.31	3.38	0.78
22			
23		1.02	
24			
25	2.13	1.16	0.54
26	1.44	2.39	1.66
27			
28			
29	1.65	0.81	0.49
30	3.71	2.06	0.56
31			
32			
33	2.69	0.98	0.36
34	20.92	1.54	0.07
35			
36			

Mean	3.41	2.65	1.22
Median	2.27	2.23	1.05
Min	0.84	0.81	0.07
Max	20.92	6.65	5.12
Sum	71.68	58.29	25.72
No	21	22	21

High: Protein at peak wall stress site, Art: Protein at arteriotomy site; Art/H Ratio: Ratio of Protein at arteriotomy site to Protein at peak wall stress site

Table 23. Correlations - tissue and plasma MMP2 active

Correlations – tissue and plasma MMP2 active

			MMP2 High Active	MMP2 Art Active	MMP2 Plasma Act
Kendall's tau_b	MMP2 High Active	Correlation Coefficient	1.000	.117	-.345*
		Sig. (2-tailed)	.	.446	.039
		N	22	22	19
	MMP2 Art Active	Correlation Coefficient	.117	1.000	.111
		Sig. (2-tailed)	.446	.	.506
		N	22	22	19
	MMP2 Plasma Act	Correlation Coefficient	-.345*	.111	1.000
		Sig. (2-tailed)	.039	.506	.
		N	19	19	19
Spearman's rho	MMP2 High Active	Correlation Coefficient	1.000	.171	-.498*
		Sig. (2-tailed)	.	.446	.030
		N	22	22	19
	MMP2 Art Active	Correlation Coefficient	.171	1.000	.137
		Sig. (2-tailed)	.446	.	.576
		N	22	22	19
	MMP2 Plasma Act	Correlation Coefficient	-.498*	.137	1.000
		Sig. (2-tailed)	.030	.576	.
		N	19	19	19

*. Correlation is significant at the 0.05 level (2-tailed).

Table 24. Correlations – tissue and plasma MMP2 total

Correlations – tissue and plasma MMP2 total

			MMP2 High Total	MMP2 Art Total	MMP2 Plasma Total
Kendall's tau_b	MMP2 High Total	Correlation Coefficient	1.000	.314*	-.074
		Sig. (2-tailed)	.	.046	.650
		N	22	21	20
	MMP2 Art Total	Correlation Coefficient	.314*	1.000	-.193
		Sig. (2-tailed)	.046	.	.248
		N	21	21	19
	MMP2 Plasma Total	Correlation Coefficient	-.074	-.193	1.000
		Sig. (2-tailed)	.650	.248	.
		N	20	19	20
Spearman's rho	MMP2 High Total	Correlation Coefficient	1.000	.440*	-.050
		Sig. (2-tailed)	.	.046	.835
		N	22	21	20
	MMP2 Art Total	Correlation Coefficient	.440*	1.000	-.172
		Sig. (2-tailed)	.046	.	.482
		N	21	21	19
	MMP2 Plasma Total	Correlation Coefficient	-.050	-.172	1.000
		Sig. (2-tailed)	.835	.482	.
		N	20	19	20

*. Correlation is significant at the 0.05 level (2-tailed).

Table 25. Correlations – tissue and plasma MMP8 active

Correlations – tissue and plasma MMP8 active

			MMP8 High Active	MMP8 Art Active	MMP8 Plasma Active
Kendall's tau_b	MMP8 High Active	Correlation Coefficient	1.000	.385*	-.418
		Sig. (2-tailed)	.	.012	.073
		N	22	22	11
	MMP8 Art Active	Correlation Coefficient	.385*	1.000	-.273
		Sig. (2-tailed)	.012	.	.243
		N	22	22	11
	MMP8 Plasma Active	Correlation Coefficient	-.418	-.273	1.000
		Sig. (2-tailed)	.073	.243	.
		N	11	11	11
Spearman's rho	MMP8 High Active	Correlation Coefficient	1.000	.523*	-.555
		Sig. (2-tailed)	.	.012	.077
		N	22	22	11
	MMP8 Art Active	Correlation Coefficient	.523*	1.000	-.382
		Sig. (2-tailed)	.012	.	.247
		N	22	22	11
	MMP8 Plasma Active	Correlation Coefficient	-.555	-.382	1.000
		Sig. (2-tailed)	.077	.247	.
		N	11	11	11

*. Correlation is significant at the 0.05 level (2-tailed).

Table 26. Correlations – tissue and plasma MMP8 total

Correlations – tissue and plasma MMP8 total

			MMP8 High Total	MMP8 Art Total	MMP8 Plasma Total
Kendall's tau_b	MMP8 High Total	Correlation Coefficient	1.000	.381*	-.023
		Sig. (2-tailed)	.	.016	.889
		N	21	21	19
	MMP8 Art Total	Correlation Coefficient	.381*	1.000	.340*
		Sig. (2-tailed)	.016	.	.042
		N	21	21	19
	MMP8 Plasma Total	Correlation Coefficient	-.023	.340*	1.000
		Sig. (2-tailed)	.889	.042	.
		N	19	19	20
Spearman's rho	MMP8 High Total	Correlation Coefficient	1.000	.508*	-.012
		Sig. (2-tailed)	.	.019	.960
		N	21	21	19
	MMP8 Art Total	Correlation Coefficient	.508*	1.000	.475*
		Sig. (2-tailed)	.019	.	.040
		N	21	21	19
	MMP8 Plasma Total	Correlation Coefficient	-.012	.475*	1.000
		Sig. (2-tailed)	.960	.040	.
		N	19	19	20

*. Correlation is significant at the 0.05 level (2-tailed).

Table 27. Correlations – tissue and plasma MMP9 active

Correlations – tissue and plasma MMP9 active

			MMP9 High Active	MMP9 Art Active	MMP9 Plasma Active
Kendall's tau_b	MMP9 High Active	Correlation Coefficient	1.000	.083	-.248
		Sig. (2-tailed)	.	.592	.127
		N	22	22	20
	MMP9 Art Active	Correlation Coefficient	.083	1.000	-.090
		Sig. (2-tailed)	.592	.	.581
		N	22	22	20
	MMP9 Plasma Active	Correlation Coefficient	-.248	-.090	1.000
		Sig. (2-tailed)	.127	.581	.
		N	20	20	20
Spearman's rho	MMP9 High Active	Correlation Coefficient	1.000	.154	-.320
		Sig. (2-tailed)	.	.493	.168
		N	22	22	20
	MMP9 Art Active	Correlation Coefficient	.154	1.000	-.141
		Sig. (2-tailed)	.493	.	.554
		N	22	22	20
	MMP9 Plasma Active	Correlation Coefficient	-.320	-.141	1.000
		Sig. (2-tailed)	.168	.554	.
		N	20	20	20

Table 28. Correlations – tissue and plasma MMP9 total

Correlations – tissue and plasma MMP9 total

			MMP9 High Total	MMP9 Art Total	MMP9 Plasma Total
Kendall's tau_b	MMP9 High Total	Correlation Coefficient	1.000	.457**	-.053
		Sig. (2-tailed)	.	.004	.753
		N	21	21	19
	MMP9 Art Total	Correlation Coefficient	.457**	1.000	-.100
		Sig. (2-tailed)	.004	.	.552
		N	21	21	19
	MMP9 Plasma Total	Correlation Coefficient	-.053	-.100	1.000
		Sig. (2-tailed)	.753	.552	.
		N	19	19	20
Spearman's rho	MMP9 High Total	Correlation Coefficient	1.000	.629**	-.090
		Sig. (2-tailed)	.	.002	.713
		N	21	21	19
	MMP9 Art Total	Correlation Coefficient	.629**	1.000	-.033
		Sig. (2-tailed)	.002	.	.892
		N	21	21	19
	MMP9 Plasma Total	Correlation Coefficient	-.090	-.033	1.000
		Sig. (2-tailed)	.713	.892	.
		N	19	19	20

**. Correlation is significant at the 0.01 level (2-tailed).

Table 29. Correlations – tissue and plasma TIMP1

Correlations – tissue and plasma TIMP1

			TIMP1 High	TIMP1 Art	TIMP1 Plasma
Kendall's tau_b	TIMP1 High	Correlation Coefficient	1.000	.203	-.121
		Sig. (2-tailed)	.	.185	.455
		N	22	22	20
	TIMP1 Art	Correlation Coefficient	.203	1.000	.111
		Sig. (2-tailed)	.185	.	.495
		N	22	22	20
	TIMP1 Plasma	Correlation Coefficient	-.121	.111	1.000
		Sig. (2-tailed)	.455	.495	.
		N	20	20	20
Spearman's rho	TIMP1 High	Correlation Coefficient	1.000	.342	-.181
		Sig. (2-tailed)	.	.120	.446
		N	22	22	20
	TIMP1 Art	Correlation Coefficient	.342	1.000	.169
		Sig. (2-tailed)	.120	.	.476
		N	22	22	20
	TIMP1 Plasma	Correlation Coefficient	-.181	.169	1.000
		Sig. (2-tailed)	.446	.476	.
		N	20	20	20

Table 30. Correlations – tissue and plasma TIMP2

Correlations

			TIMP2 High	TIMP2 Art	TIMP2 Plasma
Kendall's tau_b	TIMP2 High	Correlation Coefficient	1.000	.104	-.016
		Sig. (2-tailed)	.	.498	.922
		N	22	22	20
	TIMP2 Art	Correlation Coefficient	.104	1.000	.058
		Sig. (2-tailed)	.498	.	.721
		N	22	22	20
	TIMP2 Plasma	Correlation Coefficient	-.016	.058	1.000
		Sig. (2-tailed)	.922	.721	.
		N	20	20	20
Spearman's rho	TIMP2 High	Correlation Coefficient	1.000	.146	.011
		Sig. (2-tailed)	.	.518	.965
		N	22	22	20
	TIMP2 Art	Correlation Coefficient	.146	1.000	.099
		Sig. (2-tailed)	.518	.	.677
		N	22	22	20
	TIMP2 Plasma	Correlation Coefficient	.011	.099	1.000
		Sig. (2-tailed)	.965	.677	.
		N	20	20	20

8. Appendix 5: Characteristics of MMP and TIMP ELISA kits

Characteristics for MMP and TIMP detection kits as well as the protein concentration determinisation kit as provided by kit manufacturers are provided below.

8.1. MMP2 test kit details

Specificity

The assay recognises the active form of MMP2 and the total form via activation of pro-MMP2. It has no substantial cross-reactivity with other MMPs or TIMPs.

Table 31. Cross-reactivity of MMP2 kit

Compound	% Cross-reactivity
Pro-MMP2	100
Activated MMP2	77*
ProMMP2/TIMP2 complex	43
Active MMP2/TIMP2 complex	8.6
Active MMP2/TIMP1 complex	21
MMP1	0.068
MMP9	0.64
MMP3	<0.05
MMP8	<0.05
TIMP1	<0.05
TIMP2	<0.05

MMP2 activity assay system, Amersham Biosciences, Buckinghamshire 2004. *pre-activated MMP2 which was pre-activated, captured & washed. Losses may have occurred during activation or activation may not have followed through to completion.

Reproducibility – within-assay precision

The within-assay precision for duplicate determination was calculated by measuring controls within the same assay.

Table 32. MMP2 kit within-assay precision

Control	Mean +/- SD	% CV	n
A	4.75 +/- 0.33	7.0	10
B	9.54 +/- 0.52	5.4	10
C	15.63 +/- 0.68	4.4	10

MMP2 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Reproducibility – between assay precision

This is measured by the same sample being analysed in subsequent plates.

Table 33. MMP2 kit between assay precision

Control	Mean +/- SD	% CV	n
A	2.6 +/- 0.44	16.9	12
B	5.48 +/- 0.98	17.9	12
C	9.49 +/- 1.76	18.5	12

MMP2 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Sensitivity

The sensitivity was defined as 2 standard deviations above the mean optical density of 10 zero standard replicates. The mean zero and standard values were then used to

calculate the sensitivity. This was determined as 0.5ng/ml for a 3 hour incubation period.

Parallelism

The parallelism of the kit explores the effect of dilution on the expected versus observed concentrations of MMP2.

Table 34. MMP2 kit parallelism

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	% Observed/ Expected
Cell culture media	1:4	8.07	-	-
	1:8	4.30	4.04	106.4
	1:16	2.30	2.02	113.9
	1:32	0.95	1.01	94.1
Heparin plasma	1:100	1.53	-	-
	1:200	0.85	0.77	110.4
	1:400	0.44	0.38	115.8
Serum	1:100	1.69	-	-
	1:200	0.95	0.85	112.4
	1:400	0.49	0.42	116.1

MMP2 activity assay system, Amersham Biosciences.

Recovery

The recovery of the kit measured the amount of MMP2 detected after various preparations were spiked with additional known levels of MMP2 standard.

Table 35. MMP2 kit recovery

Sample type	Added concentration (ng/ml)	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Cell culture medium	0	1.58	-	-
	0.38	1.68	1.96	85.7
	0.75	1.74	2.33	74.7
	1.5	1.96	3.08	63.6
Heparin plasma (1:200)	0	0.95	-	-
	0.38	1.02	1.33	76.7
	0.75	1.10	1.71	64.7
	1.5	1.33	2.45	54.3
	3	1.74	3.95	44.1
Serum (1:200)	0	0.78	-	-
	0.38	1.01	1.16	87.1
	0.75	1.32	1.53	86.3
	1.5	1.74	2.28	76.3
	3	2.65	3.78	70.1

MMP2 activity assay system, Amersham Biosciences.

Expected values

Normal human MMP2 levels in serum and plasma were measured using the assay.

Table 36. MMP2 kit normal human levels

Sample	Mean (ng/ml)	Range (ng/ml)	N
Heparin plasma	139	114-170	5
Serum	211	156-254	5

MMP2 activity assay system, Amersham Biosciences.

8.2. MMP8 test kit details

MMP8 activity assay kit contents

This kit contained;

- 12 x 8 well microplate, pre-coated with $F(ab')_2$ goat anti-mouse.
- Assay buffer containing 10ml Tris-HCl buffer concentrate. Made up with 100ml distilled water to give 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% by volume (v/v) BRIJ 35.
- Standard contains 48ng of lyophilised (dried by freezing in a high vacuum) human pro MMP8. On reconstitution with 1ml of buffer, this gives a concentration of 48ng/ml pro MMP8 in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.

- Antibody. This contains lyophilised mouse anti-MMP8, which on reconstitution with 11ml of assay buffer gives mouse anti-MMP8 in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.

- p-Aminophenylmercuric acetate (APMA). Bottle contains 352mg of APMA in powder form, made up with 1ml dimethylsulphoxide (DMSO) to 1M concentrated APMA solution. This is further diluted using 15μl of concentrated APMA (1M) added to 10ml of assay buffer to form a ready to use APMA solution (1.5mM) (note different concentration of APMA to MMP2 assay).

- Detection enzyme. Tube containing 100μl of concentrated solution of modified urokinase in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.

- Substrate. Bottle containing lyophilised S-2444 peptide substrate , reconstituted with 5.1ml of assay buffer to form a ready to use solution of S-2444 substrate in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.

- Wash buffer. Bottle contains 12.5ml of phosphate buffer concentrate which when made up to 500ml with distilled water gives a 0.01M sodium phosphate buffer pH7.0 containing 0.05% Tween 20. Tween 20 (also known as polysorbate 20) is a stable relatively non-toxic polysorbate surfactant used here as a detergent.

The kit was stored between –15 and –30°C until needed. Kit instructions were fully followed as detailed including critical parameters. Kits were equilibrated at room temperature, particularly the microplates, as condensation affects the performance of the antibody with which the plates are coated.

MMP8 detection procedure protocol

The assay protocol for the assay range 0.75 – 24ng/ml of MMP8 was followed (higher endogenous levels). The extended detection range (for lower endogenous levels – 0.09-6ng/ml) was occasionally used and involved more standards, and standards of a lower concentration than in the normal protocol.

Day 1 reagent preparation

Assay buffer concentrate, anti-MMP8 coated microplate, and wash buffer concentrate were allowed to equilibrate to room temperature before use. Other components were removed from storage just before use. Assay buffer and wash buffer were prepared according to kit protocol, using distilled water. Standard was prepared using assay buffer. Both buffers were stored in a closed vessel at room temperature, and standard was stored on ice until needed.

Day 1 procedure

6 standards of known concentration of human pro-MMP8 (0.75, 1.5, 3, 6, 12 and 24ng/ml)(note 1 more standard than MMP2 assay kit) were freshly prepared by successive dilution with assay buffer and vortex mixing. 1 sample of assay buffer only was also prepared ('blank' - 0ng/ml MMP8 content). 100µl of anti-MMP8 was pipetted into all wells, covered and incubated at 37°C for 2 hours. The microplate was aspirated and washed 4 times with wash buffer. 100µl of blank, all standard

concentrations and unknown samples were put in wells on the microplate in positions as suggested (figure below), in duplicate. The microplate was covered and incubated at 2-8°C overnight.

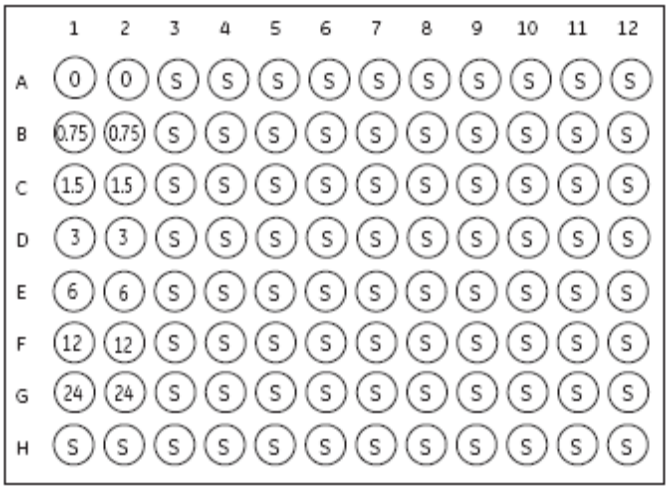


Figure 45. Recommended positioning of standard and sample wells (Amersham Biosciences)

Day 2 reagent preparation

APMA, detection enzyme, substrate, detection reagent was prepared according to kit protocol. APMA was prepared as instructed using fresh dimethylsulphoxide (DMSO)(in a fume cupboard, with skin and eye protection), and then diluted again to form the ready-to-use dilution. Detection enzyme was stored on ice after thawing, but prior to use. Substrate was also stored on ice prior to use. Detection reagent was 100µl of detection enzyme added to the reconstituted substrate. This was prepared immediately prior to addition to wells and vortex mixed.

Day 2 procedure

All wells were washed with wash buffer and aspirated 4 times using a microplate washer ensuring each well was fully filled and emptied each time. 50µl of ready-to-use APMA was pipetted into all standard wells, and wells needing the quantification of total MMP2. 50µl of assay buffer was added to wells for quantification of active MMP2 only. The plate was covered and incubated at 37°C for 1 hour, then all wells then had 50µl of detection reagent added. The microplate was shaken in a microplate shaker for 20 seconds and the initial (T_0) spectrophotometer reading at 405nm was taken. The microplate was then covered and incubated at 37°C for 2 hours, shaken and a subsequent spectrophotometer reading at 405nm taken (T_2).

Other kit details

Specificity

The assay recognises the active form of MMP8 and the total form via activation of pro-MMP8. It has no substantial cross-reactivity with other MMPs or TIMPs.

Table 37. Cross-reactivity of MMP8 kit

Compound	% Cross-reactivity
Pro-MMP8	100
Activated MMP8	100*
MMP1	7.9
MMP2	1.2
MMP3	1.5
MMP9	1.3
MMP13	2.2
TIMP1	0.5

MMP8 activity assay system, Amersham Biosciences, Buckinghamshire 2004. *pre-activated MMP8 which was pre-activated, captured & washed without further APMA activation.

Reproducibility – within-assay precision

The within-assay precision for duplicate determination was calculated by measuring controls within the same assay.

Table 38. MMP8 kit within-assay precision

Control	Mean +/- SD	% CV	n
L	4.71 +/- 0.62	13.2	9
M	9.62 +/- 0.79	8.2	9
H	18.69 +/- 1.37	7.3	9

MMP8 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Reproducibility – between assay precision

This is measured by the same sample being analysed in subsequent plates.

Table 39. MMP8 kit between assay precision

Control	Mean +/- SD	% CV	n
L	3.73 +/- 0.41	19.0	18
M	9.26 +/- 0.78	8.4	18
H	18.7 +/- 1.41	7.5	18

MMP8 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Sensitivity

The sensitivity was defined as 2 standard deviations above the mean optical density of 10 zero standard replicates. The mean zero and standard values were then used to calculate the sensitivity. This was determined as 1.2ng/ml for a 2 hour incubation period.

Recovery

The recovery of the kit measured the amount of MMP8 detected after various preparations were spiked with additional known levels of MMP8 standard.

Table 40. MMP8 kit recovery

Sample type	Added concentration (ng/ml)	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Cell culture medium	0	0.10	-	-
	0.38	0.55	0.48	118
	0.75	0.97	0.85	117
	1.5	1.95	1.60	123
	3.0	3.77	3.10	123
Heparin plasma (1:20)	0	0.34	-	-
	0.38	0.56	0.72	57
	0.75	0.79	1.09	59
	1.5	1.30	1.84	64
	3.0	2.43	3.34	70
Serum (1:20)	0	3.04	-	-
	0.38	3.15	3.42	27
	0.75	3.40	3.79	48
	1.5	4.03	4.54	66
	3.0	5.07	6.04	68

MMP8 activity assay system, Amersham Biosciences.

8.3. MMP9 test kit details

Kit contents

This kit contained;

- 12 x 8 well microplate, pre-coated with anti-MMP9.
- Assay buffer containing 10ml Tris-HCl buffer concentrate. Made up with 100ml distilled water to give 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% by volume (v/v) BRIJ 35.
- Standard contains 32ng of lyophilised (dried by freezing in a high vacuum) human pro MMP9. On reconstitution with 1ml of buffer, this gives a concentration of 32ng/ml pro MMP9 in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35.
- p-Aminophenylmercuric acetate (APMA). Bottle contains 352mg of APMA in powder form, made up with 1ml dimethylsulphoxide (DMSO) to 1M concentrated APMA solution. This is further diluted using 10μl of concentrated APMA (1M) added to 10ml of assay buffer to form a ready to use APMA solution (1mM) (note different concentration of APMA to MMP2 & MMP8 assays).
- Detection enzyme. Tube containing 100μl of concentrated solution of modified urokinase in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, 0.01% (v/v) BRIJ 35. .

- Substrate. Bottle containing lyophilised S-2444 peptide substrate , reconstituted with 5.1ml of assay buffer to form a ready to use solution of S-2444 substrate in 50mM Tris-HCl buffer pH7.6 containing 1.5mM sodium chloride, 0.5mM calcium chloride, 1μM zinc chloride, and 0.01% (v/v) BRIJ 35.
- Wash buffer. Bottle contains 12.5ml of phosphate buffer concentrate which when made up to 500ml with distilled water gives a 0.01M sodium phosphate buffer pH7.0 containing 0.05% Tween 20. Tween 20 (also known as polysorbate 20) is a stable relatively non-toxic polysorbate surfactant used here as a detergent.

The kit was stored between –15 and –30°C until needed. Kit instructions were fully followed as detailed including critical parameters. Kits were equilibrated at room temperature, particularly the microplates, as condensation affects the performance of the antibody with which the plates are coated.

MMP9 detection procedure protocol

The assay protocol for the assay range 0.5 – 16ng/ml of MMP9 was followed (normal endogenous levels). The extended detection range (for lower endogenous levels – 0.125-4ng/ml) was occasionally used and involved more standards, and standards of a lower concentration than in the normal protocol.

Day 1 reagent preparation

Assay buffer concentrate, anti-MMP2 coated microplate, and wash buffer concentrate were allowed to equilibrate to room temperature before use. Other components were removed from storage just before use. Assay buffer and wash buffer were prepared

according to kit protocol, using distilled water. Standard was prepared using assay buffer. Both buffers were stored in a closed vessel at room temperature, and standard was stored on ice until needed.

Day 1 procedure

6 standards of known concentration of human pro-MMP9 (0.5, 1, 2, 4, 8 and 16ng/ml) were freshly prepared by successive dilution with assay buffer and vortex mixing. 1 sample of assay buffer only was also prepared ('blank' - 0ng/ml MMP9 content). 100µl of blank, all standard concentrations and unknown samples were put in wells on the microplate in positions as suggested (figure above), in duplicate. The microplate was covered and incubated at 2-8°C overnight.

Day 2 reagent preparation

APMA, detection enzyme, substrate, detection reagent was prepared according to kit protocol. APMA was prepared as instructed using fresh dimethylsulphoxide (DMSO)(in a fume cupboard, with skin and eye protection), and then diluted again to form the ready-to-use dilution. Detection enzyme was stored on ice after thawing, but prior to use. Substrate was also stored on ice prior to use. Detection reagent was 100µl of detection enzyme added to the reconstituted substrate. This was prepared immediately prior to addition to wells and vortex mixed.

Day 2 procedure

All wells were washed with wash buffer and aspirated 4 times using a microplate washer ensuring each well was fully filled and emptied each time. 50µl of ready-to-use APMA was pipetted into all standard wells, and wells needing the quantification of total MMP9. 50µl of assay buffer was added to wells for quantification of active

MMP9 only. The plate was then shaken and incubated at 37°C for 1.5hours. All wells then had 50µl of detection reagent added. The microplate was shaken in a microplate shaker for 20 seconds and the initial (T_0) spectrophotometer reading at 405nm was taken. The microplate was then covered and incubated at 37°C for 1 hour, shaken and a subsequent spectrophotometer reading at 405nm taken (T_1).

Other kit details

Specificity

The assay recognises the active form of MMP9 and the total form via activation of pro-MMP9. It has no substantial cross-reactivity with other MMPs or TIMPs. Both pro and active MMP9/TIMP1 complexes as well as active MMP9/TIMP2 complexes have shown a degree of cross-reactivity in the assay. The non-specific protease inhibitor α_2-macroglobulin does not interfere with the assay.

Table 41. Cross-reactivity of MMP9 kit

Compound	% Cross-reactivity
Pro-MMP9	100
Activated MMP9	65.88*
ProMMP9/TIMP1 complex	38.53
Active MMP9/TIMP1 complex	21.64
Active MMP9/TIMP2 complex	7.04
MMP1	<0.1
MMP2	<0.1
MMP3	<0.1
MMP8	<0.133
TIMP1	<0.05
TIMP2	<0.05

MMP9 activity assay system, Amersham Biosciences, Buckinghamshire 2004. *pre-activated MMP9 which was pre-activated, captured & washed. Losses may have occurred during activation or activation may not have followed through to completion.

Reproducibility – within-assay precision

The within-assay precision for duplicate determination was calculated by measuring controls within the same assay.

Table 42. MMP9 kit within-assay precision

Control	Mean +/- SD	% CV	n
A	6.03 +/- 0.26	4.3	10
B	13.00 +/- 0.4	3.1	10
C	21.15 +/- 5.11	3.4	10

MMP9 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Reproducibility – between assay precision

This is measured by the same sample being analysed in subsequent plates.

Table 43. MMP9 kit between assay precision

Control	Mean +/- SD	% CV	n
A	3.59 +/- 0.73	20.2	10
B	12.51 +/- 2.72	21.7	10
C	24.75 +/- 5.11	20.7	10

MMP9 activity assay system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Sensitivity

The sensitivity was defined as 2 standard deviations above the mean optical density of 10 zero standard replicates. The mean zero and standard values were then used to

calculate the sensitivity. This was determined as 0.5ng/ml for the 1 hour incubation period.

Parallelism

The parallelism of the kit explores the effect of dilution on the expected versus observed concentrations of MMP9.

Table 44. MMP9 kit parallelism

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	% Observed/ Expected
Cell culture media	1:32 1:64	14.1 9.4	- 7.1	- 132
Heparin plasma	1:16 1:32	1.35 0.95	- 0.68	- 140
Serum	1:16 1:32	2.72 1.66	- 1.36	- 122

MMP9 activity assay system, Amersham Biosciences.

Recovery

The recovery of the kit measured the amount of MMP9 detected after various preparations were spiked with additional known levels of MMP9 standard.

Table 45. MMP9 kit recovery

Sample type	Added concentration (ng/ml)	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Cell culture medium	0	0	-	-
	0.5	0.4	0.3	133
	1	0.6	0.6	100
	2	1.4	1.4	108
	4	2.9	2.9	112
	8	6.3	6.3	121
Heparin plasma (1:32)	0	1.0	-	-
	0.5	1.4	1.5	93.3
	1	1.8	2.0	90.0
	2	2.6	3.0	86.7
	4	4.3	5.0	86.0
	8	7.2	9.0	80.0
Serum (1:32)	0	1.0	-	-
	0.5	1.3	1.5	86.7
	1	1.6	2.0	80.0

	2 4 8	2.4 4.2 7.9	3.0 5.0 9.0	80.0 84.0 87.8

MMP9 activity assay system, Amersham Biosciences.

Expected values

Normal human MMP9 levels in serum and plasma were measured using the assay.

Table 46. MMP9 kit normal human levels

Sample	Mean (ng/ml)	Range (ng/ml)	N
Heparin plasma	18.7	4.4-27.2	9
Serum	43.8	-	1

MMP9 activity assay system, Amersham Biosciences.

8.4. TIMP1 test kit details

Specificity

The assay recognises total TIMP1, ie free TIMP1 and that complexed to MMPs. The assay will cross react with TIMP1 in complexes with MMP1, MMP2, MMP3, and MMP9. It does not cross-react with TIMP2.

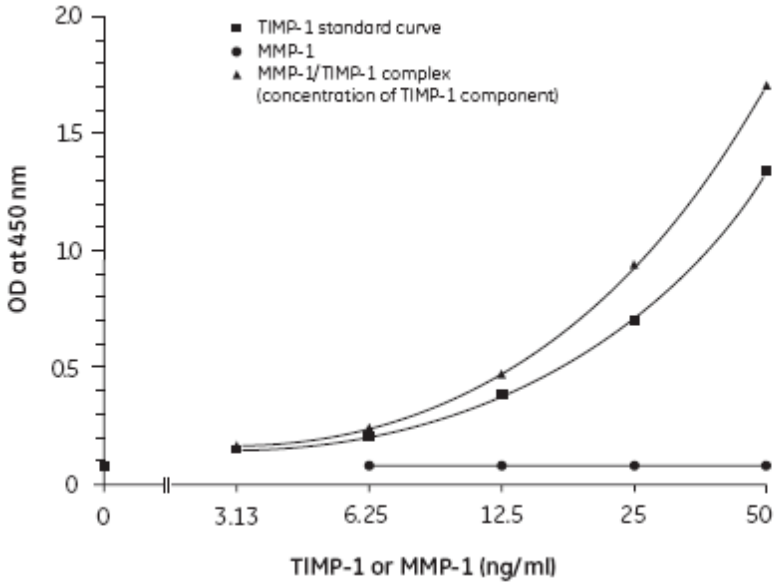


Figure 46. Cross-reactivity of TIMP1 assay with MMP1 and MMP1/TIMP1 complex. Amersham Biosciences.

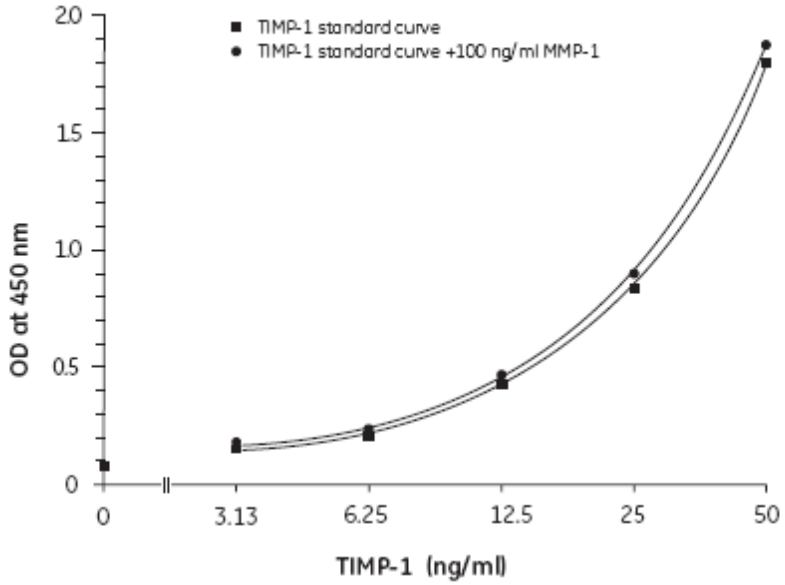


Figure 47. Non-interference of MMP1 in TIMP1 assay. Amersham Biosciences.

Reproducibility – within-assay precision

The within-assay precision for duplicate determination was calculated by measuring controls within the same assay.

Table 47. TIMP1 kit within-assay precision

Control	Mean +/- SD	% CV	n
A	10.3 +/- 1.18	11.4	11
B	23.1 +/- 2.14	9.3	11
C	39.4 +/- 3.50	8.9	11

TIMP1 ELISA system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Reproducibility – between assay precision

This is measured by the same sample being analysed in subsequent plates.

Table 48. TIMP1 kit between assay precision

Control	Mean +/- SD	% CV	n
A	12.5 +/- 1.9	15.2	24
B	24.9 +/- 3.1	12.4	24
C	47.3 +/- 6.2	13.1	24

TIMP1 ELISA system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Sensitivity

The sensitivity was defined as 2 standard deviations above the mean optical density of 10 zero standard replicates was determined for each of 4 batches of reagents. The corresponding concentration was calculated from a standard curve set up in quadruplicate for each batch. The grand mean zero and standard values were then used to calculate the sensitivity. This was determined as 1.25ng/ml.

Parallelism

The parallelism of the kit explores the effect of dilution on the expected versus observed concentrations of TIMP1.

Table 49. TIMP1 kit parallelism

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	% Observed/ Expected
Cell culture media	Neat	39.0	-	-
	1:5	9.3	7.8	119
	1:10	4.7	3.9	120
	1:20	2.8	1.95	143
Heparin plasma	1:10	10.7	-	-
	1:20	5.5	5.4	102
	1:40	3.4	2.7	126
	1:80	2.2	1.4	157
Serum	1:10	11.0	-	-
	1:20	7.2	5.5	131
	1:40	4.8	2.8	171
	1:80	3.1	1.4	221

TIMP1 ELISA system, Amersham Biosciences.

Recovery

The recovery of the kit measured the amount of TIMP1 detected after various preparations were spiked with additional known levels of TIMP1 standard.

Table 50. TIMP1 kit recovery

Sample type	Added concentration (ng/ml)	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Cell culture medium (1:10)	0	0.37	-	-
	46	40.78	46.37	88
	22	18.05	22.37	81
	11	8.40	11.37	74
Heparin plasma (1:40)	0	4.2	-	-
	46	42.4	50.0	85
	22	22.6	26.2	86
	11	13.7	15.2	90
Serum (1:40)	0	5.0	-	-
	46	41.9	51.0	82
	22	22.2	27.0	82
	11	13.6	16.0	85

TIMP1 ELISA system, Amersham Biosciences.

8.5. TIMP2 test kit details

Specificity

The assay recognises free TIMP2, and TIMP2 complexed with active MMPs, but not TIMP2 complexed with pro-MMP2. The immunoreactivity is shown in the table below.

Table 51. Immunoreactivity of TIMP2 ELISA system

Form	Immunoreactivity (%)
Free TIMP2	100
TIMP2 / proMMP2	~5
TIMP2 / active MMP1	330
TIMP2 / active MMP2	450
TIMP2 / active MMP3	105
TIMP2 / active MMP7	100
TIMP2 / active MMP8	~33
TIMP2 / active MMP9	300

TIMP2 ELISA system. Amersham Biosciences.

The cross-reactivity of the ELISA system with other TIMPs is as detailed in the table below.

Table 52. Specificity towards TIMPs (TIMP2 ELISA system)

Standard (ng/ml)	TIMP2	TIMP1	TIMP3
0	0.036	0.036	0.036
8	0.087	0.049	0.049
16	0.142	0.043	0.045
32	0.352	0.040	0.029
64	1.145	0.041	0.028
128	3.576	0.034	0.024

TIMP2 ELISA system. Amersham Biosciences. (values as optical density)

The assay also detects TIMP2 from other species including mouse, rat, guinea pig, rabbit, and calf. The immunoreactivity between human and rat purified TIMP2 is equivalent.

Reproducibility – within-assay precision

The within-assay precision for duplicate determination was calculated by measuring controls within the same assay.

Table 53. TIMP2 kit within-assay precision

Control	Mean +/- SD	% CV	n
A	16.3 +/- 0.88	5.4	12
B	31.6 +/- 1.10	3.5	12
C	50.9 +/- 1.36	2.8	12

TIMP2 ELISA system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Reproducibility – between assay precision

This is measured by the same sample being analysed in subsequent plates.

Table 54. TIMP2 kit between assay precision

Control	Mean +/- SD	% CV	n
A	16.0 +/- 0.94	5.9	12
B	29.5 +/- 1.76	6.0	12
C	50.1 +/- 1.25	2.5	12

TIMP2 ELISA system, Amersham Biosciences. (mean values as ng/ml, %CV; percentage coefficient of variation: (sd/mean)*100) (The coefficient of variation is a way of reporting measurement error)

Sensitivity

The sensitivity was defined as 2 standard deviations above the mean optical density of 20 zero standard replicates was determined. The corresponding concentration was

calculated from a standard curve. The mean zero and standard values were then used to calculate the sensitivity. This was determined as 3.0ng/ml.

Parallelism

The parallelism of the kit explores the effect of dilution on the expected versus observed concentrations of TIMP2.

Table 55. TIMP2 kit parallelism

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	% Observed/ Expected
Cell culture media	1:2	109	-	-
	1:4	53.8	54.5	98.7
	1:8	27.3	27.3	100
	1:16	15.8	13.6	116
EDTA plasma	1:2	104	-	-
	1:4	57.8	52.0	111
	1:8	28.1	26.0	108
	1:16	14.3	13.0	110
Serum	1:4	91.9	-	-
	1:8	48.3	46.0	105
	1:16	25.7	23.0	112
	1:32	13.5	11.5	118

TIMP2 ELISA system, Amersham Biosciences.

Recovery

The recovery of the kit measured the amount of TIMP2 detected after various preparations were spiked with additional known levels of TIMP2 standard.

Table 56. TIMP2 kit recovery

Sample type	Added concentration (ng/ml)	Measured (ng/ml)	Expected (ng/ml)	Recovery (%)
Cell culture medium	0	49.0	-	-
	8	59.6	57.0	105
	16	66.2	65.0	102
	32	85.0	81.0	105
	64	118	113	104
Heparin plasma	0	50.4	-	-
	8	60.9	58.4	104
	16	65.8	66.4	99.1
	32	90.3	82.4	110
	64	117	114	102
Heparin plasma	0	17.3	-	-
	8	22.6	25.3	89.3
	16	31.9	33.3	95.8
	32	47.4	49.3	96.1
	64	88.8	81.3	109

Serum	0 8 16 32 64	49.5 58.5 67.1 82.7 118	- 57.5 65.5 81.5 114	- 102 102 101 104
Serum	0 8 16 32 64	20.1 26.3 35.1 52.1 88.2	- 28.1 36.1 52.1 84.1	- 93.6 97.2 100 105

TIMP2 ELISA system, Amersham Biosciences.

Expected values

Normal serum and plasma samples were evaluated in the assay.

Table 57. Normal levels of TIMP2

Sample	Mean (ng/ml)	Range (ng/ml)	n
Plasma (EDTA)	50	21-108	40
Serum	56	29-108	127

TIMP2 ELISA system, Amersham Biosciences.

8.6. Protein concentration test kit details

Typical standard curve

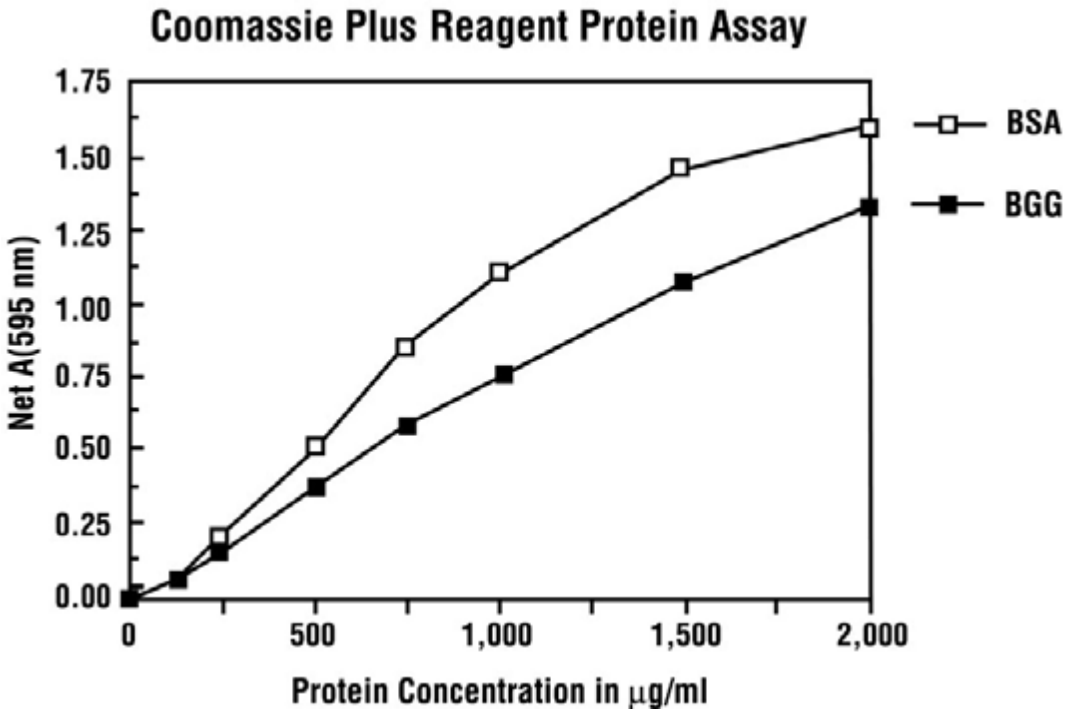


Figure 48. Coomassie plus reagent typical standard curve (Piercenet.com)

Compatible substances

Table 58. Compatible substances with Coomassie plus reagent

Substance	Compatible Concentration
Salts/Buffers	
ACES, pH 7.8	100 mM
Ammonium sulfate	1.0 M
Asparagine	10 mM
Bicine, pH 8.4	100 mM
Bis-Tris, pH 6.5	100 mM
Borate (50 mM), pH 8.5 (# 28384)	undiluted
B-PER® Reagent (#78248)	1/2 dilution*
Calcium chloride in TBS, pH 7.2	10 mM
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted
Cesium bicarbonate	100 mM
CHES, pH 9.0	100 mM
Na-Citrate (0.6 M), Na-Carbonate (0.1 M), pH 9.0 (#28388)	undiluted
Cobalt chloride in TBS, pH 7.2	10 mM
EPPS, pH 8.0	100 mM
Ferric chloride in TBS, pH 7.2	10 mM
Glycine	100 mM
Guanidine•HCl	3.5 M
HEPES, pH 7.5	100 mM
Imidazole, pH 7.0	200 mM
MES, pH 6.1	100 mM
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	undiluted
MOPS, pH 7.2	100 mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted
Nickel chloride in TBS, pH 7.2	10 mM
PBS; Phosphate (0.1 M), NaCl (0.15 M), pH 7.2 (#28372)	undiluted
PIPES, pH 6.8	100 mM
RIPA lysis buffer; 50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	1/40 dilution*
Sodium acetate, pH 4.8	180 mM
Sodium azide	0.5%
Sodium bicarbonate	100 mM
Sodium chloride	5.0 M
Sodium citrate, pH 4.8 or pH 6.4	200 mM
Sodium phosphate	100 mM
Tricine, pH 8.0	100 mM
Triethanolamine, pH 7.8	100 mM
Tris	2.0 M
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	undiluted
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	undiluted
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	1/4 dilution*
Zinc chloride in TBS, pH 7.2	10 mM

Substance	Compatible Concentration
Detergents	
Brij®-35	0.062%
Brij®-56 (Brij®-58)	0.031%(0.016%)
CHAPS, CHAPSO	5.0%
Deoxycholic acid	0.4%
Lubrol® PX	0.031%
Octyl β-glucoside	0.5%
Nonidet P-40 (NP-40)	0.5%
Octyl β-thioglucopyranoside	3.0%
SDS	0.016%
Span® 20	0.5%
Triton® X-100, X-114	0.062%
Triton® X-305, X-405	0.125%(0.025%)
Tween®-20	0.031%
Tween®-60	0.025%
Tween®-80	0.016%
Zwittergent® 3-14	0.025%
Chelating agents	
EDTA	100 mM
EGTA	2 mM
Sodium citrate	200 mM
Reducing & Thiol-Containing Agents	
N-acetylglucosamine in PBS, pH 7.2	100 mM
Ascorbic acid	50 mM
Cysteine	10 mM
Dithioerythritol (DTE)	1 mM
Dithiothreitol (DTT)	5 mM
Glucose	1.0 mM
Melibiose	100 mM
2-Mercaptoethanol	1.0 M
Potassium thiocyanate	3.0 M
Thimerosal	0.01%
Misc. Reagents & Solvents	
Acetone	10%
Acetonitrile	10%
Aprotinin	10 mg/L
DMF, DMSO	10%
Ethanol	10%
Glycerol (Fresh)	10%
Hydrochloric Acid	100 mM
Leupeptin	10 mg/L
Methanol	10%
Phenol Red	0.5 mg/ml
PMSF	1 mM
Sodium Hydroxide	100 mM
Sucrose	10%
TLCK	0.1 mg/L
TPCK	0.1 mg/L
Urea	3.0 M
o-Vanadate (sodium salt), in PBS, pH 7.2	1 mM

Pierce, Rockford, IL, USA (Piercenet.com)

Protein to protein variation

Table 59. Protein-to-protein variation

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)	
Protein Tested	**Ratio**
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.74
α-Chymotrypsinogen, bovine	0.52
Cytochrome C, horse heart	1.03
Gamma globulin, bovine	0.58
IgG, bovine	0.63
IgG, human	0.66
IgG, mouse	0.62
IgG, rabbit	0.43
IgG, sheep	0.57
Insulin, bovine pancreas	0.67
Myoglobin, horse heart	1.15
Ovalbumin	0.68
Transferrin, human	0.90
Average ratio	**0.73**
Standard Deviation	**0.21**
Coefficient of Variation	**28.8%**

Absorbance at 595nm relative to BSA (Bovine serum albumin) with standard test tube protocol with Coomassie plus (Pierce, Rockford, IL, USA)

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