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Studies into the Effects of Hypoxia on Matrix Metalloproteinases within the Abdominal Aortic Aneurysm

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Studies into the Effects of Hypoxia on Matrix Metalloproteinases within the Abdominal Aortic Aneurysm

Susan Pegrum

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department for Health

December 2016

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Abbreviations

2-OG	2-Oxoglutarate
α 2M	α 2- Macroglobulin
AA	Amino acid
AAA	Abdominal Aortic Aneurysm
AAAP-40	Aortic aneurysm-associated protein-40
ABC	Avidin-biotin complex
ABC-AP	Avidin-biotin complex-alkaline phosphatase
ACE	Angiotensin-converting enzyme
ADCY7	Adenylylcyclase type 7
ADM	Adrenomedullin
ADSCs	Adipose derived stem cells
ADU	Arbitrary density units
ALD-A	Aldolase-A
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ang-II	Angiotensin II
AngIIIR	Angiotensin II receptor
AoAF	Aortic adventitial fibroblasts
AOD	Aortic Occlusive Disease
AP-1	Activating protein-1
ARD1	Activator of RNA decay
ARE	AU-rich elements
ARL4C	ADP-ribosylation factor (ARF)-like 4c
ARNT	Aryl hydrocarbon nuclear translocator
ATH	Atherosclerotic

AT1	Angiotensin II type 1
AU-rich	Adenylate/uridylate-rich
bFGF	Basic fibroblast growth factor
bHLH	Basic-helix-loop-helix
BLNK	B - cell linker protein
BNip3	Bcl2/adenovirus E1B 19kD-interacting protein 3
BpA	Bisphenol A
BSA	Bovine serum albumin
CA	Cysteine array
CA9	Carbonic anhydrases-9
CATHD	Cathepsin D
CBP	CREB - binding protein
CETP	Cholesterol-ester transfer protein
cGMP	Cyclic guanosinemonophosphate
CIRP	Cold-inducible RNA-binding protein
CMV	Cytomegalovirus
CO	Carbon monoxide
CO ₂	Carbon Dioxide
COL	Collagen
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CREB	cAMP - response element binding protein
CT	Computerized tomography
CTA	Computerized tomographic angiogram
CTAD	C-terminal domain
Cys	Cysteine residue
Cu	Copper

DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
DPI	Diphenyliodonium Chloride
DSCG	Disodium cromoglycate
E	Eosin
EBP	Elastin binding protein
ECE	Endothelin converting enzyme
ECM	Extracellular matrix
ECs	Endothelial cells
EDPs	Elastin derived peptides
EDS	Ehlers-Danlos syndromes
EDTA	Ethylenediaminetetraacetic acid disodium salt
EGF	Epidermal growth factor
ELN	Elastin
EMILIN	ElastinMicrofibril Interface Located Protein
EMMPRIN	Extracellular matrix metalloproteinase inducer
ENA	Epithelial neutrophil activating
EPAS1	Endothelial PAS domain protein 1
ET	Endothelin
EVAR	Endovascular aneurysm repair
FBN	Fibrillin
FBLN	Fibulin
FCS	Foetal calf serum
FDP	Fibrin degradation products
FIH	Factor inhibiting HIF-1
FLS	Fibroblast-like synoviocytes
GAG	Glycosaminoglycan

GPI	Glycosylphosphatidylinositol
GRO	Growth related oncogene
GWAS	Genome-Wide Association Studies
GZMB	Granzyme B
H	Haematoxylin
H ₂ O	Water
HASMC	Human aortic smooth muscle cells
HPX	Hemopexin
HDAC	Histone deacetylase
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HLF	HIF-like factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRE	Hypoxia response element
HRF	HIF-related factor
HSP90	Heat shock protein 90
IAAA	Infrarenal abdominal aortic aneurysm
IFN- γ	Interferon-gamma
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
ILT	Intraluminal thrombus
IMS	Industrial methylated spirit
INOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	Jun N terminal kinase

LDH	Lactate Dehydrogenase
LDL-RP	Low density lipoprotein receptor related protein
LEP	Leptin
LLE	Lipophilicligand efficiency
MAGP	Microfibril-associated Proteins
MAPK	Mitogen activated protein kinases
MCP	Monocytechemoattractant protein
MD	Molecular dynamics
MDR	Multidrug resistance
MDUS	Meta-dynamics and umbrella sampling
MHC	Major histocompatibility antigen
MIC	Microneme protein
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MOP	Member of the PAS superfamily
MR	Magnetic Resonance
MRA	Magnetic Resonance angiogram
MRI	Magnetic resonance imaging
MT-MMPs	Membrane-type MMPs
NAD ⁺	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NMFS	Neonatal Marfan Syndrome
NO	Nitric oxide
NOS	Nitric oxide synthase
NTAD	N-terminal transactivation domain

OAc	Acetylation
OB	Oligosaccharide/oligonucleotide binding-fold
ODD	Oxygen-dependent degradation
ODNs	Oligodeoxynucleotides
OH	Hydroxyl radical
ONOO	Peroxynitrite
OPG	Osteoprotegerin
PAEC	Pulmonary artery endothelial cells
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitors
PAS	PER-ARNT-SIM
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidinedithiocarbamate
PGE2	Prostaglandin E2
PGI ₂	Prostacyclin
PGs	Prostaglandins
PHD	Proline-hydroxylases
PMN	Polymorphonuclearneutrophils
pO ₂	Partial pressure of oxygen
PPARs	Peroxisomeproliferator-activated receptors
Pro	Propeptide
PTFE	Polytetrafluoroethylene
pVHL	Von- Hippel-Lindau tumour suppressor gene
RACK	Receptor of activated protein C kinase
RANTES	Regulated on activation normal T-cell exp and secreted

RAS	Renin-Angiotensin System
RASI	Rheumatoid arthritis synovial inflammation
RGD	Arginylglycylaspartic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSM	Rainbow size marker
RT-PCR	Reverse transcriptase–polymerase chain reaction
SDS	Sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid.
SMA	Smooth muscle actin
SMC	Smooth muscle cell
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
SORT1	Sortilin-1
STAT	Signal transducers and activators of transcription
TAA	Thoracic aortic aneurysm
TE	Tropoelastin
TFPI	Tissue-factor pathway inhibitor
TGF- β	Transforming growth factor- β
TIMPs	Tissue Inhibitor of Metalloproteinases
TMJ	Temporomandibular joint
TNF	Tumor necrosis factor
t-PA	Tissue plasminogen activators
TRAIL	TNF-related apoptosis-inducing ligand
Ub	Ubiquitin
u-PAR	Urokinase-type plasminogen activator receptor
UTRs	Untranslated regions

VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WT	Wild type
WSS	Wall shear stress
XO	Xanthine oxidase
Zn	Zinc

Abstract

Abdominal aortic aneurysm (AAA) is a permanent and irreversible localised dilation of the aortic wall which if left untreated can rupture leading to severe haemorrhage and death affecting over 5% of males over 50 years of age.

The pathology of AAA is poorly understood, however research has shown that increased activity of matrix metalloproteinases (MMPs) leads to the destruction of major structural elements such as elastin and collagen within the aortic wall leading to weakening and resulting in aneurysm formation.

Arterial wall hypoxia, which has been investigated in other pathological conditions may also play role in the pathogenesis of AAA. Research has also shown that hypoxia exists in vivo in the aneurysms of patients. This study has investigated the possible role of aortic wall hypoxia as a contributing factor for the increased expression of MMPs and also the affects if any on the endogenous inhibitors of MMPs known as TIMPs (tissue inhibitors of metalloproteinases). The major hypoxia transcription factor HIF-1 α , along with Ets-1 and VEGF are also being investigated as mediating factors.

Results demonstrated significant increases in expression of MMP-2 levels when human aortic smooth muscle cells were exposed to severe hypoxia over 48 hours. Increased levels of Tissue Inhibitor of Metalloproteinases (TIMPs) 1 and 2 were also noted in the HASMC supernatant of hypoxic VHASMC samples. Ets-1 and VEGF demonstrated raised levels in hypoxic HASMC whole cell lysate samples.

Interestingly when HASMCS were treated with Bisphenol A (BpA) to inhibit the transcription factor HIF-1 α during exposure to severe hypoxia, results showed an effect on the expression of MMP-2 levels in BpA treated hypoxic samples. Conclusions from this study could support a role for hypoxia and the involvement of HIF-1 α in the signalling pathway.

Chapter 1. Introduction: Vascular Biology

1.Introduction: Vascular Biology

1.1 Structural Components of the Normal Aorta

The aorta is characteristically highly elastic allowing it to accommodate large fluctuations in arterial blood pressure created by the heartbeat. Histologically the vessel wall is composed of concentric layers, the tunica intima, tunica media and tunica adventitia. The innermost layer, the tunica intima consists of a monolayer of simple squamous endothelial cells that border the lumen, underlain by the basal lamina and a subendothelial layer of connective tissue composed of collagenous tissue and elastin. Separated from the intima by a fenestrated layer of elastin known as the internal elastic lamina, the tunica media is the thick middle layer containing circumferentially arranged smooth muscle cells, collagenous fibres and elastic lamellae arranged as concentric fenestrated sheets. The outer collagenous tunica adventitia prevents the artery from stretching beyond its physiological limits and is separated from the media by the external elastic lamina (Ross *et al.*, 1989).

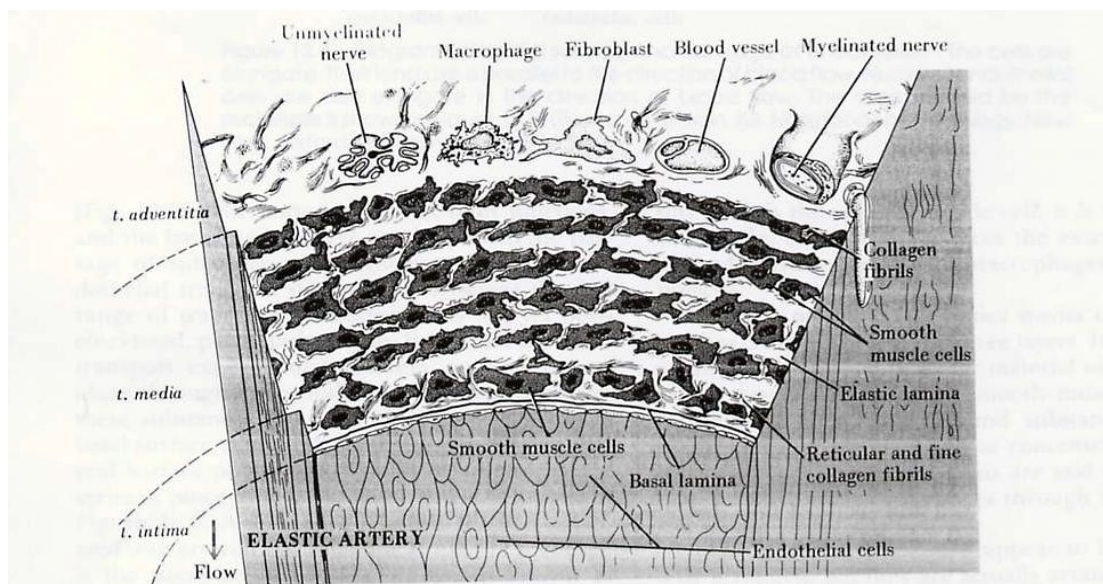


Figure 1 Schematic Diagram of an Elastic Artery

The tunica intima, tunica media and outer tunica adventitia are illustrated along with their cellular and extracellular components. (Adapted from Rhodin, 1963).

Although most of the aortic media is avascular and receives nourishment from the luminal blood supply, in the thoracic aorta, the tunica adventitia contains vasa vasorum (small blood vessels) that penetrate from the adventitia into the outer layers of the aortic media to supply oxygen and nutrients to cells in the vessel wall too far from the lumen to be nourished by diffusion (Heistad *et al.*, 1978). In contrast the abdominal aorta is largely devoid of medial vasa vasorum and relies on luminal blood flow to a greater extent for oxygenation and nutrition of the tissues (Zarins *et al.*, 2001). The arterial extracellular matrix (ECM) consists of the structural proteins collagen and elastin (which confer resistance to aneurysmal dilatation under haemodynamic stress), and specialised proteins such as fibrillin, fibronectin, laminin, proteoglycans and plasma components) which are secreted at a local level by cells within the ECM, in particular smooth muscle cells, endothelial cells and fibroblasts (Stary *et al.*, 1992).

1.1.1 Elastin

Elastin is the major component of elastic fibres, giving the extensibility and elastic recoil to blood vessels essential for their physiological function. (Hofmann *et al.*, 2005). Formation of elastin by smooth muscle cells occurs early during the development of the aorta, with little or no synthesis in adults due to destabilisation of elastin mRNA which appears to be an important factor in the regulation of this protein (Hew *et al.*, 1999) and as a consequence there is a decrease in the elastin content relative to collagen with increasing age (Hosoda *et al.*, 1984). The human elastin gene (ELN) is a single copy gene localised to the long arm of chromosome 7 within the q11.1-21.1 region and consists of 34 exons occupying ~47kb of genomic DNA (Fazio *et al.*, 1991). An intron: exon ratio of 20:1 indicates that relatively small exons are interspersed within large introns, furthermore the exon-intron boundaries always split codons in the same manner (Bashir *et al.*, 1989) allowing extensive alternative splicing of the transcripts without

disrupting the reading frame resulting in multiple isoforms of tropoelastin (Rosenbloom *et al.*, 1993). Elastin consists of two morphologically distinct components, the initial product of the ELN gene is soluble tropoelastin, a 72 kDa polypeptide rich in glycine, proline and hydrophobic amino acids, which is secreted by cells into the extracellular space (Rosenbloom *et al.*, 1993). The second component, 10-15 nm microfibrils are thought to provide a scaffold that facilitates elastin molecular alignment and subsequent formation of intermolecular cross-links known as desmosines (Mecham and Davis, 1994). Cross-linking of tropoelastin requires the oxidation of lysyl residues catalyzed by the copper-dependent enzyme lysyl oxidase and subsequently forms highly insoluble elastin (Sato *et al.*, 2007).

Recently Halm *et al.*, (2016) developed techniques for visualizing tropoelastin (TE) production and its fibre assembly in a long-term human elastic fibre cell culture model expressing fluorescence-labelled TE. This will allow the tracking of TE produced under various conditions and serve as a tool for investigating fibre degradation processes in a disease-in-a-dish-model which may prove useful in AAA research where elastin is degraded during aneurysm formation.

Elastin is a vital protein for maintaining stability of the aortic wall which led researchers to evaluate a novel small-molecular-weight elastin-specific MR probe for the *in vivo* assessment of aortic wall integrity in AAAs. They were able to visualize and quantify changes in elastin content at different stages of aneurysmal disease which could enable more accurate risk stratification and guide treatment options (Makowski *et al.*, 2014).

The degradation of elastin is an important factor in the formation of AAA and, in an attempt to address this, Tian *et al.*, (2014) investigated whether adipose derived stem cells (ADSCs) could promote the secretion of elastin by SMCs in a rat model of AAA. Results demonstrated that ADSCs promote the expression of elastin in SMCs and contribute to the reconstruction of elastic fibre which may provide new ideas for treating AAA.

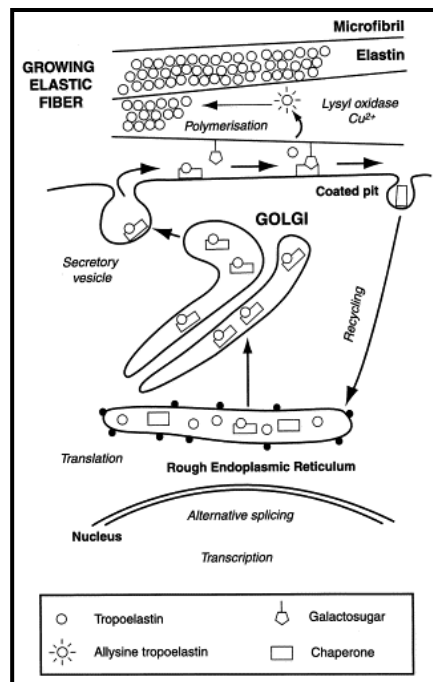


Figure 2 Formation of Elastin

After translation the tropoelastin molecule is bound by an elastin binding protein (EBP) in the rough endoplasmic reticulum. The EBP acting as a chaperone prevents premature aggregation of the tropoelastin until the EBP/tropoelastin complex is secreted onto the cell surface where the chaperone interacts with the galactosugars of the microfibrils and releases the tropoelastin molecule. The EBP is recycled and the tropoelastin is aligned and modified by lysyl oxidase to be incorporated into the growing elastin network. (Adapted from Hinek, 1997).

1.1.2 Fibrillins

Fibrillins-1, 2 and 3 are large (~350kDa) cysteine-rich glycoproteins that are a known component of microfibrils (Saki *et al.*, 1991) Fibrillin aligns in a head to tail fashion as parallel bundles of six to eight molecules to form most of the microfibrillar structure (Gibson *et al.*, 1996). Fibrillin-1 mutations result in Marfan syndrome, a connective tissue disorder affecting many structures, including the skeleton, lungs, eyes, heart and blood vessels, including aortic

dilatations, while fibrillin-2 mutations result in congenital arachnodactyly (Lee *et al.*, 1991). Additionally a novel fibrillin-1 gene missense mutation associated with neonatal Marfan Syndrome (nMFS), a rare and severe form of Marfan Syndrome leading to death during early childhood, was reported recently (Peng *et al.*, 2016). Several other proteins also associate with microfibrils including the microfibril-associated proteins (MAGPs), fibulins and Elastin Microfibril Interface-Located Protein-1 (EMILIN-1) (Kielty *et al.*, 2002).

1.1.2.1 Microfibril-associated Proteins (MAGPs)

Microfibril-associated glycoprotein-1 (MAGP-1) is a small (~31kDa) glycoprotein that has been shown to bind to both tropoelastin and fibrillin (Jenson *et al.*, 2001). It is thought that MAGP-1 may stabilise the head to tail interactions of fibrillin-1 within the beaded region of microfibrils by disulfide linkages (Henderson *et al.*, 1996) and mediate the binding and alignment of tropoelastin onto the microfibril template (Gibson *et al.*, 1991). MAGP-2 has also been shown to bind fibrillin but has a more restricted pattern of tissue localization than MAGP-1 suggesting it may have developmental stage-specific expression (Gibson *et al.*, 1998).

Fibulins are a six-member family of glycoproteins associated with elastic fibres and believed to function as molecular 'bridges' that participate in the assembly, organization and stabilization of extracellular matrix structures (Argraves *et al.*, 2003). Fibulins-1, 2 and 5 all bind to tropoelastin (Sasaki *et al.*, 1999), while fibulin-3 is expressed only in capillaries Hanada *et al.*, (2007) demonstrated that reduced expression of fibulin-4 leads to aneurysm formation in mice. Fibulin-5 (FBLN5) mediates cell-ECM interactions and elastic fibre assembly and is critical for ECM remodelling. Orriels *et al.*, (2016) found FBLN5 expression was significantly decreased in human aneurysmatic aortas compared with healthy aortas and identified a SOX9/HDAC dependent mechanism involved in the down-regulation of FBLN5 by inflammation. They concluded that HDAC inhibitors or

pharmacological approaches aimed to preserve FBLN5 could be useful to prevent the disorganisation of ECM induced by inflammation in AAA.

EMILIN-1 is a cysteine-rich extracellular matrix glycoprotein abundantly expressed in elastin-rich tissues such as blood vessels and skin and is localised to the interface between amorphous elastin and surrounding microfibrils. Bressan and colleagues (1993) found that antibodies against EMILIN-1 inhibited elastin deposition by smooth muscle cells in vitro suggesting that the protein may play a role in elastogenesis. EMILIN-1 was also found to bind elastin and fibulin-5 and the association of fibulin-5 with elastin is altered in the absence of EMILIN-1 (Zanetti *et al.*, 2004). EMILIN-1 is encoded by the *EMILIN1* gene and recently Capuano *et al.*, (2016) identified a heterozygous missense alteration in exon 1 of this gene. Their findings may represent a new disease gene associated with an autosomal-dominant connective tissue disorder which interestingly can also present in ascending and descending aortic aneurysms in affected patients.

1.1.3 Collagen

Collagen is the second major structural protein of the aortic wall, accounting for approximately 20% of the total protein in the normal aorta and is responsible for maintaining the structural integrity of the aortic wall.

Twenty-five types of collagen α chain have now been identified resulting in at least sixteen types of collagen which are synthesized as longer precursor proteins called procollagen (Alberts *et al.*, 1994). The regulation of procollagen synthesis appears to occur predominantly at the level of gene transcription (Vuorio and De Crombrughe, 1990). All commonly share a triple helical structure composed of three polypeptide α chains consisting of Gly-X-Y repeats, where X is any amino acid, and Y is frequently proline or hydroxyproline. These three left-handed helices wind around each other to form a right-handed superhelix (Sato *et al.*, 2002). During collagen biosynthesis, stability of the helical structure is significantly increased post-

translationally by conversion of proline residues by the enzyme prolyl-4 hydroxylase to 4-hydroxyproline in the Y position in the nascent procollagen chain. When lysine is present in the Y position of the Gly-X-Y triplet, it can be converted to hydroxylysine residues by lysyl hydroxylase which can then serve as sites for further modification of procollagen (Salo *et al.*, 2006).

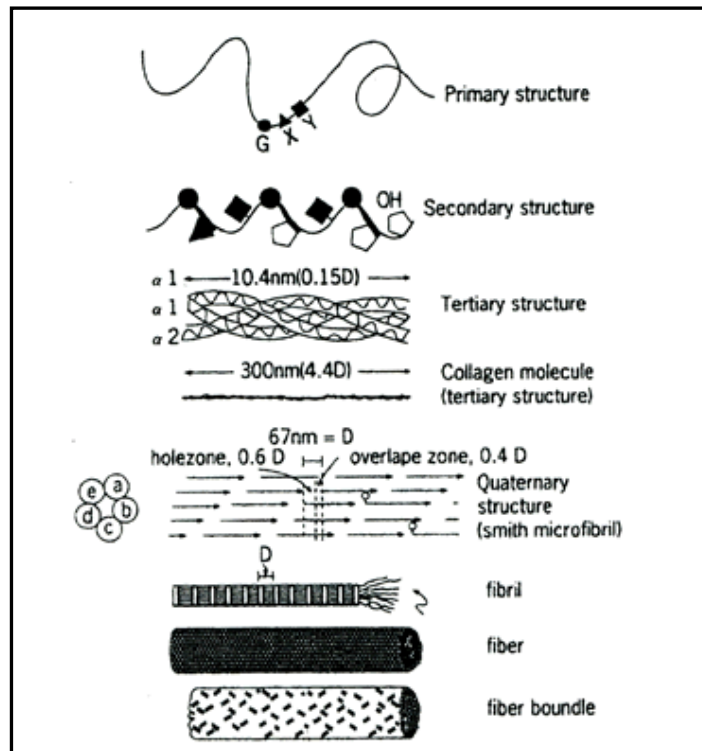


Figure 3 Formation of Collagen

The primary structure shows the repetitive triplet sequence Gly-X-Y characteristic of collagen and the left-handed helix of the secondary structure. The tertiary structure of this type-I collagen molecule is composed of three intertwined peptide chains, (2 $\alpha 1$ and 1 $\alpha 2$), each triple-stranded helix is 300 nm in length and 1.5 nm in diameter, and approximately 300 kilodaltons in molecular weight. The collagen molecules are assembled in a 'head-to-tail' alignment in a staggered side by side arrangement to form fibrils, adjacent molecules are displaced from one another by 67nm producing the striated effect seen in electron micrographs. The fibrils then aggregate into larger bundles to form collagen fibres which can be several micrometers in diameter.

In the normal adult artery the majority of collagen exists in the form of two interstitial collagens, type I and type III, although other collagen types including IV, V and VI are also present to a lesser extent (Stary *et al.*, 1992). Smooth muscle cells (SMCs) within the media are the major cell type responsible for collagen synthesis in the vessel wall and it is thought SMC hyperplasia alters the ratio of types I and III collagen in favour of type I in the aging aorta (Morton and Barnes, 1982).

The collagen fibres lie between the elastic lamellae and the surrounding SMC within the aortic media, in the adventia they form a loose mesh-like network where they are thought to be able to align more effectively during higher degrees of tensile loading to accommodate the physical forces imposed by blood pressure and avoid aortic vessel rupture (Thompson *et al.*, 2002).

Table 1 Major Types of Collagen

TYPE	α CHAINS	GENES	LOCALIZATION
I	$[\alpha 1(\text{I})_2[\alpha(\text{I})]$	COL1A1 COL1A2	Skin, tendon, bone, artery walls (most abundant collagen)
II	$[\alpha 1(\text{II})]_3$	COL2A1	Cartilage, vitreous humor
III	$[\alpha 1(\text{III})]_3$	COL3A1	Skin, artery walls, organs
IV	$[\alpha 1(\text{IV})_2[\alpha 2(\text{IV})]$	COL4A1 COL4A2 COL4A3 COL4A4 COL4A5 COL4A6	All basal lamina, highly vascularised tissues e.g. placental villi
V	$[\alpha 1(\text{V})][\alpha 2(\text{V})\alpha 3(\text{V})]$	COL5A1 COL5A2 COL5A3	Bone, skin, tendons, ligaments, cornea
VI	$[\alpha 1(\text{VI})][\alpha 2(\text{VI}) [\alpha 3(\text{VI})]$	COL6A1 COL6A2 COL6A3	Most connective tissue
VII	$[\alpha 1(\text{VII})]_3$	COL7A1	Epithelia
VIII	$[\alpha 1(\text{VIII})]_3$	COL8A1 COL8A2	
IX	$[\alpha 1(\text{IX})][\alpha 2(\text{IX})][\alpha 3(\text{IX})]$	COL9A1 COL9A2 COL9A3	Tissues associated with Type II
X	$[\alpha 1(\text{X})]_3$	COL10A1	Hypertrophic cartilage
XI	$[\alpha 1(\text{XI})][\alpha 2(\text{XI})][\alpha 3(\text{XI})]$	COL11A1 COL11A2	Cartilage
XII	$\alpha 1(\text{XII})$	COL12A1	Tissue associated with Type 1

Adventitial collagen structure provides the aorta with tensile strength and its degradation is a key characteristic of AAA. Urabe *et al.*, (2016) investigated whether the structural characteristics of aortic adventitial collagen changed with aging, location or aneurysm formation. Results showed that, although the basic structure was maintained in abdominal aortas regardless of age or location, the molecular structure at the subfibril level changed and was enhanced in AAA; they concluded that alterations within the molecular structure of collagen such as in cross linkages may be associated with aneurysm formation. Change in collagen structure has also been studied by other researchers such as Dai *et al.*, (2015) who demonstrated that Smad3 deficiency promotes vessel wall remodelling, and collagen fibre reorganization in an inflammatory AAA mouse model after their earlier study found Smad3-mediated TGF- β signalling (which is an important mechanism in the pathogenesis of aneurysms) plays a protective role in the pathogenesis of AAA (Dai *et al.*, 2013). Furthermore, other research has established that the degradation of decorin, a key regulator of collagen organization by the pro-apoptotic protease Granzyme B (GZMB) results in reduced collagen organization and tensile strength, possibly resulting in aortic rupture (Granville *et al.*, 2013). Gregoli and colleagues, (2015) using a AAA mouse model discovered collagen degradation through increased MMP activity could be altered through inhibition of microRNA (miR)-181b (a validated repressor of TIMP-3 protein expression) by promoting collagen preservation and accumulation via TIMP-3 modulation stabilizing AAA formation.

Of concern, Daneman *et al.*, (2015) carried out a study which reported a novel and important association between fluoroquinolones, a family of frequently prescribed antibiotics, and aortic aneurysms. They concluded fluoroquinolone-associated collagen degradation may affect the aortic wall and could have the potential to contribute to aortic aneurysms.

1.1.4 Fibronectin and Laminin

The glycoproteins fibronectin and laminin are also present in the extracellular matrix (ECM) of the normal intima of the aorta and other blood vessels. Cotrufo *et al.*, (2005) found that in AAA the basal lamina of the aortic wall exhibited important changes to some of its major components, including the laminin alpha2 chain and fibronectin which lost their regular arrangement in pathological specimens along with decreased expression in comparison to normal aortic wall specimens.

1.1.4.1 Fibronectin

Fibronectin exists in both soluble and fibrillar forms and in humans there are up to twenty different isoforms arising from alternative splicing of mRNA from a single fibronectin gene (Potts and Campbell, 1994). Fibronectin is a dimeric glycoprotein composed of two 250 kDa subunits joined by disulphide bonds at the carboxyl terminal end. The rod-like structure contains three different types of homologous repeating modules (type 1, II and III) which are folded into at least six tightly folded domains each with a high affinity for a different substrate such as heparin sulphate, collagen, fibrin and cell-surface receptors (Peterson *et al.*, 1983). The most abundant module is the fibronectin type III repeat which contains synergy sequences and a specific tripeptide sequence (*Arg-Gly-Asp*) or RGD receptor recognition sequence known to be involved in cell adhesion (Main *et al.*, 1992).

Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting, cell migration and serves as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. In addition, it can organize cellular interaction with the ECM by binding to different components of the extracellular matrix and to membrane-bound fibronectin receptors on cell surfaces (Potts and Campbell, 1996). Studies have shown that fibronectin production by many cell types has been enhanced by a variety of cytokines in particular interleukin 1 (IL1) and

Transforming Growth Factor- β (TGF-beta). Recent reports have also shown fibronectin affects proliferation, morphology, migration and differentiation of the smooth muscle cells during the development of atherosclerosis (Yurdagul *et al.*, 2014) which is frequently associated with AAA.

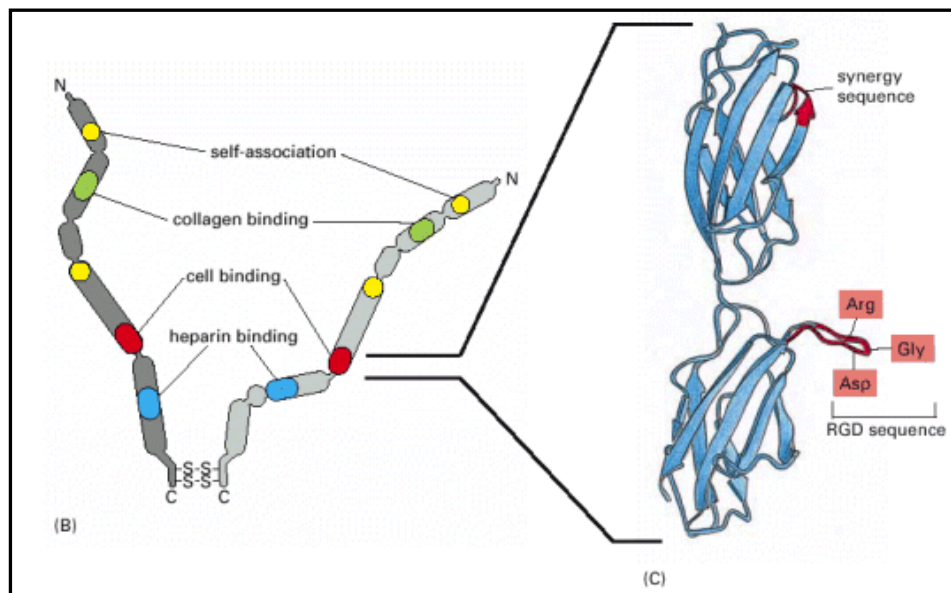


Figure 4 Structure of a Fibronectin Dimer

B: Fibronectin is a V-shaped dimer made up of two polypeptide chains which are similar but not identical due to alternative splicing and are joined by two disulfide bonds near the C-termini. Fibronectin has several binding domains which can bind heparin, collagen, cells (via integrins) or itself. **C:** The three-dimensional structure of two type III fibronectin repeats which are the main repeating module in fibronectin and depicts the *Arg-Gly-Asp* (RGD) and the “synergy” sequences as part of the major cell-binding site. (Adapted from Leahy, 1997).

1.1.4.2 Laminin

Laminin is a major basement membrane glycoprotein composed of three polypeptide chains, alpha (α), beta (β) and gamma (γ) that are disulfide-bonded into an asymmetric crosslike structure (Rhodes and Simons, 2007). Several different laminin isoforms have been identified, assembled from the five different α chains, three β chains and three γ chains, each with a

characteristic tissue distribution. The ends of the 'cross' can bind cell receptors and the crosspieces allow laminin to bind other laminin molecules, additionally there are binding sites available for matrix components nidogen and perlecan which are necessary for the correct functioning of basal lamina (Timpl and Brown,1994). The major activity of laminin is the mediation of cell attachment (via integrin receptors) but it also has other roles in cellular proliferation, differentiation and migration. More recently its ability to promote the contractile phenotype of smooth muscle cells has been reported (Chuang *et al.*, 2014).

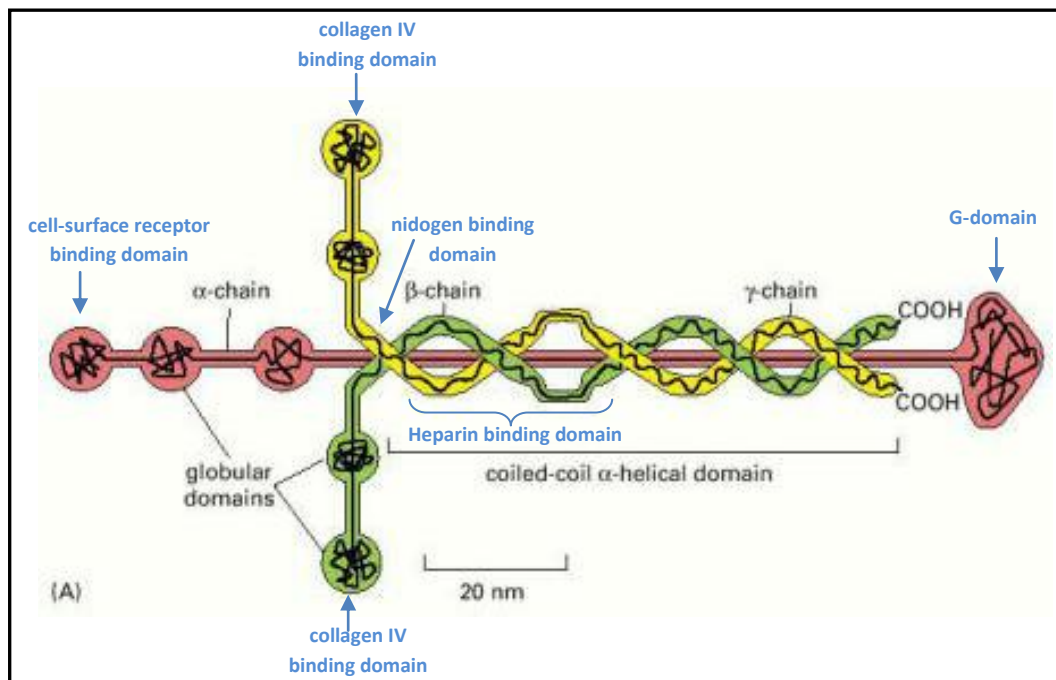


Figure 5 The General Structure of Laminin

Laminin is a heterotrimer of the three subunits α , β and γ joined by interchain disulfide bonds to create a cross-like structure. The three short arms of the 'cross' are formed by the N-terminal regions of the chains and the α -helical coiled-coiled long arm is formed by the C-terminal region where a large globular domain known as the G domain is located. Cysteine-rich EGF (epidermal growth factor)-like repeats separate the globular domains of the short arms. The sites of some binding domains have been marked on the diagram. (Adapted from Molecular Biology of the Cell).

1.1.5 Proteoglycans

Proteoglycans are a group of macromolecules consisting of a core protein with one or more glycosaminoglycan (GAG) chain(s) covalently attached through O-glycosidic linkages to serine residues. Proteoglycans are synthesized by the endothelial and SMCs of the arterial wall and are vital for functions such as cell proliferation, migration and adhesion, viscoelasticity, permeability, ion exchange, lipid metabolism and filtration in these vessels. They are also thought to be important in the stabilization of the spatial arrangement of collagen fibres (Starey *et al.*, 1992, Bosman and Stamenkovic, 2003, Kresse and Schonher, 2001).

They are generally categorized by size and the nature of their GAG chains and those identified in the arterial wall include hyaluronic acid, dermatan sulfate (biglycan) associated with collagen fibrils, heparin sulfate (syndecan, perlecan) present in arterial basement membranes and associated with elastin fibres and chondroitin 4- and 6-sulfate (versican) found in the interstitial matrix of the arterial ECM (Volker *et al.*, 1987). Proteoglycans are minor components of the normal arterial wall, but their accumulation in intimal lesions of blood vessels is a typical event in the early phases of atherosclerotic occlusive disease and AAA development (Wight, 1980,1989). This suggested to Melrose *et al.*, (1998) that proteoglycans may influence aortic physicochemical properties and subsequently a study was undertaken to compare proteoglycans synthesized by smooth muscle cells from AAA tissue or atherosclerotic occlusive disease sufferers to normal controls to determine whether variations in these proteoglycans can be correlated with disease processes in the vessel wall. Results showed that the levels of perlecan, versican, and biglycan synthesized by AAA smooth muscle cells in monolayer culture were significantly higher than synthesized by the other smooth muscle cells, but were unable to explain to what extent this cellular behaviour contributes to AAA development *in vivo*. In contrast Tamarina *et al.*, (1998) found a marked decrease in biglycan mRNA levels in aneurysmal aorta, which could reflect important regulatory changes specific for AAA and have a broad impact on the physiology and matrix architecture of the

aorta. This also correlates with other researchers who have shown that GAG content was decreased and their molecular differentiation was changed in the AAA wall (Sobolewski *et al.*, 1995, Theocharis *et al.*, 1999, Theocharis *et al.*, 2002). More recently in 2015, Ueda *et al* investigated the role of decorin, a small leucine-rich repeat proteoglycan in the pathogenesis of AAA. Their results suggested that adventitial decorin in normal aorta may protect against the development of AAA, but macrophages expressing decorin in AAA walls may facilitate the progression of AAA by up-regulating MMP-9 secretion.

1. 2 Physiology of the Aorta

1.2.1 Haemodynamic Forces in the Aorta

The aortic wall is continuously subjected to mechanical forces created by blood flow and it is now recognized that these haemodynamic forces directly influence vascular cell biology and play a role in modulating vascular tone and vascular remodelling. Fluid flow across the endothelial cells (ECs) lining the intimal surface of the aortic vasculature results in shear stress whereas both endothelial, SMCs and the underlying matrix are subjected to cyclic strain (stretch) resulting from transmural pressure gradients and smooth muscle tone (Patrick and McIntire, 1995). These physical forces can affect a number of EC functions; in particular it is well known that they respond to increases in flow by modulating the release of bioactive molecules including the vasorelaxants nitric oxide (NO) (Busse and Fleming, 1998), prostacyclin, (PGI₂), the vasoconstrictor endothelin-1 and tissue plasminogen activator (Carosi and McIntire, 1994). NO is a diatomic gas formed by the conversion of L-arginine to L-citrulline by NO synthases (NOS) and plays a major role in regulating blood flow by inhibiting smooth muscle contraction, platelet adhesion, aggregation (Moncada and Higgs, 1993) as well as SMC proliferation (Schwartz and Liaw, 1993). Activation of constitutive NOS in ECs by inhibitory G-proteins and subsequent release of NO results in the stimulation of soluble guanylyl cyclase leading to a rise in guanosine 3', 5'-

cyclic monophosphate (cGMP) in SMCs and platelets (Stamler *et al.*, 1992). Stimulation of these G-proteins can initiate a number of signalling cascades including activation of potassium channels, phospholipase C, phospholipase A2, protein kinase C and adenylyl cyclase (Wand *et al.*, 1993). The effect of cyclic strain on SMCs has shown it induces the production of platelet-derived growth factor and stimulates cell proliferation, furthermore under inflammatory conditions SMCs can express iNOS (inducible NOS) which also is capable of synthesizing large quantities of NO (Busse and Mulsch, 1990). Wagner *et al.*, (1997) also reported that SMCs exposed to shear stress express another diatomic gas carbon monoxide (CO) with similar properties to NO causing blood vessel relaxation and inhibiting platelet reactivity through a cGMP-dependent mechanism.

Analysis of haemodynamics has demonstrated that when the pulse wave generated by the heartbeat is transmitted from the aortic root to the inguinal region, the pulse pressure increases with the largest pulsatile load being localized to the infrarenal aorta. This localization of haemodynamic stress is due to the distal tapering of the aorta, the progressive stiffening of the aortic wall resulting from the decrease in the number of elastic lamellae (especially as it enters the abdomen) and the additive effects of retrograde pressure waves that reflect from the iliac bifurcation (Dobrin, 1989). These factors appear to correlate with the propensity for AAAs to form in this segment of the aorta.

Dobrin and colleagues, (1984) looked at the relative contributions made by elastin and collagen to the biophysical properties of the aortic wall by treating arteries with elastase or collagenase. Treatment with elastase led to marked arterial dilatation and stiffening at physiologic pressures, whereas treatment with collagenase led to arterial rupture with little dilatation demonstrating that elastin degradation was a key step in the development of aneurysmal dilatation but that collagen degradation was ultimately required for aneurysm rupture.

Recently researchers suggested that haemodynamic forces and intraluminal thrombus (ILT), another feature of AAA, interact and affect each other. Their

study looked at the relationship between aneurysm expansion, wall shear stress (WSS) and ILT accumulation. Comparison between AAAs with and without thrombus showed that aneurysm with ILT recorded lower values of WSS and higher values of AAA expansion than those without thrombus. Results suggested that low WSS may promote ILT accumulation and that, by increasing WSS levels, ILT accumulation may be prevented (Zambrano *et al.*, 2016).

Haemodynamic forces appear to play an influential role in the evolution of aneurysms and studies have usually focused on WSS, attempting to find correlations between patterns of haemodynamic indices and regions subjected to disease formation and progression. Poelma *et al.*, (2015) instead demonstrated the role of transitional flow in aneurysms which can lead to significantly different WSS distributions in consecutive cardiac cycles. Therefore accurate determination of time-averaged haemodynamic indices may thus require simulation of a large number of cycles, which contrasts with the common approach to determine parameters using data from a single cycle.

1.2.2 Endothelin-1

Endothelin (ET)-1 is a 21 amino acid peptide that is produced by the vascular endothelium from a 39 amino acid precursor, big ET-1, through the actions of an endothelin converting enzyme (ECE) (Yanagisawa *et al.*, 1988). There are three isoforms known as ET-1, -2 and -3 which are encoded by separate genes and are implicated in a wide variety of physiological and pathological processes in the body. Endothelins are the most potent vasoconstrictors known and although vascular endothelial cells are the major source of endothelin it is also produced by a wide variety of other cell types including vascular SMCs (Inoue *et al.*, 1989).

AAA is associated with increased ET-1 levels both systemically and locally within the aorta. Tresca *et al.*, (1999) discovered plasma levels of ET-1 were elevated in patients with AAA which also correlated with aneurysm

diameter and suggested a role for ET-1–induced vascular remodelling in the pathogenesis of AAA. Chew and colleagues, (2003) proposed increased ET-1 and elastase activities in AAA patients modulated the effects of elastase on the vasoconstrictive actions of ET-1 during AAA formation by elastase inhibition of ET-1 induced aortic contraction.

In 2013, Li *et al* concluded that ET-1 is implicated in the progression of atherosclerosis and AAA formation by decreasing high-density lipoprotein and increasing oxidative stress, inflammatory cell infiltration and MMP-2 in perivascular fat, vascular wall and atherosclerotic lesions.

1.2.3 Cells of the Arterial Intima

1.2.3.1 Smooth muscle cells

SMCs are the major cell type of the tunica media of medium and large sized arteries, contributing to lipid metabolism, vascular tone, vascular remodelling and are responsible for the synthesis and deposition of important components of the extracellular matrix including collagen, elastin and proteoglycans. Vascular SMCS normally exhibit a contractile phenotype (Munro and Cotran, 1988) but under certain pathological conditions the SMCs may become exposed to growth factors and cytokines that induce a transformation from the contractile to synthetic state allowing the SMCs to proliferate and accumulate within the intimal layer (Ross *et al.*, 1989). Importantly, like other vascular cells SMCs have the capacity to express components of the ECM, growth factors and proteases including matrix metalloproteinases (MMPs), enzymes that selectively digest components of the ECM during normal events such as angiogenesis or after stimulation with various cytokines associated with vascular homeostasis or in pathological conditions such as AAA (Lesauskaite *et al.*, 2003).

Zhang *et al.*, (2015) sought to determine the role of Smad4 in SMCs in the pathogenesis of aortic aneurysms. Smad4 is the central mediator of the

canonical TGF- β signalling pathway which is known to be involved in aneurysm formation. Their research showed Smad4-deficient SMCs directly triggered aortic wall inflammation via the excessive production of chemokines to recruit macrophages and further demonstrated Smad4-dependent TGF- β signalling in SMCs protects against aortic aneurysm formation.

1.2.3.2 Endothelial cells

The vascular endothelial cells of the tunica intima are also involved in many aspects of vascular biology including synthesis of ECM components (Jaffe, 1987) control of platelet adhesion, maintaining a balance of fibrinolytic and prothrombic activity, regulating vascular tone and a critical role in regulating the recruitment of leukocytes into inflammatory sites (Springer, 1995). The arterial endothelium is also permeable to all plasma proteins allowing the transport of lipoproteins and other macromolecules across the endothelium through a complex system of interendothelial cell junctions (Schwartz and Benditt, 1972).

Endothelial cells respond to a number of stimulating factors, including smoking, hypertension and non-uniform distribution of wall stress so their ability to produce NO is crucial in order to adapt. Endothelial cells contribute to AAA development due to increased oxidative stress which is partly mediated by impaired NO bioavailability due to endothelial dysfunction and NADPH oxidase overexpression (Siasos *et al.*, 2015).

1.2.3.3 Fibroblasts

The fibroblast is the principal cell type in the adventitia and increasingly it is thought that they play a significant role in normal vascular homeostasis, the vascular response to injury and vascular remodelling (Stenmark *et al.*, 2002). The vascular adventitia could be considered the principle injury-sensing

tissue of vessel walls and in response to environmental stresses resident fibroblasts are activated and can rapidly modulate their functions, including cell proliferation, expression of ECM proteins and secretion of factors affecting vascular tone and growth (Stenmark *et al.*, 2006).

Aortic adventitial fibroblasts (AoAF) are a rich source of cytokines and ECM and they participate in arterial wall remodelling and have been implicated in the pathophysiology of AAA and atherosclerosis. Although little is known about signalling pathways that regulate fibroblasts in cardiovascular diseases Zhao *et al.*, (2013) examined the role of Notch signalling in adventitial fibroblasts in normal human aorta, AAA and atherosclerotic samples and the effect of Notch signalling on cytokines and ECM production by fibroblasts. Significantly increased Notch activity was observed in fibroblasts from AAA samples and decreased Notch activity was found in fibroblasts from atherosclerotic aorta whilst Notch activation also inhibited cell proliferation and induced differentiation of the cells to myofibroblasts. They concluded Notch signalling is activated in aortic fibroblasts at the site of aortic aneurysms and results in increased production of TGF- β by these cells, implicating involvement of Notch-TGF- β signalling in the formation of aortic aneurysms.

1.3 Matrix metalloproteinases (MMPs)

MMPs are a family of zinc-mediated endopeptidases present in the ECM that are involved in a variety of physiological processes including inflammation, wound healing, embryogenesis and remodelling of structural proteins such as elastin and collagen.) Additionally, MMPs play a central role in diseases such as cancer, arthritis, atheroma and tissue ulceration (Woessner., 1998). The important role MMPs play in the pathogenesis of AAA will be discussed in detail later in Chapter 2 (Vascular Pathology) Section 2.2.1. They are produced in an inactive form by various mesenchymal cells such as fibroblasts, endothelial cells and SMCs as well as cells involved in the

inflammatory cascade (neutrophils, macrophages) and can be activated by a number of proteinases including plasmin and other MMPs. Activation occurs by removal of the signal sequence and the propeptide domain which contains a conserved cysteine which chelates the active zinc site. Under normal circumstances MMPs are strictly regulated at the level of transcription and through activation of precursor zymogens or inhibition by endogenous inhibitors termed tissue inhibitors of matrix metalloproteinases (TIMPs) (Nigase and Woessner, 1999).

1.3.1 General Structure of MMPs

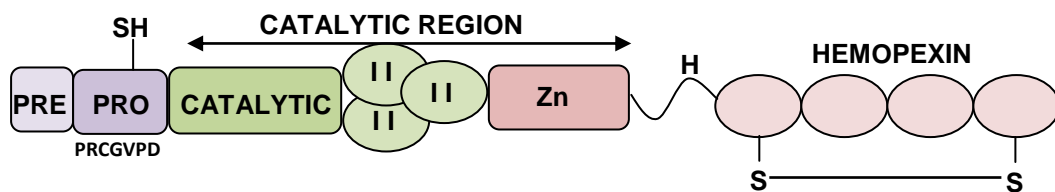


Figure 6 The Domain Structure of MMP-2 (Gelatinase)

Secreted MMPs contain an amino-terminal signal sequence (Pre) that directs them to the endoplasmic reticulum, a propeptide (Pro) domain containing the highly conserved sequence PRCGVPD around the chelating cysteine known as the 'cysteine switch' that maintains them as inactive zymogens. The simple hemopexin-domain thought to confer much of the substrate specificity to MMPs as well as mediating interactions with TIMPs is connected to the catalytic domain by the small proline-rich hinge region which allows folding of the C-terminal domain onto the catalytic domain. The first and the last of the four repeats in the hemopexin-like domain are linked by a disulphide bond (S-S). The gelatin-binding MMPs also contain inserts that resemble collagen-binding type II repeats of fibronectin and are required to bind and cleave collagen and elastin.

The MMP family consists of at least 23 distinct proteases in humans and are usually defined by their homologous structure at the protein and DNA levels

as well as their substrate specificity by which they are categorized into 6 main groups as shown in Table 2 (Visse and Nigase, 2003). All members of the MMP family share several basic features within their five-domain structure including a pre-domain which contains a signal peptide responsible for secretion, the pro-domain which keeps the enzyme inactive by the cysteine residue interacting with the Zn^{2+} from the catalytic domain to prevent binding and cleavage of the substrate, and the haemopexin-like domain which is linked to the catalytic domain by a flexible hinge region (Pei and Kang, 2000).

Although a general proteolytic mechanism of activation is understood for MMPs, the mechanism of the inceptive period of protein insertion into the active site of the MMP prior to activation remains unknown. Crystallographic data continues to suggest an active site that is too narrow to encompass the respective substrate. Nash *et al.*, (2016) undertook a fully parameterised Molecular Dynamics (MD) study of the structural properties of an MMP-1-collagen crystallographic structure followed by an exploration of the free energy surface of a collagen polypeptide chain entering the active site using a combined meta-dynamics and umbrella sampling (MDUS) approach. They concluded that interactions between MMP-1 and the collagen substrate indicated an energetic barrier for a local uncoiling and insertion event and this knowledge would help to elucidate future collagen-peptide non-bonded association steps with the active site prior to proteolytic mechanisms to provide a better understanding of the role of the enzyme in the ECM. Another research group examined the Importance of the linker region between the catalytic (CAT) and hemopexin-like (HPX) domains in matrix metalloproteinase-1 and results of MD revealed that interactions between the collagen-model triple-helical peptide with both the CAT and HPX domains of MMP-1 are dynamic in nature, and the linker region of MMP-1 influences the interactions and dynamics of both the CAT and HPX domains and collagen binding to MMP-1 (Singh *et al.*, 2016).

1.3.2 Classification of MMPs

Table 2 Classification of MMPs and Substrates

Classification	MMP
Collagenases	1, 8, 13, 18
Gelatinases	2,9
Stromelysins	3, 10, 11
Matrilysins	7, 26
Membrane-type MMPs	14, 15, 16, 17,24,25
Other MMPs	12, 19, 20,21,23,28

1.3.2.1 Collagenases

MMP-1, MMP-8, MMP-13, and MMP-18 are in this group and have the ability to cleave native fibrillar collagen types I, II, III, V and IX and can also digest a number of other ECM and non-ECM molecules (Knäuper *et al.*, 1996). The collagenases differ in their substrate specificities and functional roles. MMP-1 preferably degrades collagen III whilst MMP-8 prefers type I collagen and MMP-13 collagen II (Massova *et al.*, 1998, Knäuper *et al.*,1996).

Recently Fischer and Riedl, (2016) used MMP-13 as the target enzyme for the structure-based design and synthesis of inhibitors able to recognize the catalytic zinc ion in addition to an allosteric binding site in order to increase the affinity of the ligand. They optimized an initial allosteric inhibitor by addition of linker fragments and weak zinc binders for recognition of the catalytic centre and improved the lipophilic ligand efficiency (LLE) of the initial inhibitor by adding zinc ions thereby elevating the affinity of all the synthesized inhibitors.

1.3.2.2 Gelatinases

Gelatinase A (MMP-2) and gelatinase B (MMP-9) are able to digest denatured collagens and gelatin due to three fibronectin type II repeats within the catalytic domain that bind to gelatin, collagen and laminin. They also digest a number of ECM molecules including type IV, V, XI collagens and aggrecan core protein. Additionally MMP-2 but not MMP-9 is able to digest Type I, II, and III collagens in a similar way to collagenases (Aimes and Quigley, 1995, Patterson *et al.*, 2001). Both gelatinases contain Zn²⁺ and Ca²⁺ binding sites, but studies on MMP-9 by Tobwala and Srivastava, (2013) have also revealed that calcium ions are not just a structural requirement but also have a role in regulating the catalytic activity of MMP-9 by modifying the active site.

1.3.2.3 Stromelysins

MMP-3, MMP-10 and MMP-11 have a domain arrangement similar to that of collagenases although they do not cleave interstitial collagens. MMP-3 and MMP-10 are similar in structure and substrate specificity and digest a number of ECM molecules. In addition MMP-3 participates in the activation of a number of proMMPs and is critical for the generation of fully active MMP-1 (Suzuki *et al.*, 1990). MMP-11 contains a furin recognition motif RX[R/K]R at the C-terminal end of the propeptide and therefore is activated intracellularly (Pei and Weiss, 1995). MMP-11 in comparison to MMP-3 and MMP-10 has very weak activity toward ECM molecules, but readily cleaves serpins (Murphy *et al.*, 1993, Pei *et al.*, 1994).

1.3.2.4 Matrilysins

MMP-7 and MMP-26 are characterized by the lack of a hemopexin domain (Uria and López-Otín, 2000) and both digest a number of ECM components.

MMP-7 also processes cell surface molecules such as pro- α -defensin, Fas-ligand, pro-tumor necrosis factor (TNF)- α , syndecan 1 and E-cadherin (Parks *et al.*, 2004). MMP-26, also known as endometase (Park *et al.*, 2000) cleaves gelatin and β -casein (de Coignac *et al.*, 2000) type IV collagen, fibronectin, fibrinogen, vitronectin (Marchenko *et al.*, 2001, Uria and López-Otín., 2000), alpha 1-antitrypsin (Li *et al.*, 2004), alpha 2-macroglobulin (Ahokas *et al.*, 2005), alpha (1)-proteinase inhibitor (Park *et al.*, 2000, Li *et al.*, 2004) and insulin-like growth factor-binding protein 1 (Park *et al.*, 2002). Moreover MMP-26 also has the ability to cleave and fully activate MMP-9 (Uria and López-Otín, 2000).

1.3.2.5 Membrane-type MMPs (MT-MMPs)

MT-MMPs include four type I transmembrane proteins (MMP-14, -15, -16, and -24) and two glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and -25). They all have a furin recognition sequence RX[R/K]R at the C-terminus of the propeptide and are therefore activated intracellularly, and active enzymes are likely to be expressed on the cell surface (Zucker *et al.*, 2003, Shiomo and Okada, 2003), Folgueras *et al.*, 2004). Apart from MMP-17, all MT-MMPs are capable of activating pro-MMP-2 (Thomas, 2002, Zucker *et al.*, 2003, Zhao *et al.*, 2004). MMP-14 (MT1-MMP) also activates MMP-13 and has collagenolytic activity on type I, II, III collagens and is able to cleave gelatin, fibronectin, laminin-1, vitronectin, cartilage, proteoglycans and fibrillin-1. MMP-15 (MT2-MMP) can also activate MMP-13 and can degrade laminin, fibronectin, and tenascin. MMP-16 (MT3-MMP) hydrolyzes gelatin, casein, type III collagen, and fibronectin (Zucker *et al.*, 2003). MMP-17 (MT4-MMP) has tumor necrosis factor-alpha convertase activity but does not activate MMP-2, but may have a role in the regulation of cell surface proteins, inflammatory processes and has the ability to degrade fibrinogen and fibrin (English *et al.*, 2000). MMP-24 (MT5-MMP) is believed to function both as a membrane-bound and soluble proteinase and can degrade several ECM components, such as inhibitory chondroitin sulfate

proteoglycans (Wang *et al.*, 1999, Hayashita-Kinoh *et al.*, 2001) and mediates the cleavage of the cell-adhesion molecule N-cadherin in heterologous cells (Monea *et al.*, 2006). MMP-25 (MT6–MMP) cleaves collagen type-IV, gelatin, fibronectin and fibrin (English *et al.*, 2001) as well as cleaving and inactivating α 1-proteinase inhibitor (Nie and Pei, 2004).

Invasion of most normal cells across basement membranes and collagen-rich interstitial tissues involves degradation of the ECM by membrane type-1 matrix metalloproteinase (MT1-MMP/MMP14) (Willis *et al.*, 2013). To achieve this activity MT1-MMP is transported to podosomes, the specialized ECM-degrading membrane protrusions found in highly migratory cells (Murphy and Courtneidge, 2011).

More recently, an unexpected function independent of its proteolytic activity has been described for the MT1-MMP/MM-14 cytoplasmic domain in imprinting spatial memory for podosome reformation via assembly in membrane islets acting as cellular memory devices (Gucciardo *et al.*, 2016, El Azzouzi *et al.*, 2016).

1.3.2.6 Other MMPs

MMP-12 (Metalloelastase) is expressed primarily in macrophages and digests elastin, type IV collagen, type I gelatin, fibronectin, laminin, vitronectin, proteoglycans, myelin basic protein, and α 1-antitrypsin (Shiomi and Okada, 2003). Zheng *et al.*, (2015) looked at the role of the innate immune cells in the pathobiology of aneurysms and demonstrated that inactivation of p110 δ expressed by leukocytes and which regulates immune function leads to extracellular matrix degradation in vessels and promotes aneurysm development via dysregulation of MMP-12 expression.

MMP-19 digests many ECM molecules including the components of basement membranes (Stracke *et al.*, 2000). It is also known as RASI-1 (rheumatoid arthritis synovial inflammation-1) and is recognised as an autoantigen in patients with rheumatoid arthritis and systemic lupus erythematosus (Sedlacek *et al.*, 1998). MMP-20 (Enamelysin) is primarily

located within newly formed tooth enamel and digests amelogenin (Ryu *et al.*, 1999). MMP-21, MMP-23 and MMP-28 have a furin recognition sequence before the catalytic domain and therefore they are likely to be activated intracellularly and secreted as active enzymes. MMP-21 is known to digest gelatin but its activity on the other components of the ECM is largely unknown. MMP-23 is a unique member of the MMP family, devoid of structural features distinctive of the diverse MMP subclasses but instead has a novel cysteine array motif and an immunoglobulin-like C2 type fold domain (Pei *et al.*, 2000) and appearing to digest only gelatin (Pei, 1999). MMP-28 also named Epilysin belongs to the MMP-19 subfamily of the MMP superfamily and is reported to only digest casein (Lohi *et al.*, 2001).

1.4 MMP Regulation

The proteolytic activities of MMPs influence essential cellular processes like cell proliferation, migration and adhesion, as well as many fundamental physiological events involving tissue remodelling, such as angiogenesis, bone development, wound healing, and uterine and mammary involution (Page-McCaw *et al.*, 2007, Rowe and Weiss., 2008). The major function of MMPs is the degradation and removal of ECM molecules from tissues and to achieve this they must tightly regulated. MMP activity is controlled at four different levels including gene transcription, compartmentalisation, proenzyme activation and endogenous inhibition.

1.4.1 Transcriptional regulation of MMPs

MMP gene expression is primarily regulated at the transcriptional level, which results in most MMPs being expressed in normal tissues at low levels but is upregulated during certain physiological and pathological processes. Several *MMP* promoters share several cis-elements in their promoter

regions which allows tight control of cell-specific expression by a diverse set of *trans*-activators including AP-1, PEA3, Sp-1, β -catenin/Tcf-4, and NF- κ B (Benbow and Brinckerhoff, 1997, Westermarck and Kahari, 1999, Chakraborti *et al.*, 2003). Induction or stimulation at the transcriptional level is mediated by a variety of inflammatory cytokines, hormones and growth factors such as interleukin-1(IL-1), interleukin-6 (IL-6) (Mauviel, 1993), tumour necrosis factor- α (TNF- α) (Ito *et al.*, 1990), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) (Fabunmi *et al.*,1996) and CD40 (Malik *et al.*, 1996).

Furthermore, extracellular matrix metalloproteinase inducer (EMMPRIN) a cell surface protein that induces MMP expression has been identified in both normal and diseased human tissues (Spinale *et al.*, 2000). Moreover, other factors such as heparin, transforming growth factor- β (TGF- β), interleukin-4 (IL-4), retinoic acid and corticosteroids have been shown to have an inhibitory effect on MMP expression (Mauviel, 1993).

This response at the transcriptional level occurs several hours after exposure to a stimulus which suggests *MMP* promoters are downstream targets within signalling pathways of early response genes, which are induced shortly after cellular stimulation. These early response genes encode signalling proteins that phosphorylate the different transcription factors, which are then able to bind the promoters of *MMP* genes. These signaling intermediates involved in the activation of transcription factors include the nuclear factor kappa B (NF- κ B), the mitogen activated protein kinases (MAPK), the signal transducers and activators of transcription (STAT) and the Smad family of proteins (Vincenti *et al.*, 2007).

Studies have also shown that the p65 subunit of NF- κ B can regulate MMP-1, MMP-2, MMP-3, and MMP-9 transcription during the initiation and progression of AAA and intracranial aneurysms (Cheng and Wang, 2013).

1.4.2 Post-transcriptional regulation of MMPs

Although MMPs are mainly regulated at the transcriptional level, post-transcriptional gene regulation can be instigated by cytosolic mRNA stability mediated by trans-acting RNA binding proteins that interact with multiple AU-rich elements (ARE) mostly located in their 5'- or 3'-untranslated regions (UTRs), which are potential targets of different UTR-binding proteins with ability to stabilize or destabilize these mRNAs. The expression of MMP-2, MMP-9 and MMP-13 has been shown to be regulated through mRNA stability as demonstrated by researchers who found TGF- β increases MMP-2 and -9 levels by extending the half-life of MMP mRNAs in human gingival fibroblasts and prostate cancer cells (Overall *et al.*, 1991; Hsu *et al.*, 1992), and inducing MMP-13 expression by both inducing transcription and stabilizing the transcript in mouse osteoblasts and fibroblasts (Delany and Canalis, 2001).

1.4.3 Compartmentalization of MMPs

It is essential that cells are unable to indiscriminately release proteases which lead to the unwanted degradation of proteins. *In vitro* studies have demonstrated significant overlap in the substrates MMPs can cleave within the ECM, although substrate selectivity can be honed by enzyme affinity and compartmentalization. (Sternlicht and Werb, 2001). Kinetic studies have shown that specific enzymes degrade some substrates more efficiently than others. One example was demonstrated by Mackay *et al.*, (1990) who established that both MMP-2 and MMP-9 act on cleaved collagen more efficiently than other MMPs. Compartmentalization refers to where and how in the pericellular environment an MMP is released and held and is an essential mechanism for regulating the substrate specificity and efficiency of any proteinase (Ra and Park, 2007). Secreted MMPs require a cellular compartment-specific localization mechanism to function properly. Through

their motifs and modules they are directed to extracellular compartments and are likely to be anchored to cell membranes which targets their catalytic activity by locating and concentrating them close to potential substrates within the pericellular space (Hadler-Olsen *et al.*, 2011). A number of mechanisms have been described to compartmentalize MMPs. In addition to the membrane-bound MMPs, several examples of specific cell-MMP interactions have been reported, such as the binding of MMP-2 to the $\alpha\beta3$ integrin (Brooks *et al.*, 1996) MMP-1 to the $\alpha2\beta1$ integrin (Dumin *et al.*, 2001, Stricker *et al.*, 2001), MMP-9 to CD44 (Yu and Stamenkovic, 2000) and MMP-7 to surface proteoglycans (Yu and Woessner, 2000). All these processes increase the likelihood that proteolysis will be highly regulated and specific.

1.4.4 Activation of ProMMPs

Although transcriptional regulation is essential for MMP production, the third level of control is the extracellular activation of the secreted latent enzymes by cleavage of the propeptide.

The cysteine residue (Cys73) is present within the propeptide domain which functions as a stabilizer of the inactive form by forming a bond with the active Zn^{2+} site in the catalytic domain rendering the enzyme inactive. This mechanism has been referred to as the “cysteine switch” The cysteine residue is replaced by a water molecule which binds to the Zn^{2+} ion converting it from non-catalytic zinc to catalytic zinc and resulting in the intermediate active enzyme following which the pro-domain of the MMP is removed by autolytic cleavage or by other proteases to produce the fully active enzyme (Springman *et al.*, 1990, Van Wart and Birkedal-Hansen, 1990).

1.4.4.1 Chemical Activation of ProMMPs

MMPs can be activated by a number of proteinases *in vivo*, or *in vitro* by non-proteolytic agents such as SH-reactive agents, mercurial compounds, reactive oxygen and denaturants (Nagas and Woessner, 1999). These agents most likely work through the disturbance of the cysteine-zinc interaction of the cysteine switch (Van Wart and Birkedal-Hansen, 1990, Chen *et al.*, 1993). ProMMP-3 activation with a mercurial compound have indicated that the initial cleavage occurs within the propeptide and that this reaction is intramolecular whilst the subsequent removal of the rest of the propeptide is due to intermolecular reaction of the generated intermediates (Okada *et al.*, 1988, Nagase *et al.*, 1990). Interestingly Gu *et al.*, (2002) demonstrated the chemical activation of a proMMP *in vivo* by showing that NO activates proMMP-9 during cerebral ischemia by reacting with the thiol group of the cysteine switch and forming an S-nitrosylated derivative.

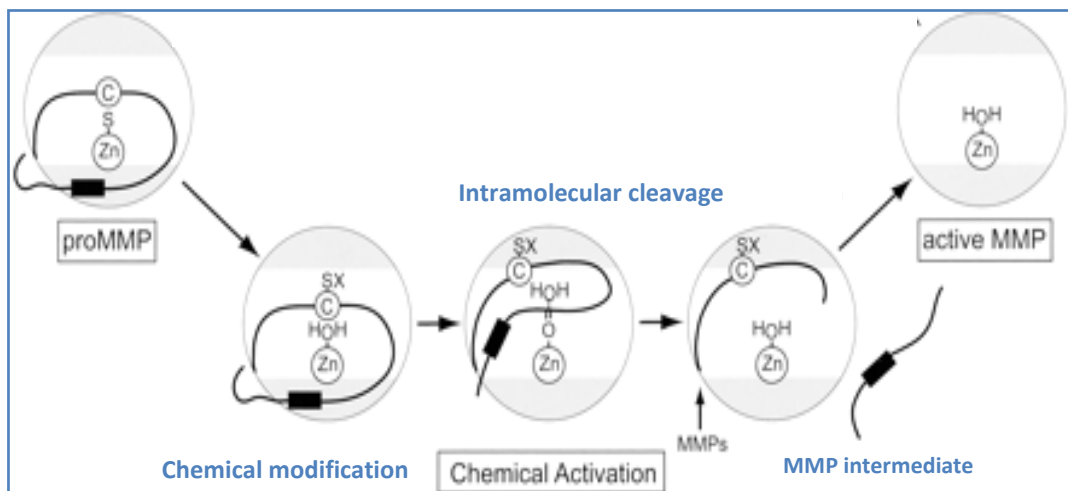


Figure 7 Example of Chemical Activation of a ProMMP

Chemical activation of MMPs relies on modification of the cysteine switch sulfhydryl (SX) resulting in partial activation of the MMP and intramolecular cleavage of the propeptide after which removal of the propeptide by intermolecular processing results in full activity of the MMP. The catalytic domain is depicted as a grey circle, the active-site cleft is shown in white containing the zinc binding site (Zn). The propeptide is represented as a black line containing the bait region (black filled rectangle) and the cysteine switch (C). SH indicates the sulfhydryl of the cysteine (Adapted from Visse and Nigase, 2003).

1.4.4.2 Furin Intracellular Activation of ProMMPs

Generally most proMMPs are secreted from cells and activated extracellularly. However in 1995, Pei and Weiss demonstrated that proMMP-11 (stromelysin 3) is activated intracellularly by the Golgi-associated subtilisin-like serine proteinase furin and secreted as an active enzyme propeptide which is subsequently activated intracellularly before secretion. Later Sato *et al.*, (1996) established that MT1-MMP expressed in *Escherichia coli* was also activated by furin and proposed that all MT-MMPs contained a similar furin recognition sequence RXKR or RRKR and were likely to be activated intracellularly although the precise mechanism was unknown. About a third of MMPs including all membrane-bound MMPs are now known to contain a furin recognition sequence at the C-terminal end (Marchenko and Ratvnikov, 2001, Sternlicht and Werb, 2001, Kang *et al.*, 2002, Illman *et al.*, 2003). As these MMPs are secreted with enzyme activity, regulation by gene expression or endogenous inhibition is vital to control their proteolytic action.

1.4.4.3 Activation of proMMPs by Plasmin

The remaining MMP members are expressed and secreted as inactive pro-forms, which need to be activated. Proteolytic activation of MMPs is stepwise in many cases and the first step usually involves proteinases such as plasmin, trypsin, furin, chymase, elastase or kallikrein, of which plasmin appears to be the major physiological activator via the cysteine switch *in vivo* (Wart and Hansen-Birkedal, 1990, Murphy *et al.*, 1994). At the cell surface, plasmin is derived from plasminogen by the action of urokinase plasminogen activators (uPAs) and tissue plasminogen activators (tPAs) which are both membrane-associated thereby creating localized proMMP activation and subsequent ECM turnover. Plasmin is reported to activate proMMP-1(interstitial collagenase), proMMP-3 (stromelysin 1), proMMP-7 (matrilysin), proMMP-9 (gelatinase B), proMMP-10 (stromelysin 2) and

proMMP-13 (collagenase 3) and subsequently some may participate in processing other MMPs (Eeckhout and Vaes, 1977, HE *et al.*, 1989, Lijnen., 2001). Activation of proMMP-2 is different, in being a two-step process involving hydrolysis of MT1-MMP and generating a 64 kDa intermediate which is then activated by plasmin (Baramova *et al.*, 1997).

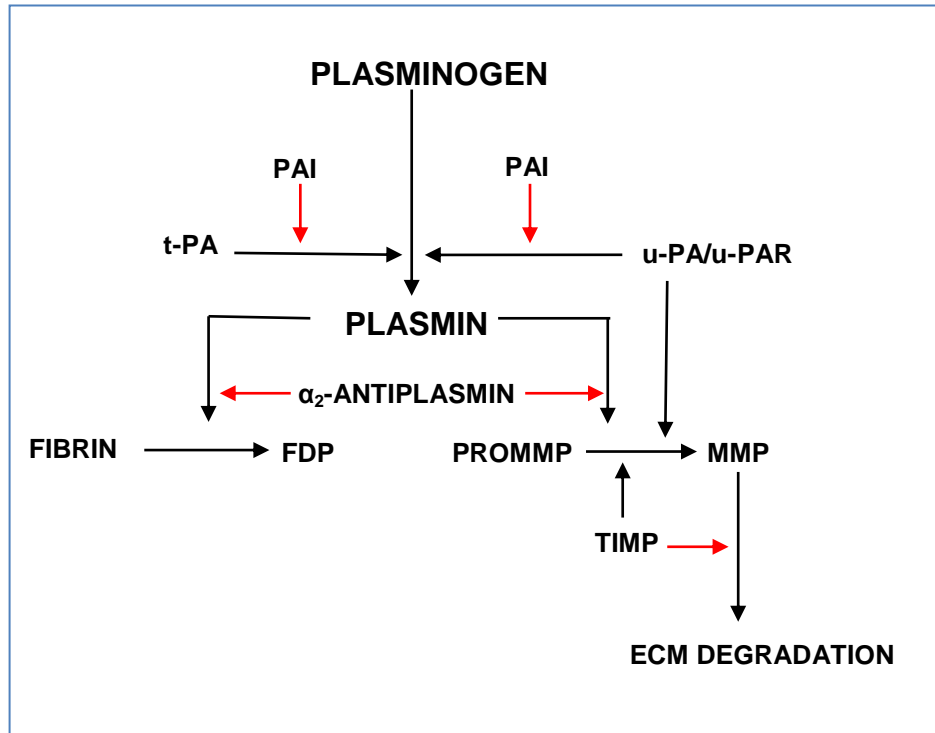


Figure 8 Plasminogen Activation

Tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) bound to its cellular receptor (u-PAR) converts plasminogen to the active enzyme plasmin. Plasmin's primary role is to convert fibrin in blood clots to soluble degradation products (FDP) but in addition to this has the ability to convert some proMMPs into active MMPs resulting in ECM degradation. The plasminogen activators t-PA and u-PA are inhibited by plasminogen activator inhibitors (PAI) and plasmin is mostly inhibited by α_2 -antiplasmin while MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs).

1.4.4.4 Activation of proMMPs by MMPs

As well as the plasminogen system a number of MMPs can cleave the prodomain of other MMP zymogens leading to activation. In 1994, Sato *et al* cloned a membrane-type MMP (MT1-MMP) and identified it as an activator of latent MMP-2.

Since, at least six MT-MMPs have been cloned and demonstrated to activate other MMPs including MT1-MMP, MT2-MMP (Butler *et al.*, 1997, Takino *et al.*, 1997, MT3-MMP (Takino *et al.*, 1995, Zhao *et al.*, 2004), MT5-MMP (Llano *et al.*, 1999, Pei, 1999) and MT6-MMP (Velasco *et al.*, 2000). Knauper *et al.*,(1996) also demonstrated that MMP-2 together with MT1-MMP were able to process human procollagenase-3 (MMP-13) to generate the fully active enzyme via an activation cascade mechanism. MT1-MMP-mediated activation of proMMP-2 has been studied the most extensively and is unique in the fact that TIMP-2 is crucial for MMP-2 activity (Wang *et al.*, 2000) unlike proMMP-2 activation by MT2-MMP which is direct and independent of TIMP-2 (Morrison *et al.*, 2001).

1.4.4.4.1 Cell Surface Activation of ProMMP-2 by MT1-MMP

ProMMP-2 forms a tight complex with TIMP-2 (Howard and Banda, 1991) through their C-terminal domains allowing the N-terminal inhibitory domain of TIMP-2 to bind to MT1-MMP on the cell surface which is subsequently activated by MT1-MMP that is free of TIMP-2 thus generating an intermediate form that is further processed to the fully activated form (Strongin *et al.*, 1995, Kinoshita *et al.*, 1998).

Alternatively, MT1-MMP inhibited by TIMP-2 can act as a “receptor” of proMMP-2. This MT1-MMP–TIMP-2–proMMP-2 complex is then presented to an adjacent free MT1-MMP for activation (Strongin *et al.*, 1995, Butler *et al.*, 1998, Cao *et al.*, 1995, Morgunova *et al.* , 1999). Research has shown

that TIMP-2 promotes pro-MMP-2 activation by MT1-MMP through this trimolecular complex formed from pro-MMP-2, TIMP-2 and MT1-MMP, although excess amounts of TIMP-2 inhibit activation by blocking MT1-MMP activity. Therefore *in vivo*, efficient pro-MMP-2 activation may be dependent on the balance between the amounts of MT1-MMP and TIMP-2 (English *et al.*, 2006).

Although proMMP-2 activation was expected to occur only at low TIMP-2 concentrations relative to MT1-MMP, a study by Kudo *et al.*, (2007) showed that proMMP-2 was activated by MT1-MMP which was mostly saturated with TIMP-2, and as a result TIMP-2 appeared to inhibit cleavage of other direct MT1-MMP substrates even at the level that induces proMMP-2 activation. In light of these results they speculated that TIMP-2 concentration dictates MT1-MMP substrate choice, direct cleavage of its own substrates or proMMP-2 activation.

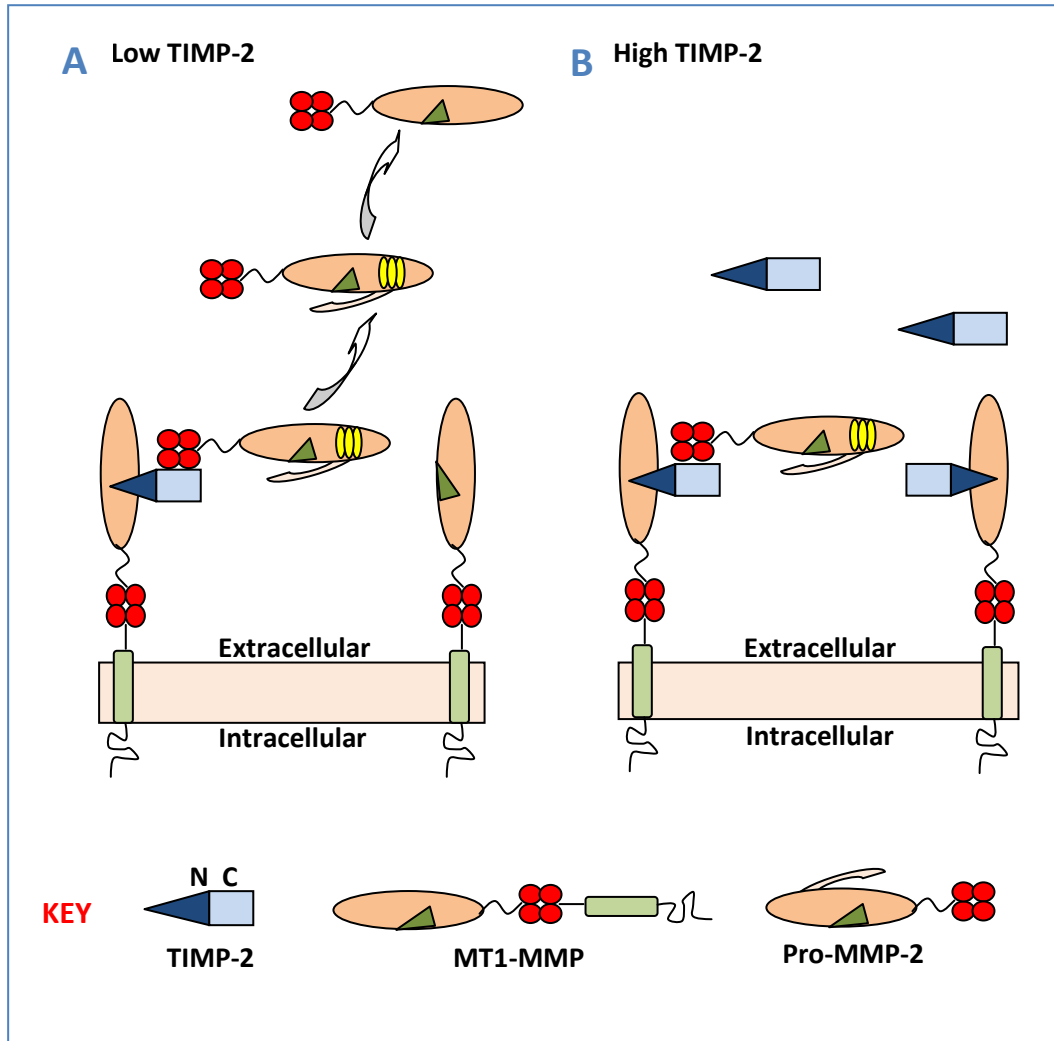


Figure 9 Cell Surface Activation of ProMMP-2

A. MT1-MMP on the cell surface acts as a receptor for TIMP-2 which binds via its N-terminal domain to the active site of the MT1-MMP. This binary complex then acts as a receptor for pro-MMP-2, with the TIMP-2 free MT1-MMP molecule in close proximity can then cleave the propeptide of pro-MMP-2, generating an intermediate species. Further proteolysis of the propeptide through an autocatalytic mechanism results in the generation of the fully active enzyme. Activation of pro-MMP-2 is only possible if the TIMP-2 concentration is low, with sufficient TIMP-2 to generate the trimeric complex but not enough to saturate all the MT1-MMPs required for proteolysis of the propeptide.

B. If high levels of TIMP-2 are present, TIMP-2 will bind to and inhibit all MT1-MMP activity, preventing pro-MMP-2 activation (Adapted from Lafleur *et al.*, 2003).

More recently Otto *et al.*, (2016) looked at aortic stiffness in cardiovascular disease using a co-culture model of rat aortic endothelial cells and smooth muscle cells stimulated with Ang II which resulted in activation of latent pro-MT1- MMP and pro-MMP-2 exclusively in SMCs when co-cultured with ECs. They concluded that the endothelium under ANGII stimulation acts as a novel and key activator of latent pro-MT1- MMP and pro-MMP2 in SMCs of rat aorta and therefore the endothelium may critically contribute to the pathophysiology of aortic stiffness which is also a factor in AAA.

1.4.5 Endogenous Inhibition of MMPs

In normal healthy tissues MMPs are tightly regulated by naturally occurring inhibitors of which TIMPs (tissue inhibitor of metalloproteinases) are the dominant source while α_2 -macroglobulin and tissue-factor pathway inhibitor-2 (TFPI-2) and calcium-binding proteoglycans inhibit MMPs to a lesser extent. Disruption of the balance between the production of active enzymes and their inhibition may result in diseases such as arthritis, atherosclerosis, tumour growth and metastasis.

1.4.5.1 Tissue-Factor Pathway Inhibitor-2

TFPI-2 is a 32 kDa matrix-associated Kunitz-type serine proteinase inhibitor consisting of a short amino-terminal region with three tandem Kunitz-type domains and a positively charged carboxy-terminal tail (Chand *et al.*, 2005). Research has shown TFPI-2 can diminish the proteolytic activity of MMP-1, MMP-2, MMP-9 and MMP-13 (Herman *et al.*, 2001). TFPI-2 can also weakly inhibit the coagulation proteins factor Xa and factor VIIa/TF complex in addition to its established targets the serine proteins kallikrein, trypsin, chymotrypsin, and plasmin, (Sprecher *et al.*, 1994, Petersen *et al.*, 1996 Rao *et al.*, 1999).

1.4.5.2 Calcium-binding Proteoglycans

The testicans form a subgroup within the BM-40/SPARC/osteonectin family of modular extracellular proteins (Hartmann and Maurer., 2001) and are composed of highly conserved, extracellular, calcium-binding, sulfate proteoglycans. The Testican family is composed of highly conserved, extracellular, calcium-binding, sulfate proteoglycans. Testicans are detected in a variety of tissues and family members include Testican-1, Testican-2, Testican-3 and an amino-terminal splice variant of Testican-3, designated NTes. Most Testicans are able to inhibit MT-MMPs thereby inhibiting the activity of pro-MMP-2 as shown by Nakada *et al.*, (2001) who demonstrated that N-Tes, testican-1 and testican-3 but not testican-2 are able to inhibit MT1- or MT3-MMP mediated proMMP-2.

1.4.5.3 α_2 -Macroglobulin

Blood plasma derived α_2 -macroglobulin (α_2 M) is a 772 kDa protein comprised of four nearly identical subunits bound together by -S-S- bonds. α_2 M is a broad spectrum proteinase inhibitor of tissue fluids and blood. and able to inactivate a variety of proteinases including serine, cysteine, aspartic acid and MMPs. It is synthesised mainly in the liver by hepatocytes but other cell types such as macrophages are also believed to produce α_2 M. Whilst α_2 M is considered to be the main inhibitor of MMPs in serum/tissue fluids, the major tissue inhibitors of active MMPs are thought to be the TIMP family where they may act more locally (Cawston and Mercer, 1986, 1987). α_2 -Macroglobulin inhibits endopeptidases by cleaving one or more bonds in the 'bait' region initiating a conformational change that leads to entrapment of the whole enzyme which becomes covalently anchored by transacylation, preventing interaction with its natural substrates. The α -macroglobulin/proteinase complex is then quickly cleared by low density lipoprotein receptor-related protein (LDL-RP) mediated endocytosis (Sotturp-Jensen, 1989, Strickland *et al.*, 1990, Birkedal-Hansen *et al.*, 1993). It is

probable that MMPs are similarly internalised and degraded by the same process as demonstrated by Barmina *et al.*, (1999) who established that MMP-13 associates with the LDL-RP through a specific receptor on osteoblastic cells and Hahn-Dantona *et al.*, (2001) who showed MMP-9 and its TIMP-1 complex could be internalised by binding to LDL-RP. α_2 -Macroglobulin also plays a role in the irreversible clearance of MMPs as macroglobulin/MMP complexes are removed by scavenger receptor-mediated endocytosis in contrast to TIMPs which inhibit MMPs in a reversible manner (Sternlicht and Werb, 2001).

More recently, Satoh *et al.*, (2013) examined potential serum protein biomarkers for aortic aneurysms by comparing protein profiles from pre-surgical and post-surgical sera in AAA patients. Their findings suggested that α_2 -macroglobulin may be a potential serum biomarker for AAA.

1.4.5.4 Tissue Inhibitors of Metalloproteinases (TIMPs)

TIMPs are endogenous inhibitors of MMPs found in most tissues and body fluids and by inhibiting MMP activity are consequently important regulators of ECM turnover, tissue remodelling and cellular behaviour. Within the aortic wall MMPs are able to degrade most proteins of the ECM but are counteracted by TIMPs which inhibit MMP activity and restrict ECM breakdown and thus play an important role in maintaining the integrity of the healthy aorta. The balance between MMP and TIMP activities is involved in both normal and pathological events such as AAA, wound healing, tissue remodelling, angiogenesis, tumorigenesis and metastasis (Gomez *et al.*, 1997) In addition to their primary role of MMP inhibition, recent studies have suggested that TIMPs themselves may have roles independent of MMPs that include direct modulation of cell growth and differentiation (Lambert *et al.*, 2004).

Currently four members of the TIMP family have been identified in humans and are designated TIMP-1, 2, 3 and 4 (Brew *et al.*, 2000). They are

homologous proteins of 21–29 kDa in size and each TIMP molecule consists of around 190 amino acids composed of two distinct domains, a larger N-terminal and a smaller C-terminal domain, each stabilised by three conserved disulfide bonds. The N-terminal domain containing the conserved VIRAK amino acid consensus sequence is able to inhibit MMPs by chelating their catalytic zinc atom whilst the function of the C-terminal domain is not fully understood, but it has been shown that it can bind tightly to the haemopexin domain of latent MMPs (Williamson *et al.*, 1990, Murphy *et al.*, 1991, Nagase, 2002).

In general most mesenchymal and epidermal cells are able to produce TIMPs but additionally TIMP-1, 2, and 3 are also produced by white blood cells (Rowe *et al.*, 1997). TIMPs form non-covalent 1:1 stoichiometric complexes with MMPs most of which can be inhibited by all four TIMPs, although differences in binding affinity have been reported (Olson *et al.*, 1997) and membrane-type (MT) MMPs form a distinct group since they are bound to the cell membrane and are rarely inhibited by TIMP-1 (English *et al.*, 2001, Baker *et al.*, 2002).

X-ray crystallographic studies have shown that TIMP-1 and 2 molecules have an elongated wedge-like shape (consisting of the N-terminal and the C-terminal halves of the polypeptide chains opposing each other) that fits into the entire length of the active-site cleft of an MMP just like a substrate molecule. Subsequently the conserved cysteine in the N-terminal domain will chelate the active-zinc site and expel the water molecule, thereby inactivating the MMP protein (Gomis-Rüth *et al.*, 1997). Next, the N-terminal domain is folded into a five-stranded β -pleated sheet rolled into a β -barrel common to the oligosaccharide/oligonucleotide binding-fold (O-B fold) found in certain DNA-binding proteins (Williamson *et al.*, 1994). TIMP-1 is a 25 kDa secreted polypeptide that is capable of inhibiting most MMPs, whereas TIMP-2, a 21 kDa nonglycosylated protein is predominantly active against MMP-2 although it also appears more effective than TIMP-1 at inhibiting MMP-9. TIMP-1 and TIMP-3 expression is inducible, whereas TIMP-2 expression is largely constitutive. Often the production of TIMPs is

elevated by the same stimuli that promote MMP expression (Thompson *et al.*, 2002). TIMP-1 and TIMP-2 are not only able to bind to the active forms of MMPs but TIMP-1 can bind to proMMP-9 and TIMP-2 to proMMP-1 and 2, inhibiting the intramolecular autoactivation of these proenzymes (Howard *et al.*, 1991, De Clerck *et al.*, 1991, Okada *et al.*, 1992). Another important feature of TIMP-2 is its role in the activation of proMMP-2 by MT1-MMP which was described earlier in section 1.4.4.4.1 and Figure 9.

TIMP-4 inhibits MMP-1, MMP-3, MMP-7 and MMP-9, showing a particular interaction with MMP-2. TIMP-4 transcripts appear abundant in the human heart but occur only at low levels in other organs (Liu., 1997). In mice, TIMP-4 mRNA was restricted to neural tissue, Sertoli cells, ovaries, cardiac, breast and skeletal muscle tissues which also applied in part to humans (Young *et al.*, 2002, Lambert *et al.*, 2004).TIMP-3 appears to be unique in that it exists primarily in association with the extracellular matrix (Gomez *et al.*, 1997), its ability to form complexes with sulphated glycosaminogens (Yu *et al.*, 2000) and its inhibitory action against other metalloendopeptidases such as the ADAM family (Amour *et al.*, 1998, Loechel *et al.*, 2000, Kashiwagi *et al.*, 2001). Presently there is no evidence that TIMPs are able to inhibit aspartic, serine, and cysteine proteinases (Uitto *et al.*, 2003).

Table 3 Summary of Common Features of TIMPs

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Amino acid residues	184	194	188	194
Protein kDa	28	22	24/27	22
Glycosylation sites	2	0	1	0
Protein localization	Soluble/cell surface	Soluble/cell surface	ECM/cell surface	Soluble/cell surface
Synapsin gene	<i>SYN1</i>	N/A	<i>SYN3</i>	<i>SYN2</i>
Pro-MMP binding	ProMMP-9	ProMMP-2	ProMMP-2/9	ProMMP-2
MMP inhibition	Weak for MMP-14 -16 -19 and -24	All	All	Most
Binding partners	CD63, LRP1/MMP9	$\alpha3\beta1$integrin LRP1	EFEMP1, AngIIIR, VEGFR	
ADAM inhibition	ADAM10	ADAM12	ADAM10, ADAM12, ADAM17, ADAM19, ADAM33, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS2 (weak)	ADAM17, ADAM28

Adapted from Baker *et al.*, 2002

Chapter 2. Introduction: Vascular Pathology

2. Introduction: Vascular Pathology

2.1 The Abdominal Aortic Aneurysm (AAA)

An abdominal aortic aneurysm (AAA) is a swelling (aneurysm) of the aorta which is the main artery that leads away from the heart, through the abdomen to the rest of the body. The abdominal aorta is the largest blood vessel in the body and is usually around 2cm wide. An abdominal aortic aneurysm is defined as permanent and irreversible localised dilation of the aorta involving all three layers of the aortic wall that exceeds the normal diameter by 50% or greater than 3 cm in total (Johnston *et al.*, 1991).

Abdominal aortic aneurysm (AAA) represents a common degenerative disease of the aortic wall with a life-threatening risk of rupture and is generally associated with old age. The most common location of arterial aneurysm formation is the abdominal aorta, specifically the segment of the abdominal aorta below the renal arteries ending above the bifurcation of the iliac arteries and are termed infrarenal abdominal aneurysms (IAAA) and typically exhibit a fusiform morphology with symmetrical circumferential enlargement of the wall or less frequently a saccular form affecting only part of the aortic circumference. Although most aortic aneurysms occur in the abdominal aorta, aneurysmal degeneration that occurs in the thoracic aorta is termed a thoracic aortic aneurysm (TAA). However, this study will only focus on AAAs and not TAAs for several reasons, including different anatomical locations and differing aetiologies which are explained more fully in the discussion section.

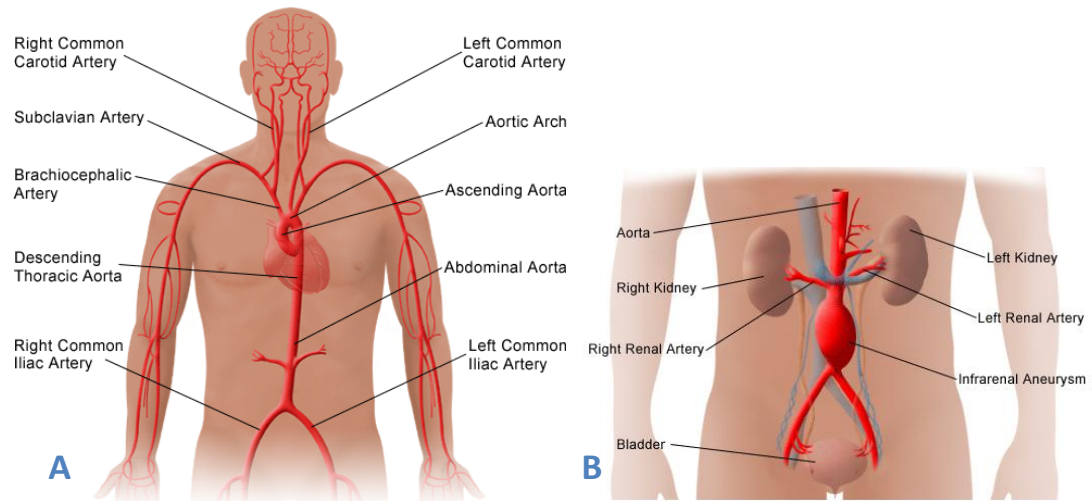


Figure 10 Diagrams of the Normal Anatomy of the Aorta and Location of an Infrarenal Abdominal Aortic Aneurysm

A: The aorta extends upward from the top of the left ventricle of the heart in the chest area (ascending thoracic aorta), then curves (aortic arch) downward through the chest area (descending thoracic aorta) into the abdomen (abdominal aorta). The aorta delivers oxygenated blood pumped from the left side of the heart to the rest of the body. **B:** The location of a typical infrarenal abdominal aneurysm.

2.1.1 Aetiology of Abdominal Aortic Aneurysms (AAAs)

AAA formation is not well understood and is a complex multifactorial condition involving genetic and environmental factors as well as mechanical influences such as turbulent blood flow and hypertension. AAA is characterized by destruction of elastin and collagen in the media and adventitia, depletion of medial smooth muscle cells with thinning of the vessel wall, and transmural infiltration of lymphocytes and macrophages. Atherosclerosis is often a common underlying feature of aneurysms and is considered a risk factor along with hypertension, cigarette smoking, advanced age, genetic predisposition and male gender.

2.1.2 Prevalence of AAA in UK

Screening studies in the UK have estimated a prevalence of 1.3 - 12.7% depending on the age group studied. The incidence of AAA in men is approximately 25 per 100,000 at age 50 increasing to 78 per 100,000 in those older than 70 years. (NICE Technology Appraisal Guidance., 2009). Men are six times more likely to be diagnosed with AAA than women and that likelihood increases with age and other risk factors such as smoking, hypertension or having a first degree relative affected by AAA. During 2016 the NHS Screening Programme also stated that 54 out of every 10,000 men screened will eventually have surgery to repair an aneurysm (NHS AAA Screening Programme, 2016). Interestingly the incidence of ruptured AAA rose from the 1970s to 2000 but now appears to be declining which may be attributed to changes in smoking habits and increases in elective AAA repair in those aged 75 years and over (Anjum *et al.*, 2012).

The NHS AAA Screening Programme (NAAASP) screened more than 260,000 men (65 years of age or over) in the UK during 2013/14 and detected nearly 3,700 aneurysms of 3cm or larger which resulted in 491 elective AAA repairs. (<http://aaa.screening.nhs.uk>).

2.1.3 Summary of Histological Features of AAA

Table 4 Major Histological Features of AAA

Inflammatory infiltrate	Koch,1990, Freestone,1995
Extensive extracellular matrix remodelling	Golledge, 2008
Aortic advential thickening and neovascularisation	Holmes,1995 Thompson,1996
Thickening of the tunica intima	Holmes,1995 Thompson,1996
Fragmentation/decrease in aortic medial elastin	Campa,1987, Baxter,1992
Medial neovascularisation	Holmes, 1995
Fragmented internal elastic lamina	Morimoto, 1993
Intraluminal thrombus	Harter,1982
SMC reduction/apoptosis	Lopez-Candales,1997

2.1.4 Infrarenal Adominal aortic Aneurysm

The propensity for aneurysms to develop in this part of the aorta is multifactorial. Firstly, the number of elastic lamellae is greatest in the ascending aorta, containing approximately 55 to 60 elastic lamellae (elastic fibres and interspersed SMCs constitute a 'lamellar unit'), which gradually decrease in number across the descending thoracic aorta down the length of the aorta to reach approximately 26 at the aortic bifurcation (Wolinsky and Glagov,1967). Despite this regional variation in medial thickness, there appears to be a relatively consistent relationship between the number of elastic lamellae and the estimated hemodynamic stress typically placed on the vessel wall. This relationship is maintained in a number of mammals

however, it is particularly notable that the human, infrarenal aorta represents one of the few exceptions to this rule, because it contains fewer elastic lamellae than would otherwise be predicted for its estimated haemodynamic load, therefore the elastin content is markedly decreased resulting in this segment becoming thinner and stiffer (Wolinsky and Glagov, 1969). Additionally, with increasing age there is a corresponding decrease and degeneration of the elastic lamellae due to elastin synthesis essentially ceasing in the adult aorta, and partial replacement with collagen resulting in the some of the histological changes listed in Table 4.

Secondly, the oxygen and nutrient supply to the aortic media is generally derived by simple diffusion from the lumen in segments that contain up to approximately 29 elastic lamellae (Wolinsky and Glagov, 1967). Aortic segments from humans and other mammals that contain a greater number of elastic lamellae require an additional nutrient supply which provided by *vasa vasora* that extend from the adventitia to the outer layers of the elastic media. The human thoracic aorta contains a rich *vasa vasorum*, however the infrarenal aorta is normally devoid of medial *vasa vasorum* and as the infrarenal aorta may contain more than 29 elastic lamellae, the lack of *vasa vasorum* in this region represents another unique feature of this aneurysm-prone segment (Zarins *et al.*, 2001).

Another consideration is localization of increased haemodynamic load in the infrarenal aorta due to the distal tapering and progressive stiffening (due to decreased elastin) in this region as well the additive effects of retrograde pressure waves that reflect from the iliac bifurcation combined with incoming antegrade pressure waves (Dobrin, 1989 and Newman, 1973). Overall this culminates in the infrarenal aorta having a lower capacity to withstand the haemodynamic demands placed on it and hence the predisposition for AAAs to occur in this region of the aorta.

2.1.5 Genetic Associations of AAA

Clustering of AAA in families, raising the possibility of an inherited genetic predisposition, was reported as early as 1977 (Clifton, M.A). Numerous studies since that time have analysed possible candidate genes, including matrix metalloproteinases, and these are summarised in Table 5.

Table 5 Most Significant AAA Associated Candidate Genes

Candidate gene	Functional class	Polymorphism	Reference
<i>SORT1</i>	Lipid metabolism	rs599839	Jones <i>et al.</i> , 2013
<i>IL6R</i>	Inflammation	rs7529229	Harrison <i>et al.</i> , 2012
<i>LPA</i>	Lipid metabolism	rs10455872 rs3798220	Helgadottir <i>et al</i> 2012
<i>AGTR1</i>	Renin-angiotensin system	rs5186	Jones <i>et al.</i> , 2008
<i>TGFBR2</i>	TGFB signalling	rs1036095	Biros <i>et al.</i> , 2011
<i>TGFBR2</i>	TGFB signalling	rs764522	Biros <i>et al.</i> , 2011
<i>ACE</i>	Renin-angiotensin system	rs4646994	McColgan <i>et el.</i> ,2009
<i>MMP3</i>	Degradation of ECM	rs3025058	Morris <i>et al.</i> , 2014
<i>MMP13</i>	Degradation of ECM	rs2252070	Saracini <i>et al.</i> , 2012
<i>MTHFD1</i>	Methionine metabolism	rs8003379	Giusti <i>et al.</i> , 2008
<i>MTRR</i>	Methionine metabolism	rs326118	Giusti <i>et al.</i> , 2008
<i>LRP5</i>	Lipid metabolism	rs3781590 rs4988300	Galora <i>et al.</i> , 2013

Human leukocyte antigen (HLA) alleles have also been suggested to act as genetic risk factors for AAA and studies involving the HLA-DR B1 locus found that the B1*02 and B1*04 subtypes were more prevalent in the aneurysm group compared to a control group, although the precise mechanism by which the HLA genotype affects susceptibility is unknown

(Rasmussen *et al.*, 2001). Increased frequency of the HLA-DR2 allele was described by Hirose *et al.*, (1998, 1999) who suggested this might be a genetic risk factor for AAA, as opposed to HLA-DQ3 which appeared to have a protective effect.

As technology has improved, genome-wide studies have identified certain chromosomal regions (susceptibility loci) of the human genome contributing to genetic risk for AAA (Krishna *et al.*, 2010, Hinterseher *et al.*, 2011). Within these areas, over 1800 target genes have been identified including MMP-9 (Pahl *et al.*, 2012).

Whilst there is vast evidence of genetic factors in the development of familial AAA, as yet no clear pattern of inheritance has been discovered. Many questions remain unanswered although it seems likely to be a complex interplay between genetic predisposition and environmental exposure; future studies may define subgroups of patients at risk.

2.1.6 Autoimmunity in AAA

In nonspecific AAAs infiltration of monocytes, macrophages, B-lymphocytes, plasma cells and T-lymphocytes (including both CD4 and CD8 T-cells) are commonly observed as well as the presence of Russell bodies (aggregates of immunoglobulin) in the adventitia and outer media, leading researchers to investigate the role of autoimmunity in the pathogenesis of AAA (Koch *et al.*, 1990). Using immunoglobulin G isolated from human aneurysmal tissue as the primary antibody in Western blot analysis of aortic tissue extracts, Gregory and colleagues (1996) found antibodies with specific reactivity against two major protein bands migrating at 40 kDa and 80 kDa (a dimeric form of the predominant 40-kDa species). This initial putative aortic autoantigen was designated aortic aneurysm associated protein-40 (AAP-40) and found to co-localize with elastin-associated microfibrils. Additional studies indicated that AAP-40 has a high degree of sequence homology to microfibril-associated proteins (MAGP), an important component of the

elastin-associated microfibrils, fibrinogen beta and vitronectin (Xia *et al.*, 1996, Hirose *et al.*, 1998). Furthermore, using immunohistochemistry an 80 kDa collagen-associated protein was observed in the adventitial connective tissue and is suggested as another target of an autoimmune response in AAA (Chew *et al.*, 2003). Immunoreactivity against autoimmune proteins that co-localize with structural cytoskeletal components could contribute towards the pathological features of AAA.

Molecular mimicry defined as the sharing of common antigenic epitope(s) between a microorganism (bacteria or viruses) and self (host) (Oleszak *et al.*, 2004) is another mechanism which may be responsible for the generation of autoimmunity in AAA. Microorganisms such as *Chlamydia pneumoniae*, *Treponema pallidum*, herpes simplex virus and cytomegalovirus all share variable sequence homology with epitopes of AAAP-40 thereby triggering the autoimmune response (Ozsvath *et al.*, 1996., Halme *et al.*, 1999).

Certain human leukocyte antigen (HLA) subtypes are associated with chronic inflammatory and autoimmune diseases such as rheumatoid arthritis and have been studied in relation to aortic aneurysms. The immune response within the aortic wall is regulated by the HLA system and expression of certain alleles has been associated with susceptibility to autoimmunity in AAA and was discussed earlier in section 2.1.5.

2.1.7 Infectious Agents in AAA

Mounting evidence suggests that infectious agents may play a contributory role in the pathogenesis of atherosclerosis, particularly cytomegalovirus and *Chlamydia pneumoniae* (Chung and Rivera, 1998). Studies by Tanaka and colleagues (1994) have also indicated that chronic low grade infection may be involved in the development of aneurysmal disease after finding evidence of cytomegalovirus (CMV) genomic DNA in 65% of degenerative AAA and in 86% of inflammatory AAA, but in only 31% of normal aortic tissues (Tanaka *et al.*, 1994). Furthermore in IAAA, expression of CMV immediate early mRNA (signifying active infection) was detected in macrophages, fibroblasts

and endothelial cells leading researchers to suggest frequent and active infection of CMV in IAAA plays a significant role in the induction of chronic inflammatory reaction (Yonemitsu., 1996). However, Satta and colleagues (1998) found no evidence for cytomegalovirus in degenerative human AAA tissues thus the evidence to date suggests that cytomegalovirus more likely plays a role in inflammatory AAAs, whereas the role of chronic viral infection in the more common degenerative form of aneurysm disease is open to question.

Chlamydia pneumoniae has also been associated with atherosclerosis as well as being detected in SMCs and macrophages located in AAA lesions. Petersen *et al.*, (1998) using a nested polymerase chain reaction method designed to detect a fragment of the major outer membrane protein gene of *C. pneumoniae*, discovered *C. pneumoniae*-specific DNA in 35% of patients with AAA, but only 5% in non-aneurysmal abdominal aortas. *C. pneumoniae* was also demonstrated in the walls of AAA by Juvonen *et al.*, (1997) using immunohistochemistry and PCR and again later by Karlsson *et al.*, (2000) using the same methods. In 2001 Lindholt and colleagues during serological studies reported a significant correlation between IgG and IgA titres against *C. pneumoniae* and aneurysmal expansion rate, although previously in 1998 the Viborg Study in Denmark had failed to find any correlation (Lindholt *et al.*, (1999). Interestingly, an earlier study by the Lindholt group (1997) had failed to detect any evidence of *C. pneumoniae* in the walls of symptomatic AAA using a nested polymerase chain reaction method. Furthermore, chronic obstructive pulmonary disease (COPD) and its connection with chronic *C. pneumoniae* was also considered a possible risk factor for AAA but Lederle *et al.*, (2000) found no association between COPD and AAA.

The more commonly involved organisms involved in infections of aneurysms are *Staphylococcus aureus* and *Salmonella* species which can be easily identified by culture (Sessa *et al.*, 1997). Less diagnosed is *Coxiella burnetii*, the etiologic agent of Q fever and in 2005 Sessa *et al* reported on three of the known nine cases of this infection in aneurysms, suggesting that

underlying cardiovascular disease such as valve defects or aortic aneurysms are risk factors for Q fever and can lead to severe complications if untreated (Sessa *et al.*, 2005).

The sexually transmitted disease syphilis caused by the spirochetal bacterium *Treponema pallidum* used to be a common risk factor for AAA if left untreated but is now less common due to adequate antibiotic treatment. Mycotic aortic aneurysms are unusual, accounting for less than 1% of aortic aneurysm repairs and are defined as those naturally occurring aortic aneurysms that result from or are secondarily infected by bacteria arising in a distant site of infection. In these cases of mycotic aneurysms of haematogenous origin, local invasion of the intima and media gives rise to abscess formation and aneurysmal dilation of the vessel caused by either gram-negative or more commonly gram-positive organisms and only rarely. More recently in 2008, Nyberg and colleagues investigated 119 patients with infrarenal AAA and 36 matched controls for specific IgG class antibodies against *C. pneumoniae*, *Helicobacter pylori*, Cytomegalovirus, and Herpes simplex virus. The study demonstrated no significant difference in seroprevalence for these infectious pathogens between AAA patients and controls and therefore failed to establish a connection between AAA and infectious burden (Nyberg *et al.*, 2008). Overall the association between infectious agents and the development of AAA remains controversial and unresolved and future work may provide new insights.

2.1.8 Atherosclerosis and AAA

In Section 2.1.1 atherosclerosis was described as an important risk factor and for many years AAA was considered an end-stage manifestation of atherosclerosis, but recently this view has been increasingly questioned and the specific relationship to atherosclerosis remains unknown. For many years AAA has long been considered an end-stage manifestation of atherosclerosis, but in recent years this view has been increasingly

questioned and the specific relationship to atherosclerosis still remains unknown. Most AAAs occur in association with advanced atherosclerosis, and most patients with aneurysm disease exhibit risk factors for atherosclerosis which strongly suggests that atherosclerosis and AAAs are causally linked; however there are potentially important differences in pathophysiology and morphology between the two diseases.

Table 6 Comparison of Features for AAA and Arterial Occlusive Disease (AOD)

FEATURES	AAA	AOD
Age at onset	Approx 10 years later than AOD	Approx 60 years
Gender	Predominantly affects males in a ratio approx 5:1 to females	Both sexes affected equally
Genetics	Family history of AAA is a strong risk factor	Genetic factors represent a risk for atherosclerosis e.g. Familial hypercholesterolemia
Smoking	Risk factors	Risk factors
Hyperlipidaemia	Low risk factor	High risk factor
Hypertension	Significant risk factor	Risk factor
Diabetes mellitus	Negative risk factor	High risk factor
Pathophysiology	Dilation of all layers of artery wall – loss of elastin and SMCs – weakening of adventitial collagen leading to rupture	Predominantly affects subendothelium – migration and proliferation of cells leading to rigidity and stenosis of vessel
Inflammatory response	High proteolytic activity and greater infiltration of inflammatory cells than AOD	Chronic inflammation
TNF-α, IL-1β, IL-6	Higher levels of these cytokines found in AAA tissue in comparison to AOD	

Furthermore, results have been contradictory in some areas of this research, for example Shteinberg's comparison of AAA and AOD found the risk factors male gender and smoking high in both groups with no significant difference (Shteinberg *et al.*, 2000) whereas LaMorte *et al.*, (1995) found male gender a stronger risk factor in AAA than AOD and smoking a stronger risk factor in the AOD group. Louwrens *et al.*, (1993) found AAA patients were older with higher blood pressure than patients with AOD but no difference in tobacco consumption or cholesterol levels contradicting Chan *et al.*, (1996) whose AOD patients were older with higher rates of smoking and no difference was found in regard to hypertension and lipid levels.

Recently, a retrospective study demonstrated the atherosclerotic profiles are significantly different between patients with thoracic aortic aneurysm (TAA) and AAA suggesting mechanisms underlying the development of aortic aneurysms may differ in TAA and AAA (Ito *et al.*, 2008). It is also notable that aneurysms only rarely occur in certain vessels that are commonly affected by occlusive atherosclerosis (such as the carotid and external iliac arteries) and that patients without evidence of atherosclerosis may develop AAA. Although it is difficult to exclude atherosclerosis as a likely causative factor in aneurysm disease it is difficult to define the exact etiologic relationship between the two and some researchers propose that aneurysms are a unique disease entity with localized atherosclerosis representing a nonspecific secondary response, thus the specific pathogenesis of AAA versus AOD remains ill-defined.

2.1.9 Oxidative stress in AAA

A prominent pathological feature of AAA is the inflammatory response during which oxidative stress is invariably increased as well as further contributing to the pathophysiology of inflammation. Oxidative stress can be defined as tissue damage occurring secondary to increased production and/or decreased removal of reactive oxygen species (ROS), the balance of which depends on the activity of ROS-generating systems such as NADPH oxidase

as well as levels of endogenous cellular antioxidants (McCormick *et al.*, 2007).

Oxidative stress is another proposed main player in AAA and the idea that ROS including superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$) and reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$) are associated with the pathologic progression of AAA has long been considered by researchers (Yajima *et al.*, 2002). In 1987, Dubick *et al.* reported that levels of ascorbic acid, Cu, Zn and superoxide dismutase (SOD) activity in tissue samples from patients with AAA and AOD were reduced suggesting a role for oxygen radicals or increased lipid peroxidation in occlusive and aneurysmal disease of the aorta. More recently, Zhang *et al.*, (2003) established the presence of inducible nitric oxide synthase (iNOS) (which catalyzes the production of nitric oxide (NO) in the media and adventitia of AAA patients promoting formation and activity of peroxynitrite and further contributes to oxidative tissue and cellular injury in AAA. Liao and colleagues (2001) also confirmed these findings, that expression of iNOS increased significantly in the aneurysm walls of patients with AAA compared to the healthy controls and concluded SMCs and inflammatory cells were main cellular sources of increased iNOS in AAA. Furthermore NO may regulate the development of AAA by inducing the expression of the extracellular MMP inducer EMMPRIN thereby modulating the activity of MMPs (Johanning *et al.*, 2002, Lizarbe *et al.*, 2009).

The major vascular cells found in the aortic wall, SMCs, fibroblasts and endothelial cells all produce ROS primarily via membrane-associated NADPH oxidases, xanthine oxidase (XO) and the multiple isoforms of nitric oxide synthase (NOS). Xiong *et al.*, (2009) investigated the hypothesis that ROS produced by these enzymes play an important role in aneurysm formation by selectively blockading them in a murine model of AAA. The results show that apocynin (the specific inhibitor of NADPH oxidase) but not oxypurinol (XO inhibitor) suppressed experimental aortic aneurysm formation in the murine model suggesting that NADPH oxidase but not XO contributes to ROS production. Additionally, they found that $CaCl_2$ treated homozygous

iNOS deficient mice were partly resistant to aneurysm induction in comparison to wild type mice, signifying a role for both the NADPH oxidase and iNOS pathways in AAA development (Xiong *et al.*, 2009). Significantly, ROS are reported to activate matrix metalloproteinases (MMPs) (Rajagopalan *et al.*, 1996), to inhibit plasminogen activator inhibitor-1 (PAI-1) and to induce apoptosis of SMCs (Li *et al.*, 1997) as well as augmenting the cycle of inflammation, ultimately resulting in extracellular matrix degradation as seen in AAA.

Recently, Spadaccio *et al.*, (2016) conducted a study which looked at protein oxidation and supported the involvement of oxidative stress in the pathogenesis of AAA. Using a redox proteomic approach they investigated total and specific protein carbonylation and protein-bound 4-hydroxy-2-nonenal (HNE) in the serum of AAA patients and demonstrated increased oxidative damage to protein. Emeto *et al.*, (2016) also highlighted potential treatment strategies targeting ROS in AAA but concluded that none of these were effective in clinical practice.

2.1.10 Chronic inflammation in AAA

In section 2.1.3, inflammatory infiltrate was described as a major histological feature of AAA and additionally can be another factor causing increased expression of MMPs in this disease, therefore inflammation and its mediators will be discussed in the following sections.

The specific factors that initiate chronic inflammation in AAA are undefined but whatever the factors responsible for initiating aneurysmal degeneration, chronic inflammation is one of the common histologic features of established AAAs. The inflammatory response includes cells such as polymorphonuclear neutrophils (PMN), T cells, B cells, macrophages, mast cells and NK cells and is usually transmural in distribution with dense infiltrates largely focused in the outer media and adventitia as well as elevated IgG in the tissues (Freestone *et al.*, 1990, Satta *et al.*, 1998). A subset of lesions described as

"inflammatory AAAs" (accounting for approximately 5% of AAAs) were first described by Walker *et al* in 1972; these aneurysms are characterized by an unusually thick wall with dense retroperitoneal fibrosis extending into periaortic tissues. Patients with the inflammatory variant are younger and usually symptomatic, largely from back or abdominal pain.

The pathogenesis of inflammatory AAA appears to involve an immune response localized to the vessel wall although the etiology of most inflammatory AAA cannot be established (Hellmann *et al.*, 2007). Other research groups have suggested that inflammatory AAAs probably represent the extreme end of the spectrum of the inflammatory process found in all AAAs rather than a separate clinical entity (Witz and Korzets., 2005).

Meher *et al.*, (2014) examined the role of B cells (B1 and B2 subsets) in the development of AAA and found B2 cells in the absence of other B-cell subsets, increase splenic regulatory T-cell population and suppress experimental AAA formation in mice. A study by Wang *et al.*, (2014) established an important role for Immunoglobulin E (IgE) in AAA pathogenesis through the activation of CD4+ T cells, mast cells and macrophages and proposed neutralizing plasma IgE in the treatment of human AAAs.

More recently, studies by Lai *et al.*, (2016) investigated Toll-like receptors (TLRs) which are type I transmembrane proteins that play a key role in various inflammatory responses. Their results suggested TLR4 signalling contributes to AAA formation by promoting the proinflammatory status of VSMCs thereby inducing proteinase release such as MMPs from VSMCs.

2.1.11 Chronic Inflammatory Mediators implicated in AAA

The infiltrating cells secrete various humoral inflammatory factors such as cytokines, chemokines, leukotrienes, ROS and immunoglobulins (Shimizu *et al.*, 2006). Inflammatory mediators of particular relevance to this thesis are described here.

2.1.11.1 Chemokines

Chemokines represent a superfamily of cytokines (divided into at least three structural branches: C, CC and CXC) that function as potent chemoattractants and activators of specific leukocytes, playing an important role in the inflammatory process associated with aortic aneurysms either in the initial stages of the disease or later by amplifying the inflammatory response. In 1993 Koch *et al* demonstrated that the chemotactic molecules, monocyte chemoattractant protein-1 (MCP-1) of the CC subgroup and interleukin-8 (IL-8) of the CXC subgroup are both expressed in human AAA tissue. This was later further confirmed by Middleton *et al.*, (2007) whose results established significantly higher levels of the CXC chemokines IL-8, epithelial neutrophil activating peptide-78 (ENA-78) and growth related oncogene (GRO) and the CC chemokines MCP-1, MCP-2 and RANTES (regulated upon activation, normal T-cell expressed and secreted) in AAA samples compared with controls.

Recently Jones *et al.*, (2016) investigated tissue and plasma samples from AAA patients to determine whether inflammatory cytokines could be used as biomarkers for the presence of AAA. Results showed that Eotaxin and RANTES, belonging to the C-C family of chemokines, were potentially novel independent plasma biomarkers for AAA.

2.1.11.2 Elastin derived peptides (EDPs) hypothesis

It has been proposed that the peptide fragments derived from the degradation of extracellular matrix components, including elastin, laminin and fibronectin may further induce and intensify inflammatory cell recruitment by the production of soluble products with chemotactic activity within the degenerating aortic wall. Subsequently, Hance and colleagues (2002) using *in vitro* chemotaxis assays found that soluble EDPs in AAA tissue can attract mononuclear phagocytes through ligand-receptor interactions with their cell surface receptor the 67-kD EBP. Additionally this AAA-derived chemotactic activity can be eradicated by both competition with Val-Gly-Arg-Pro-Gly (VGVAPG), a repetitive peptide found in human elastin and the presence of lactose which dissociates the 67-kD EBP.

Dale *et al.*, (2016) using a mouse model of AAA looked at the effect of EDPs on macrophage polarization as they are known to release proinflammatory cytokines, such as TNF- α and IL-1 β , additionally TNF- α has been shown to be required for experimental aneurysm formation in mice (Xiong *et al.*, 2009). Results determined that EDPs promoted a proinflammatory environment in aortic tissue by inducing proinflammatory M1 macrophage polarization which led to AAA dilation which was attenuated by neutralization of EDPs.

2.1.11.3 Proinflammatory cytokines

Cytokines are intercellular low molecular weight soluble proteins which function as chemical messengers for regulating the immune system and are important in the inflammatory process. A large number of cytokines have been identified in AAA tissue where they are known to stimulate metalloproteinase expression by a number of cell types within the aneurysm wall (including macrophages, SMCs and fibroblasts) as well as TIMP

reduction and induction of prostaglandin synthesis. In 1992, Pearce *et al.*, first demonstrated increased expression of tumour necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) in aneurysmal tissue and later Szekanecz and colleagues (1994) found a significant increase in levels of interleukin-6 (IL-6) and interferon-gamma (IFN- γ) but not IL-2 and IL-4 in supernatants of AAA explants compared to controls. A further study by Szekanecz *et al.*, (1994) proposed that interleukin (IL)-8 and TNF- α , previously found to be present in AAA tissue may be important for chemotactic activity responsible for endothelial cell migration and therefore might play a role in neovascularization within the aneurysmal wall. More recently Liu *et al.*, (2016) utilized an angiotensin II-induced AAA mouse model to determine the effect of daidzein on AAA. Daidzein, is a phytoestrogen which contains strong anti-inflammatory activity and affects various mechanism pathways including the NF- κ B, p38MAPK and TGF- β 1 pathways. The results demonstrated that in angiotensin II-induced AAA mice, daidzein significantly attenuated incidence of AAA; producing an anti-inflammatory effect by inhibiting TNF- α , IL-1 β and NF- κ B protein expression in addition to suppressing gene expression of MMP-2, COX-2 and TIMP-1.

Unexpectedly, Davis and co-workers (1998) found that in AAA tissue there was a significant increase in the level of IL-10 which is a potent inhibitor of macrophage pro-inflammatory cytokine production and can affect levels of TNF- α , IL-6 and IL-1 β which ultimately may decrease MMP levels and increase TIMP expression. However, pro-inflammatory cytokines were still increased within the AAA walls despite the increase in IL-10, and IL-13 another anti-inflammatory cytokine was undetectable in both AAA samples and controls. Moreover they found levels of TNF- α , IL-6 and IL-1 β increased in AOD in comparison to AAA, which contradicts several other notable studies which found higher levels of these cytokines in AAA than in AOD. More recently, researchers found that IL-6 was one of the most highly secreted cytokines among the 50 pro-inflammatory cytokines analyzed in AAA tissues and further research indicated that the IL-6 pathway is critically involved in the inflammation of AAA (Nishihara *et al.*, 2013). This was

corroborated by Kokje *et al.*, (2016) who tested a role for IL-6 in the initiation and progression of AAA using a murine elastase model. They concluded AAA was characterized by increased IL-6 signalling and that IL-6 appeared multi-faceted, protective upon acute injury but negatively involved in the perpetuation of the disease process.

Table 7 Cytokines Present in the Aortic Wall

Cytokine	Source	Function
Interleukin-1 β (IL-1 β)	Macrophage Endothelial cell Dendritic cells Monocytes VSMCs	Cytokine gene expression Proliferation CD4 ⁺ cells Differentiation of B cells ↑EC adhesion molecules VSMC metabolism
Tumour necrosis factor-alpha (TNF- α)	Macrophage T cells VSMCs	↑EC adhesion molecules T cell activation induces production of cytokines and MHC Class I and II
Interleukin-6 (IL-6)	Monocyte Macrophage VSMCs Endothelial cells Fibroblasts T cells	Stimulates production of B cells/neutrophils Stimulates T cell growth and differentiation
Interleukin-8 (IL-8)	Macrophage T cells Endothelial cells	Lymphocyte activation Potent angiogenic activity Chemoattractant
Monocyte chemo-attractant protein (MCP-1)	Macrophage VSMCs	Chemotactic and activating for monocytes
Interferon-gamma I(FN- γ)	T cells Dendritic cells	Upregulation of MHC Class I and II T and B cell differentiation Ig G secretion Activation of macrophages
Interleukin-10 (IL-10)	Macrophages T cells	Anti-inflammatory Inhibitory factor Downregulates MHC Class II Inc B cell proliferation

2.1.11.4 Prostaglandins (PGs)

Prostanoids are a subclass of eicosanoids consisting of the prostaglandins and thromboxane A₂ which are important mediators in the regulation of cardiovascular functions, including contraction and relaxation of vascular smooth muscle, modulation of inflammatory processes and connective tissue turnover and there is substantial evidence that they are associated with AAA pathogenesis (Dorn *et al.*, 1992). The prostanoid most commonly observed in aneurysmal tissue is prostaglandin E₂ (PGE₂), demonstrated to be 40-fold higher than in normal aortic tissue after synthesis by macrophages and SMCs and is thought to increase the production of MMPs within the vascular wall (Holmes *et al.*, 1997) as well as suppressing vascular SMC proliferation and promoting apoptosis (Walton *et al.*, 1999). PGE₂-metabolism also involves 15-hydroxyprostaglandin-dehydrogenase (15-PGDH) and a study by Solà-Vilà *et al.*, (2015) examined 15-PGDH expression in human AAA and their data showed 15-PGDH is upregulated in AAA and mainly expressed in infiltrating leukocytes.

The production of PGE₂ from arachidonic acid (AA) is catalyzed by the rate-limiting enzyme cyclooxygenase (COX) of which two main isoforms COX-1 and COX-2 have been identified.

2.1.11.5 Cyclooxygenases

Cyclooxygenase-1 (COX-1) is a constitutively expressed enzyme involved in maintaining low levels of prostaglandins (PG) whereas cyclooxygenase-2 (COX-2) is induced in response to cell activators such as growth factors and cytokines suggesting that this enzyme is involved in the generation of PG during inflammation and has emerged as a key regulator for PG synthesis (Herschman *et al.*, 1996). Walton and colleagues (1999) found that aneurysm-infiltrating macrophages consistently stained strongly for

COX-2 in aneurysm biopsies whereas expression of COX-1 in this tissue was minimal. Experimental models have suggested a role for COX-2 in AAA expansion and using rat elastase perfusion models researchers demonstrated treatment with the nonselective COX inhibitor indomethacin reduced AAA growth (Holmes *et al.*, 1996, Miralles *et al.*, 1999). A more recent study by Gitlan *et al.*, (2007) attempted to define the role of COX-2 in AAA. Using COX-2 deficient mice infused with angiotension II to induce aneurysm formation, they showed that, after 28 days of infusion there was a 54% incidence of AAA in COX-2 wild-type mice in comparison to no detection of AAA in the COX-2 deficient mice. Additionally COX-2 and PGE₂ increase both the release and activation of pro-MMP-2 and pro-MMP-9 (Cipollone *et al.*, 2001, Shankavaram *et al.*, 2001).

At present direct evidence to support a role for COX-2 is lacking, but the demonstration of COX-2 in the inflammatory infiltrate, a crucial factor in AAA development appears consistent with COX-2 promotion of AAA as well its association with revascularization, which could also further AAA development (Chapple *et al.*, 2007).

2.1.11.6 Angiotensin II

Angiotensin II (Ang II) derived from the precursor angiotensinogen through sequential cleavage by renin and angiotensin-converting enzyme (ACE) or chymase) (Inoue *et al.*, 2009), is a potent vasoconstrictor that has been implicated in vascular inflammation and atherosclerosis. Animal studies have consistently shown the ability of Ang II during infusion to promote the formation of AAAs although the mechanism of this is presently undefined (Daugherty *et al.*, 2004). Huang *et al.*, (1996) demonstrated that Ang II can alter the integrity of the aortic wall from studies using Brown Norway rats, a strain known to be susceptible to rupture of the internal elastic lamina that was abrogated by administration of ACE inhibitors or angiotensin receptor antagonists. Ang II is also known to stimulate the release of PGs in various

cells and tissues so Ohnaka and colleagues (2000) investigated whether Ang II regulates COX-2 (a key regulator of PG synthesis) expression in cultured rat VSMCs and subsequently discovered Ang II did indeed regulate COX-2 and PG production through p42/44 MAPK and p38 MAPK signalling pathways and mediated cell growth and proliferation of VSMCs.

Additionally Ang II promotes vascular inflammation by ROS production through nuclear factor kappa B (NF- κ B) mediated induction of pro-inflammatory genes and downregulation of peroxisome proliferator-activated receptors (PPARs) a family of transcription factors that modulate vascular inflammation. Furthermore Ang II via activation of NF- κ B increases secretion of IL-6, one of the principal inflammatory cytokines found within the aneurysm wall (Tham *et al.*, 2002).

Recently Wang *et al.*, (2016) demonstrated that baicalein (BAI) an alcohol soluble flavonoid known for its anti-inflammatory effect attenuates the incidence and severity of AAA in Apoe^{-/-} mice infused with AngII by inhibiting inflammatory cell accumulation and AngII-induced activation of MMP-2 and MMP-9 to maintain elastin content *in vivo*. Additionally BAI also blocks the AngII cascade by downregulating angiotensin type 1 receptor (AT1R) and MAPKs.

2.1.11.7 NF- κ B and Ets-1

The NF- κ B transcription factor has a major role in regulating pro-inflammatory genes implicated in atherosclerosis and AAA, including adhesion molecules, cytokines, chemokines, interferons, growth factors and other mediators such as iNOS and COX-2 (Tham *et al.*, 2002). NF- κ B also regulates the transcription of MMP-1, MMP-2, MMP-3 and MMP-9 (Bond *et al.*, 1999, Takeshita *et al.*, 1999, Kim *et al.*, 2000), inhibits transcription of elastin and collagen (Kuang *et al.*, 2002, Kouba *et al.*, 1999) and is important for VSMC proliferation (Bellas *et al.*, 1995). Miyake *et al.*, (2007) demonstrated its importance by successfully inhibiting the progression of

AAA in rat and rabbit models using chimeric decoy oligodeoxynucleotides (ODNs) to inhibit both NF- κ B and Ets-1 simultaneously leading to the inhibition of a number of MMPs which play a role in human AAA.

Ets-1, the founding member of the Ets oncogene family is another transcription factor that mediates vascular inflammation and remodelling and is induced in VSMCs in response to a variety of inflammatory factors (Hultgardh-Nilsson *et al.*, 1996). The induction of the chemokine MCP-1, the adhesion molecule vascular cell adhesion molecule-1 (VCAM-1), and plasminogen activator inhibitor-1 (PAI-1) by Ang II is largely dependent on Ets-1 (Zhan *et al.*, 2005). Ni *et al.*, (2007) identified a novel role for Ets-1 as a transcriptional mediator of Ang-mediated ROS generation by regulating the expression of NAD(P)H oxidase subunits such as p47^{phox}. Although the role of Ets-1 in the development of AAA has not yet been clarified, Ets-1 is an important regulator of several MMPs including MMP-2 and MMP-9 and therefore plays a substantial role in extracellular matrix turnover, is essential for angiogenesis and is involved in vascular inflammation which are all significant factors in AAA.

2.2 Proteolytic Breakdown of the Aortic Wall

One of the major pathophysiological features of AAA is considered to be the destruction of the medial elastin and collagen degradation by increased proteolysis within the aortic wall and it is now well established that among the numerous potential proteinases MMPs (with special emphasis on MMP-2 and MMP-9) play the principal role in aneurysm formation (Vine and Powell, 1991, Thompson and Parks, 1996). Other enzymes that that may participate in degeneration of connective tissue include plasmin, the plasminogen activators urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) (Reilly *et al.*, 1994), their inhibitor plasminogen activator inhibitor-1 (PAI-1) (Allaire *et al.*, 1998), serine elastases (Wassef *et al.*,

2001) and cysteine proteases particularly cathepsins S, K and L (Sukhova and Shi, 2006). Serine proteases have been hypothesized to play a role in AAA due to their potent elastase activity but currently there is no evidence to support this except for those from the plasminogen activator family which are known to activate MMPs. Cysteine proteases also appear to play a role in AAA formation. including cathepsins a class of lysosomal proteases which have been found to be overexpressed in human AAAs compared to normal aortic tissue. The protease activity of the cathepsins is normally balanced by endogenous inhibitors such as cystatin C which is normally constitutively expressed but has been found to be significantly decreased in tissue from human AAAs (Shi *et al.*, 1999).

Impaired elastogenesis by adult VSMCs limits regenerative repair of elastic fibres but Swaminathan *et al.*, (2014, 2016) recently demonstrated significant elastogenesis by bone marrow mesenchymal stem cell-derived SMCs (BM-SMCs) and their pro-elastogenesis and antiproteolytic effects on rat aneurysmal SMCs. Following on from the earlier study they are currently investigating the effects of super paramagnetic iron oxide nanoparticle (SPION) labelling of BM-SMCs necessary to magnetically guide them to the AAA wall. Results indicate the SPION labelling is noncytotoxic and does not change the ability of the BM-SMCs to stimulate elastin regeneration and attenuate proteolytic activity and may enable future *in situ* AAA regenerative repair.

2.2.1 Role of MMPs in AAA

The structure and function of MMPs has been discussed earlier in Chapter 1 in relation to their natural physiological role within the ECM and therefore their role in the pathogenesis of AAA will be examined in this section.

2.2.1.1 Gelatinases

MMP-2 and MMP-9 both have elastolytic and collagenolytic properties and appear to play a particularly critical role in AAA formation. MMP-2 is constitutively produced and expressed in small aneurysmal aortas and therefore is thought to be significant in the initiation of abdominal aortic aneurysms (AAA) whilst the inducible MMP-9 is expressed at higher levels in large aneurysms has a significant role in aneurysm expansion and rupture (Freestone *et al*, 1995). Sakalihan *et al.*, (1996) found activated MMP-9 and MMP-2 levels were raised in aneurysmal aortic walls. Davis *et al.*, (1998) reported that MMP-2 (mRNA) and protein levels were significantly higher in AAAs than in controls whilst Yamashita *et al.*, (2001) additionally demonstrated levels of both MMP-2 and MMP-9 mRNA were increased in the AAA group compared with normal subjects and that the gelatinolytic activity of both MMPs was elevated in all AAA tissues examined. McMillan *et al.*, (1997) measured aneurysmal MMP-9 mRNA levels using RT-PCR to define the relationship between AAA size and MMP-9 expression. Results demonstrated that MMP-9 mRNA expression was significantly higher in moderate-diameter AAAs (5.0cm to 6.9 cm) than either small (<4.0 cm) or large (>7.0 cm) AAAs and led researchers to summarise that increased MMP-9 expression may account for the propensity of AAAs to continue to expand in contrast to smaller aneurysms. (McMillan *et al.*,1997). Later, immunohistochemical techniques were used by Papalambros and colleagues (2003) to investigate the expression of MMP-2 and -9 in AAA tissues. Findings revealed MMP-9 levels were significantly higher in large AAAs but there that was no relation between AAA size and levels of MMP-2, leading them to propose that immunohistochemical expression of MMP-9 in AAA is correlated to the size of the aneurysm. The role of MMP-9 on AAA formation has also been studied using MMP-9 deficient mice where MMP-9 deficiency attenuated elastase-induced AAAs and reduced calcium chloride-induced AAAs in mice (Pyo *et al.*, 2000, Longo *et al.*, 2002). Interestingly, infusing macrophages from wild-type mice reversed the protection of MMP-9 deficiency in the calcium chloride model of AAA indicating that macrophage-

derived MMP-9 is required for AAA development. By contrast, infusion of competent WT macrophages into MMP-2 deficient mice did not reconstitute the aneurysms (Longo *et al.*, 2002).

Yang *et al.* (2014) also investigated the molecular mechanism underlying the role of monocyte chemoattractant protein-1 (MCP-1) in the formation and development of AAA. Results demonstrated that MCP-1 stimulates secretion of MMP-9 directly through the ERK1/2 and p38 MAPK mediated pathways in HASMCs and might be a potential therapeutic target in the treatment of AAA. Later studies looked at mesenchymal stromal cells (MSCs) found within the aneurysmal wall and found they too contribute towards increased levels of MMP-9 in AAA (Ciavarella *et al.*, 2015).

More recently, in 2013, Tefã-Silva *et al.*, used an experimental model in rats to induce AAA by combining two potential causes of MMP secretion, inflammation and turbulent blood flow. Results of morphological examination of sections of aorta included considerable destruction of elastin fibres and deposition of collagen which they concluded were directly related to the dramatic increase of the levels of MMP-2 and -9 throughout the experiment. Researchers have also speculated that the MMP-2 gene may be a candidate gene after investigating its promoter region and documenting six polymorphisms associated with differential promoter activity which may be responsible for early elastolysis and aneurysmal degeneration in AAA (Price *et al.*, 2001 Goodall *et al.*, 2001).

Smoking is known to be a significant risk factor in AAA and Ghosh *et al.*, (2015) hypothesized that cigarette smoke may increase MMP2 and MMP9 secretion through the Jak/Stat pathway in the aorta. Treating rat VASMCs with an aqueous extract of cigarette smoke resulted in a significant increase in pro-MMP-9 and a modest increase in pro-MMP-2 which was significantly reduced after transfection of the cells with small interfering RNAs (siRNAs) for Jak2, Stat3, or both Jak2 and Stat3 leading them to conclude that inhibiting the Jak/Stat pathway could be a potential therapeutic approach in the treatment of AAA.

Recently Li *et al.* (2016) investigated the role of cold-inducible RNA-binding protein (CIRP), a recently identified pro-inflammatory cytokine, in the progression of AAA. CIRP mRNA and protein expression was upregulated significantly in human AAA tissue and anti-CIRP antibody treatment significantly suppressed the dilation of experimental AAA in rats and significantly attenuated the expression of MMP-2 and MMP-9 demonstrating its potential as a novel target against AAA progression. Additionally, Bumdelger *et al.*, (2016) reported on the possible involvement of osteoprotegerin(OPG) in the prevention of AAAs through inhibition of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Results suggested both the TRAIL-induced expression of MMP-9 in SMCs and the chemoattractive effect of TRAIL on SMCs were inhibited by OPG which may play a preventive role in the development of AAA.

2.2.1.2 MT1-MMP

MT1-MMP which is involved in the processing of the MMP-2 pro-enzyme is also expressed in the aneurysm wall by both infiltrating macrophages and to a lesser extent smooth muscle cells in a similar pattern to MMP-2 and plays a dominant role in macrophage-mediated elastolysis (Xiong *et al.*, 2009) Thompson *et al.*,1996). Significantly higher levels of MT1–MMP mRNA have also been detected in AAAs in comparison to normal aortas (Nollendorfs *et al.*, 2001).

2.2.1.3 MMP-12 (Human macrophage elastase)

MMP-12 is reported to be predominately produced by infiltrating macrophages within the aortic media of aneurysmal tissue subsequently binding with high affinity specifically to residual elastin fibres (Curci *et al.*,1998). Although researchers have confirmed increased MMP-12 in AAA

compared to normal aorta, results of animal studies involving MMP-12 deficient mice found the animals still developed elastase induced aneurysms (Pyo *et al.*, 2000). Later, animal studies by Savio *et al.*, (2008) established that inhibition of MMP-12 reduces the severity of AngII-induced AAAs in apoE^{-/-} mice (Luttun *et al.*, 2004).

Zheng *et al.*, (2015) examined the link between phosphatidylinositol 3-kinases δ (p110 δ) which regulates immune function and aneurysm development. They demonstrated that p110 δ inactivation promotes aneurysm development by inducing macrophage migration and upregulating the activator protein-1/MMP-12 pathway in macrophages.

2.2.1.4 MMP-3 (Stromelysin-1)

Immunohistochemical studies have localized the expression of stromelysin-1 in AAA to macrophages within the aortic wall (Newman *et al.*, 1994). Additionally Carrell *et al.*, (2002) also demonstrated that MMP-3 and TIMP-3 were overexpressed in the wall of AAAs using reverse transcriptase–polymerase chain reaction (RT-PCR). They hypothesized that the increased MMP-3 expression may arise from a combination of genetic and environmental factors. This was supported by a Finnish study suggesting that a polymorphism (5A MMP3 allele) may be a genetic risk factor for AAA (Yoon *et al.*, 1999). MMP-3 (stromelysin-1) not only breaks down collagen and other structural proteins of the aortic wall, but it also activates other pro-MMPs such as interstitial collagenase, matrilysin, and gelatinase B by removal of their prodomain (Fanjul-Fernandez *et al.*, 2010).

2.2.1.5 MMP-13 (Collagenase -3)

Collagen degradation is important in the latter stages of AAA development as resistance to further expansion and rupture is dependent upon the tensile strength of the interstitial collagens (Tilson *et al.*, 1990). MMP-13 is a collagenase degrading enzyme with limited tissue distribution and a highly regulated pattern of expression. Mao *et al.*, (1999) using RT-PCR and Southern blotting detected increased expression of MMP-13 in AAA samples and atherosclerotic aorta (ATH) but not in normal aorta. They also found higher levels of MMP-13 in AAA samples compared to ATH and that MMP-13 was localised to SMCs in close spatial proximity to collagen.

2.2.1.6 MMP-1 (Interstitial collagenase-1)

Irizarry *et al.*, (1993) demonstrated increased immunoreactivity of MMP-1 localized in the adventitia of AAAs. MMP-1 is secreted primarily by a variety of mesenchymal and epithelial cell types but invading inflammatory cells within the aneurysm can also constitute a source of MMP-1, although its role appears more related to tissue repair than disease processes (Welgus *et al.*, 1985, Parks *et al.*, 1998). It is particularly selective in cleaving the triple-helix domain characteristic of types I and III collagen but it has little activity against type II collagen, gelatins, or other matrix proteins (Welgus *et al.*, 1980, 1981, 1985).

2.2.1.7 MMP-8 (Neutrophil collagenase)

MMP-8, also known as collagenase-2 is a product of polymorphonuclear neutrophils (PMNs), where the protein is stored in granules and released upon cellular activation (Lazarus *et al.*, 1968, Hasty *et al.*, 1990). Studies

have indicated that chondrocytes, synovial fibroblasts and vascular endothelium can all express MMP-8 (Cole *et al.*, 1996, Hanemaaijer *et al.*, 1997). Wilson *et al.*, (2005) examined the expression of the potent type-I collagenase MMP-8 in AAA and normal aorta using enzyme-linked immunosorbent assays and immunohistochemical techniques. Results demonstrated MMP-8 levels were significantly raised in AAA tissues in comparison to normal aorta and that MMP-8 was localised to the mesenchymal cells of the adventitia of the aortic adventitia (Wilson *et al.*, 2005). Additionally Reeps *et al.*, (2009) also identified MMP-8 in the inflammatory infiltrate of AAA tissue using immunostaining methods. Mao *et al.*, (1999) using RT-PCR and Southern blotting concluded that because MMP-8 was inconsistently expressed in AAA and ATH tissue and since neutrophils are not a prominent component of the chronic inflammatory response in AAA MMP-8 was not likely to be a significant factor in AAA pathogenesis.

2.2.2 The Role of TIMPs in AAA

Despite early reports that suggested that the amount of TIMP present in human AAA tissue might be decreased (McMillan *et al.*, 1995), there is now substantial evidence that the production of TIMPs is actually increased within the aneurysm wall. Studies carried out by Tamarina *et al.*, (1997) who found TIMP-1 mRNA levels in AAA tissue were in molar excess in comparison to the metalloproteinases studied, while Elmore *et al.*, (1998) identified a small but statistically significant increase in TIMP-2 mRNA in AAA tissue although for TIMP-1, mRNA levels were not significantly different. Nishimura and colleagues (2003) also discovered TIMP-1 was highly expressed in the walls of AAA arteries compared with to the controls. Eskandari *et al.*, (2004) demonstrated the protective effects of TIMP-1 by inhibiting the effects of MMPs in an experimental AAA model where TIMP-1^{-/-} mice developed significantly larger aneurysms compared to TIMP-1^{+/+} mice. Additionally, Allaire *et al.*, (1998) found local overexpression of TIMP-1

prevented aortic aneurysm degeneration and rupture in a rat model. Plasma TIMP-1 has also been demonstrated to be considerably elevated in patients with AAA compared to patients with aortoiliac atherosclerosis or in healthy individuals (Nakamura *et al.*, 2000).

Bumdelger *et al.*, (2013) found TNF-alpha significantly activated both MMP-9 and TIMP-1 expression in cultured VSMCs, which was blocked by a Jun kinase inhibitor (SP600135) and surmised inflammatory cytokines, including TNF-alpha, may simultaneously induce MMPs and TIMPs leading to remodelling of the aortic medial layer in AAA. Most recently, Bumdelger *et al.*, (2016) determined that tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) served as an inducer of MMP-9 and TIMP-1 transcription via the JNK and/or nuclear factor-kappaB (NFkB) pathways leading to aortic medial thickening and breakdown of the aortic ECM.

Baker *et al.*, (1998) compared the effects of overexpressing TIMP-1, 2 and 3 on invasion, proliferation and death in rat aortic SMCs. Their findings established that TIMP-1 overexpression did not affect cell proliferation but TIMP-2 caused a dose-dependent reduction in proliferation and interestingly TIMP-3 overexpression induced DNA synthesis and promoted SMC death by apoptosis, a common feature of AAA. Basu *et al.*, (2012) also investigated the causal role of *Timp3* in a mouse model of AAA and demonstrated the regulatory function of *Timp3* is critical in preventing adverse vascular remodelling and loss of the *Timp3* gene leads to AAA formation.

2.3 Hypoxia Within the Aortic Wall

Inside the body internal O₂ concentrations are maintained within a narrow range and changes in oxygen tension can result in differential expression of specific genes with physiological or pathological consequences (Faller, 1999). The oxygen supply of the inner aortic media depends largely on diffusion from the endothelium as the infrarenal aorta is normally devoid of medial vasa vasorum (Zarins *et al.*, 2001) and therefore conditions which

increase wall thickness affecting oxygen diffusion or reduce oxygen transmissibility produce hypoxia and steep pO_2 gradients within the vessel wall. Arterial wall hypoxia has been investigated in other conditions such as atherosclerosis, intimal hyperplasia and myointimal hyperplasia. Atherosclerosis is often associated with AAA, Björnheden and colleagues (1999) using the hypoxia marker 7-(4'-(2-nitroimidazol-1-yl)-butyl)-theophylline, demonstrated that hypoxic zones do exist in the arterial wall in rabbits *in vivo*, within atherosclerotic plaques when lesions exceed certain dimensions and at a depth that is also readily reached in humans. Heughan *et al.*, (1973) induced experimental atheromata in rabbits by a combination of injury and high cholesterol diet which resulted in the transmural oxygen tension being markedly different from normal. The lowest mean values were recorded immediately adjacent to the lumen and measured 18 mm Hg at five weeks compared with the mean low point of 22 mm Hg measured 60% of the distance from the outer surface in normal aortas. In experimental atheromata some individual low point values reached as low as 10 and 12 mm Hg which is far lower than any measured in normal aorta. Studies with experimental hypertension (which is a high risk factor in aneurysmal disease), hyperlipidaemia and balloon injury also produced medial hypoxia with steep pO_2 gradients within the vasculature (Crawford and Blankenhorn., 1991).

In 1996, Vorp *et al.*, first hypothesized that intraluminal thrombus (ILT) may be important in the natural history and pathology of AAA as a result of poor diffusion of oxygen through the ILT. Approximately 80% of AAA have ILT resulting in increased inflammation, a thinner wall underlying the thrombus and greater risk of rupture (Satta *et al*, 1996, Kazi *et al*, 2003). Later studies concluded that ILT within an AAA caused localised hypoxia so that aortic wall pO_2 differed between aneurysmal and normal aortic tissue and may lead to increased localized mural neovascularisation, inflammation and regional wall weakening. (Vorp *et al*, 2001). Hu *et al.*, (2004) studied the expression of hypoxia-inducible factor-1 alpha (HIF-1 α) and related genes in aneurysmal and normal specimens. The global oxygen sensor HIF-1 α responds to low tissue oxygen by up-regulating hypoxia responsive genes

(Laderoute, 2005). They found the expression of HIF-1 α mRNA and protein product were significantly higher in AAA tissue than in normal aorta, furthermore, the expression of VEGF and caspase-3 were also higher in AAA tissue and both had a significantly positive relationship with HIF-1 α expression (Hu *et al.*, (2004).

More recently Xu *et al.*, (2016) investigated the serum concentration of HIF-1 α and VEGF in patients with aneurysms at different stages and discovered that serum concentrations of HIF-1 α in the early stage group were significantly higher than those in the normal group and that the late stage group were significantly higher than those in the early or mid-term group. They concluded from the results that serum HIF-1 α and VEGF has clinical value of early diagnosis and assessment of disease severity. A study by Strauss *et al.*, (2015) identified polymorphisms in the HIF1A and VEGF genes as potential genetic markers that indicate the predisposition to AAA including the HIF1A 1772T allele which seems to be a genetic risk factor that determines sensitivity to cigarette smoke exposure.

2.3.1 Hypoxia-inducible factor -1 α (HIF-1 α)

The transcription factor Hypoxia-inducible factor-1 α is a heterodimeric protein composed of the hypoxia inducible 120 kDa HIF-1 α subunit and the constitutively expressed 91-94 kDa HIF-1 β subunit also known as the aryl hydrocarbon nuclear translocator (ARNT). HIF-1 α functions as a master regulator of mammalian oxygen homeostasis, controlling the expression of over seventy genes involved in intracellular adaptive responses to hypoxia, such as angiogenesis, erythropoiesis and glycolysis (Semenza, 2004).

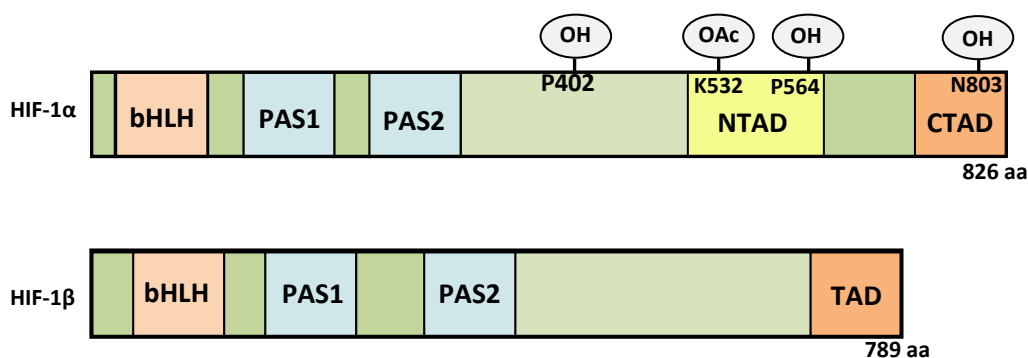


Figure 11 HIF-1 alpha and HIF-1 beta Subunits

HIF-1 α and HIF-1 β contain one basic-helix-loop-helix (bHLH) domain and two PER-ARNT-SIM (PAS1/PAS2) domains in their N-terminal regions. The positions of post-translational hydroxylation (OH) of the proline residues (P402/P564) and acetylation (OAc) of lysine (K532) of HIF-1 α are shown. This occurs within the oxygen-dependent degradation (ODD) domain (residues 401-603) close to the N-terminal transactivation domain (NTAD) and confers recognition by pVHL (von Hippel-Lindau tumour suppressor gene) leading to degradation of the subunit. At the C-terminal domain (CTAD) hydroxylation at N803 inhibits recruitment of coactivators required for HIF-1 α transcriptional activity. HIF-1 β contains one transactivation domain at its C-terminus. aa (amino acid) (Adapted from Carroll and Ashcroft., 2005).

The closely related proteins HIF-2 α (also known as endothelial PAS protein), HIF-like factor (HLF), HIF-related factor (HRF), and member of the PAS superfamily 2 (MOP2) have also been identified (Ema *et al.*, 1997; Flamme *et al.*, 1997; Hogenesch *et al.*, 1997; Tian *et al.*, 1997). In contrast to ubiquitously expressed HIF-1 α , HIF-2 α is predominantly expressed in the lung, endothelium, and carotid body (Ema *et al.*, 1997; Tian *et al.*, 1997). HIF-3 α which was discovered more recently is expressed in a variety of tissues and, like HIF-2 α is able to dimerize with HIF-1 β and bind HREs (Gu *et al.*, 1998).

2.3.2 HIF-1 α under Normoxic Conditions (Oxygen-dependent)

At normal oxygen levels the prolyl-hydroxylases PHD1, PHD2 and PHD3 hydroxylate the key proline residues (Pro402/ Pro564) within the HIF-1 α ODD domain (Epstein *et al.*, 2001). PHDs are dioxygenases that utilize oxygen as co-substrate providing the molecular basis for the oxygen-sensing function of these enzymes but 2-oxoglutarate ascorbate and iron are also required as cofactors for the prolyl hydroxylation reaction (Cioffi *et al.*, 2003). Asparagine hydroxylase FIH (factor inhibiting HIF-1) hydroxylates Asp803 inhibiting recruitment of the aforementioned coactivators required for HIF-1 α transcriptional activity. Once hydroxylated HIF-1 α interacts with an E3 ubiquitin-protein ligase complex composed of pVHL, elongin B, C and Cullen 2, targeting it for polyubiquitylation and rapid degradation by the 26S proteasome (Salceda and Caro, 1997, Maxwell *et al.*, 1999, Cockman *et al.*, 2000).

HIF-1 α also contributes to other cellular processes that occur under normoxic conditions. Many growth factors and cytokines are known to induce and activate HIF-1 α , including insulin-like growth factors, transforming growth factor, platelet-derived growth factor, epidermal growth factor and interleukin -1 β (Felser *et al.*, 1999, Hellwig-Burgel *et al.*, 1999, Stiehl *et al.*, 2002, Haddad and Land, 2002). Nitric oxide has also been reported to promote HIF-1 α expression under normoxia (Kasuno *et al.*, 2004) along with vascular hormones such as endothelin, angiotensin II, thrombin (Richard *et al.*, 2000, Gorlach *et al.*, 2001, Page *et al.*, 2002, Pisarcik *et al.*, 2008), During non-hypoxic induction of HIF-1 α the main mechanism implicated in these processes seems to be an increase in HIF-1 α protein translation rather than stabilization, and this increase alone appears sufficient to shift the balance between synthesis and degradation towards an accumulation of normoxic HIF-1 α .

2.3.3 HIF-1 α under Hypoxic Conditions

During hypoxia HIF-1 α is not hydroxylated because oxygen is unavailable allowing the unmodified protein to escape the VHL-binding and subsequent ubiquitination and degradation by the proteasome. HIF-1 α can then dimerize with HIF-1 β (ARNT) to form the HIF-1 α transcription complex which binds to its target DNA sequence within the hypoxia response element (HRE) of target genes, recruiting the coactivators p300 and CREB binding protein (CBP) to activate transcription (Semenza., 1996, Ebert and Bunn., 1998). Interestingly, PHD2 and PHD 3 are HIF targets themselves and markedly induced by hypoxia, suggesting they may participate in a negative feedback loop to suppress the HIF response to prolonged hypoxia (Marxsen *et al*, 2004, Appelhoff *et al.*, 2004, Aprelikova *et al.*, 2004, Dery *et al.*, 2005, Stiehl *et al.*, 2006).

Baek *et al.*, (2016) recently identified a new methylation-based regulation of HIF-1 α stability in the nucleus in response to cellular oxygen levels which is independent of previously identified mechanisms. This mechanism is based on the collaboration between the SET7/9 methyltransferase and the LSD1 demethylase. SET7/9 adds a methyl group to HIF-1 α which triggers degradation of the protein by the ubiquitin-proteasome system whereas LSD1 removes the methyl group, leading to stabilization of HIF-1 α under hypoxic conditions.

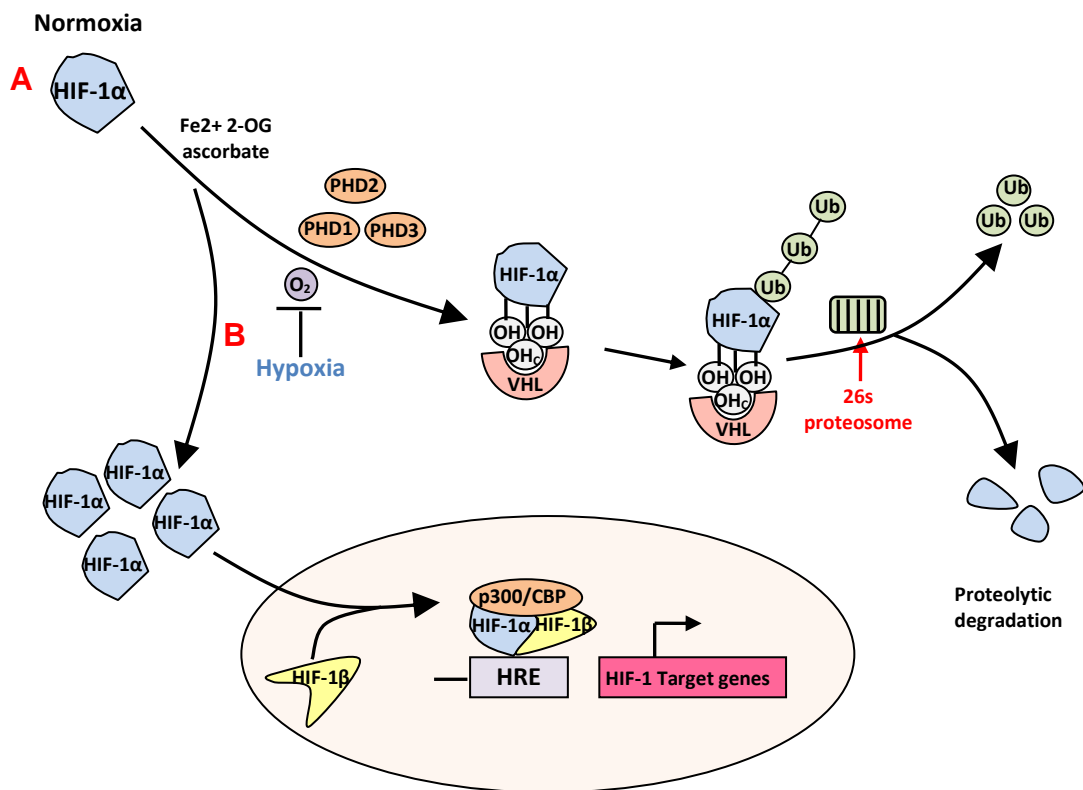


Figure 12. HIF-1 Alpha Regulation by Proline Hydroxylation

A: In normoxia, HIF-1 α is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O₂, Fe²⁺, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF-1 α (OH) is recognised by pVHL (the product of the von Hippel–Lindau tumour suppressor gene), which together with the E3 multisubunit ubiquitin ligase complex tags HIF-1 α with polyubiquitin allowing recognition by the proteasome and subsequent degradation. Acetylation of HIF-1 α (OAc) also promotes pVHL binding.

B: In response to hypoxia proline hydroxylation is inhibited. VHL is no longer able to bind and target HIF-1 α for proteasomal degradation leading to HIF-1 α accumulation and translocation to the nucleus. HIF-1 α then dimerises with HIF-1 β , binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. Abbreviation: CBP (CREB-binding protein), Ub (ubiquitin) (Adapted from Carroll and Ashcroft., 2005).

Table 8 Factors Stabilizing HIF under Normoxic Conditions

REGULATOR	FUNCTIONS/PATHWAY	CONSEQUENCE	REFERENCE
Nickel (Ni ²⁺)	Decreases cellular Fe level Inhibits PHDs	Increased HIF-1 α	Davidson <i>et al.</i> , 2005
	Down-regulates exp of FIH-1 and ARD1		Ke <i>et al.</i> , 2005
	Depletes intracellular ascorbate		Salnikow <i>et al.</i> , 2004
	P13K/Akt		Li <i>et al.</i> , 2004
Cobalt (Co ²⁺)	Replaces Fe	Increased HIF-1 α	Yuan <i>et al.</i> , 2003
	Down-regulates exp of FIH-1 and ARD1		Ke <i>et al.</i> , 2005
	Depletes intracellular ascorbate		Salnikow <i>et al.</i> , 2004
	P13K		Gao <i>et al.</i> , 2004
Arsenite	ROS	Increased HIF-1 α	Duyndam <i>et al.</i> , 2001
	p38MAPK		Duyndam <i>et al.</i> , 2003
Chromium	ROS, p38MAPK	Increased HIF-1 α	Gao <i>et al.</i> , 2004
	ROS, P13K/Akt	Increased HIF-1 α	Gao <i>et al.</i> , 2002
Vanadate	AMP-activated protein kinase (AMPK)	Increased HIF-1 α	Hwang <i>et al.</i> , 2004
Desferrioxamine	Fe chelator	Increased HIF-1 α	Wang and Semenza., 1993
Insulin, Interleukin -1 β (IL-1 β)	P13K	Increased HIF-1 α	Steihl <i>et al.</i> , 2002
Insulin-like growth factor (IGF)-1, IGF- 2	ROS	Increased HIF-1 α	Feldser <i>et al.</i> , 1999
Foetal calf serum	ROS	Increased HIF-1 α	Richard <i>et al.</i> , 2000
Angiotensin II (AngII)	ROS	Increased HIF-1 α	Gorlach <i>et al.</i> , 2001
			Page <i>et al.</i> , 2002
Thrombin, Platelet- Derived growth factor	ROS/p44/42 MAPK	Increased HIF-1 α	Gorlach <i>et al.</i> , 2001
			Page <i>et al.</i> , 2002
Nitric oxide (NO)	MAPK, P13K	Increased HIF-1 α	Kasuno <i>et al.</i> , 2004
Nitric oxide	Inhibits PHDs	Increased HIF-1 α	Metzen <i>et al.</i> , 2003
Transforming growth Factor- α (TGF- α)	ROS	Increased HIF-1 α	Haddad and Land., 2001
Tumor necrosis factor-alpha(TNF α) interleukin-1 β (IL- 1 β)	Increased HIF-1 DNA binding	Increased HIF-1 α	Hellwig-Burgel <i>et al.</i> , 1999
Endothelin-1	Increased <i>Hif1α</i> mRNA	Increased HIF-1 α	Pisarcik <i>et al.</i> , 2008

2.3.4 The Role of Hypoxia and HIF-1 α in the Expression of MMPs

The involvement of MMPs in the pathogenesis of AAA is well documented but what is unknown is the potential role of hypoxia and HIF-1 α in the regulation of MMPs in AAA, although in many other research areas HIF-1 α is known to modulate MMPs. In HCT116 human colon carcinoma cells HIF-1 α is shown to regulate the expression of many genes involved in the pathophysiology of invasion including MMP-2 (Krishnamachary *et al.*, 2003). A later study into colon adenoma and colon cancer also looked at the expression of MMP-2, HIF-1 α and VEGF and their results showed a positive correlation between levels of HIF-1 α mRNA expression and MMP-2 and VEGF mRNA (Shin *et al.*, 2007). Ahn *et al.*, (2008) examined the contribution of HIF-1 α to hypoxia-induced MMP and cytokine production in rheumatoid fibroblast-like synoviocytes transfected with a HIF-1 α overexpression vector and found a significant increase in MMP-1 and MMP-3. Lolmède and colleagues (2003), during obesity studies, subjected 3T3-F442A adipocytes to hypoxia or hypoxia mimics which stimulated the HIF-1 pathway and markedly enhanced the expression of leptin, VEGF and MMP-2 and MMP-9. Bovine cartilage disks subjected to mechanical overload (a causative factor in osteoarthritis) were strongly immunopositive for HIF-1 α , additionally MMP-1, MMP-3 and MMP-13 were detected in pressure-treated disks whereas staining in controls was low or undetectable (Pufe *et al.*, 2004). The degradation or remodelling of the ECM may play role in the pathogenesis of temporomandibular joint (TMJ) disorders, Yamaguchi *et al.*, (2001) found hypoxia and IL-1 β induced MMP-1, MMP-2, MMP-3 MMP-9 and MMP-13 mRNA in (TMJ) disc cells but concluded MMP induction was due largely to hypoxia and the involvement of HIF-1 α . An interesting study by Ahn *et al.*, (2008) was conducted to determine the contribution of HIF-1 α to hypoxia-induced MMP and cytokine production in rheumatoid fibroblast-like synoviocytes (FLS). Results demonstrated that hypoxia-induced MMP-3 expression is exclusively regulated by HIF-1 α , and hypoxia-induced MMP-1 or IL-8 expression appeared to have salvage pathways other than the HIF-1 α pathway. Most recently, in 2010 Sun *et al.*, determined the effect of hypoxia

and specifically HIF-1 α on expression of CXCR4 and MMP-1 and their role in chondrosarcoma cell invasion, finding that hypoxia and specifically HIF-1 α increased CXCR4 and MMP1 expression in a chondrosarcoma cell line and this increase could be inhibited by siRNA directed at HIF-1 α or CXCR4.

Wang *et al.*, (2015) investigated the effect of hypoxia on the proliferation and expressions of HIF-1 α , VEGF MMP-9 in keratinocytes obtained from oral lichen planus (OLP) lesions. The results demonstrated that hypoxic conditions can promote the protein expression of HIF-1 α and both the mRNA and protein expression of its downstream targets VEGF and MMP-9 in keratinocytes.

Conversely, other researchers have reported different findings, Miyoshi *et al.*, (2006), in cancer studies concluded that hypoxia accelerated metastasis by hepatoma cells by upregulating MMP expression in a HIF-1 α independent manner. Hong *et al.*, (2005) using pulmonary artery endothelial cells (PAEC) and smooth muscle cells (PASMC) also found hypoxia could inhibit the secretion and activity of MMP-2 and MMP-9 in PAEC and PASMC and proposed the HIF-1 pathway might contribute to hypoxia-induced down-regulation of MMP-2 and MMP-9. However, Herget *et al.*, (2003) found that rats exposed to chronic hypoxia demonstrated upregulation of collagenolytic MMP activity which promoted pulmonary vascular remodelling and hypertension. Furthermore these hypoxia-induced increases in collagenolytic activity in pulmonary vessels could be inhibited by treatment with Batimastat, a MMP inhibitor.

2.3.5 HIF-1 α Target Genes

The transcription factor HIF-1 α acts as a master regulator of oxygen-regulated gene expression. It is responsible for the induction of genes that facilitate adaptation and survival of cells and the whole organism from normoxia (~21% O₂) to hypoxia (~ 1% O₂) (Wang *et al.*, 1995, Semenza, 1998). HIF-1 α protein has a short half-life ($t_{1/2}$ ~ 5 min) and is highly regulated

by oxygen (Salceda and Caro, 1997) in contrast to the transcription and synthesis of HIF-1 α which are constitutive and seem not to be affected by oxygen (Wang *et al.*, 1995, Kallio *et al.*, 1997, Wiesener *et al.*, 1998). HIF-1 β is constitutively expressed and its mRNA and protein are maintained at constant levels regardless of oxygen availability (Kallio *et al.*, 1997)

During normoxia HIF-1 α proteins are rapidly degraded (Wang *et al.*, 1995) but exposure to hypoxia causes HIF-1 α become stabilized and translocates from the cytoplasm to the nucleus where it dimerizes with HIF-1 β and becomes transcriptionally active (Huang *et al.*, 1996, Kallio *et al.*, 1997). The activated HIF complex then associates with HREs in the regulatory regions of target genes and binds the transcriptional coactivators to induce gene expression (Lando *et al.*, 2002).

More than 60 putative HIF-1 target genes have been identified including secreted signalling proteins, angiogenic growth factors, cell surface receptors, extracellular matrix proteins, transcription factors, cytoskeletal proteins, pro-apoptotic, glucose transporters and glycolytic enzymes.

Table 9 HIF-1 Alpha Target Genes

Function	Gene or Abbreviation	Reference
Cell proliferation	Cyclin G2, IGF2, IGF-BP1,2 and 3, WAF-1, TGF- α , TGF- β 3	Feldser <i>et al</i> , 1999, Krishnamachary <i>et al</i> , 2003, Wykoff <i>et al</i> , 2000
Cell survival	ADM, IGF2, IGF-BP1,2 and 3, NOS2, TGF- α ,	Cormier-Regard <i>et al</i> , 1998, Feldser <i>et al</i> , 1999, Krishnamachary <i>et al</i> , 2003,
Apoptosis	Bcl-2/adenovirus E1B 19kD-interacting protein 3 (BNip3), iNip3-like protein X (NIX), RTP801	Carrero <i>et al</i> , 2000, Bruick, 2000, Shoshani <i>et al</i> , 2002
Erythropoiesis	Erythropoietin (EPO)	Semenza <i>et al</i> , 1991
Angiogenesis	VEGF, EG-VEGF, Leptin (LEP), TGF- β 3, LRP1, ENG	Levy <i>et al</i> , 1955, LeCouter <i>et al</i> , 2001, Grosfield <i>et al</i> , 2002, Scheid <i>et al</i> , 2002, Wykoff <i>et al</i> , 2000, Bernhardt <i>et al</i> , 2006
Vascular tone	NOS2, Heme oxygenase, ET1, ADM, $\alpha_{1\beta}$ -adrenergic receptor	Melillo <i>et al</i> , 1995, Lee <i>et al</i> , 1997, Hu <i>et al</i> , 1998, Nguyen and Claycomb, 1999, Eckhart <i>et al</i> , 1997
Matrix metabolism	MMPs, PAIs, , uPAR CATHD, Fibronectin 1 (FN-1)	Ben-Yosef <i>et al</i> , 2002, Kietzmann <i>et al</i> , 1999, Takahashi <i>et al</i> , 2000, Krishnamachary <i>et al</i> , 2003,
Glucose metabolism	AK-3, ALD-A and C, ENO1, LDHA, CA9 and 12, GLUT1 and 3, GAPDH, HK1 and 2, , PKM, PFKL, PGK1, PFKFB3	O'Rourke <i>et al</i> , 1996, Semenza <i>et al</i> , 1996, Wykoff <i>et al</i> , 2000, Chen <i>et al</i> , 2001, Graven <i>et al</i> , 1999, Mathupala <i>et al</i> , 2001, Semenza <i>et al</i> , 1994, Minchenko <i>et al</i> , 2002
Iron metabolism	Transferrin, transferrin receptor, Ceruloplasmin	Rolfs <i>et al</i> , 1997, Bianchi <i>et al</i> , 1999, Lok and Ponka, 1999, Chinmay <i>et al</i> , 2000
Drug resistance	Multidrug resistance 1(MDR1)	Comerford <i>et al</i> , 2002
Cell adhesion	Microneme protein 2 (MIC2)	Wykoff <i>et al</i> , 2000
Motility	LRP1, c-MET, TGF- α , autocrine motility factor (AMF)	Wykoff <i>et al</i> , 2000, Pennacchietti <i>et al</i> , 2003, , Krishnamachary <i>et al</i> , 2003, Niizeki <i>et al</i> , 2002
Aa metabolism	Transglutaminase 2	Wykoff <i>et al</i> , 2000
Cytoskeletal structure	Keratin 14, 18 and 19, vimentin	Krishnamachary <i>et al</i> , 2003, Semenza <i>et al</i> , 2003
Transcriptional regulation	ETS-1, DEC1 and 2, NUR77	Oikawa <i>et al</i> , 2001, Miyazaki <i>et al</i> , 2002, Choi <i>et al</i> , 2004
Nucleotide metabolism	Adenylate kinase 3, Ecto-5'-nucleotidase	O'Rourke <i>et al</i> , 1996, Synnestvedt <i>et al</i> , 2002
HIF activity	P35srj	Bhattacharya <i>et al</i> , 1999
Epithelial homeostasis	Intestinal trefoil factor	Furuta, <i>et al</i> , 2001

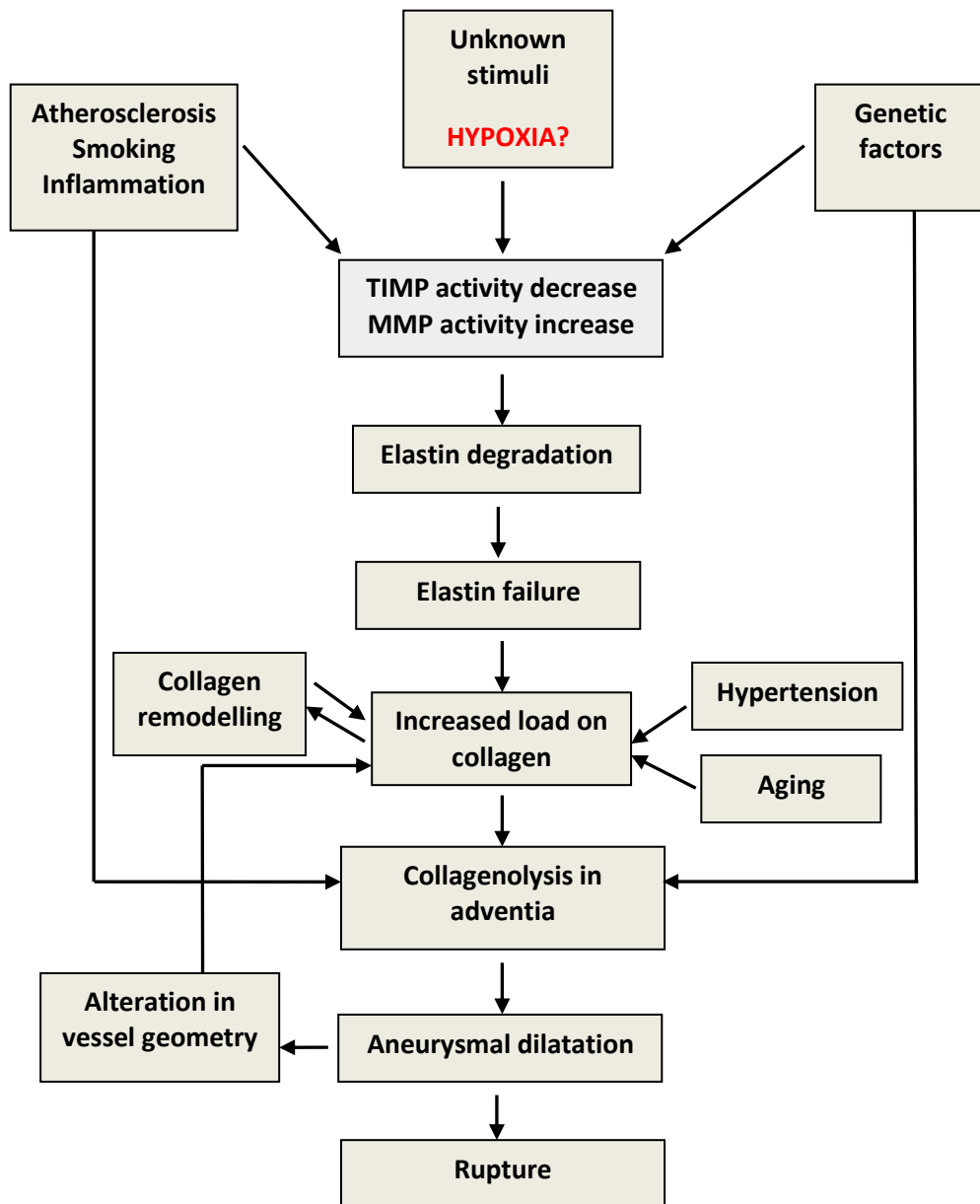


Figure 13 Overview of the Pathogenesis of AAA

This flow diagram gives a simplified overview of some of the main factors involved in the formation of AAA (Adapted from MacSweeney *et al.*, 1994).

2.4 Diagnosis of AAA

Most patients with AAA are asymptomatic and are often diagnosed during procedures such as x-rays or ultrasound scans carried out for other reasons. Sometimes an abdominal aneurysm may be discovered during a routine physical examination where a pulsatile mass is palpable in the abdomen, or through ultrasound screening programs for AAA. As the aneurysm expands, symptoms may occur and patients commonly present with abdominal, back, flank or groin pain. The risk of rupture is high in symptomatic aneurysms and mortality from AAA rupture is around 90% with 65 to 75 % of patients dying before arrival at the hospital (Brown and Powell, 1999).

2.4.1 AAA Screening

The NHS AAA Screening Programme was set up in England in 2009 and has been offered throughout the UK since the end of 2013. All men aged over 65 are invited to undergo an ultrasound scan to check the diameter of their abdominal aorta. An ultrasound scan is a painless procedure that uses high-frequency sound waves to create an image of part of the inside of the body. An ultrasound probe is used which gives off high-frequency sound waves which bounce off different parts of the body and create "echoes" that are picked up by the probe and turned into a moving image which is displayed on a monitor while the scan is carried out as shown in Figure 14.

Men with a normal sized abdominal aorta (less than 3cm in diameter) are not invited back whilst those with a small (3.0-4.4cm) or medium (4.5-5.4cm) AAA will be invited back for regular scans to check the size of the aneurysm, known as 'watchful waiting'. Annual scans are carried out for patients with small aneurysms and three monthly scans for patients with medium aneurysms. Patients are also given advice on diet, stopping smoking, weight, exercise and medication which may help to prevent the aneurysm increasing in diameter. Patients found to have a large aneurysm (5.5cm or

larger) will generally be referred to a vascular surgeon to discuss surgical options.



Figure 14 Example of an Ultrasound Scan of AAA

Longitudinal view of the abdominal aorta demonstrating a focal area of enlargement (depicted by black arrows) consistent with an abdominal aortic aneurysm (www.aafp.org).

2.4.2 Surgical Intervention

Prior to surgical intervention further imaging of the AAA may be carried out to provide greater detail for the vascular surgeon to plan surgical repair of the aneurysm. These tests may include computerized tomography (CT) or magnetic resonance imaging (MRI) scans. Also if these scans are performed with the addition of contrast medium it provides greater detail to enable enhanced evaluation of the vasculature and is known as CTA (computerized tomographic angiogram) or MRA (magnetic resonance angiogram). Occasionally a procedure known as aortography (aortogram) is carried out to evaluate the aorta, aneurysm and how the aneurysm affects the branches of blood vessels coming off of the aorta.

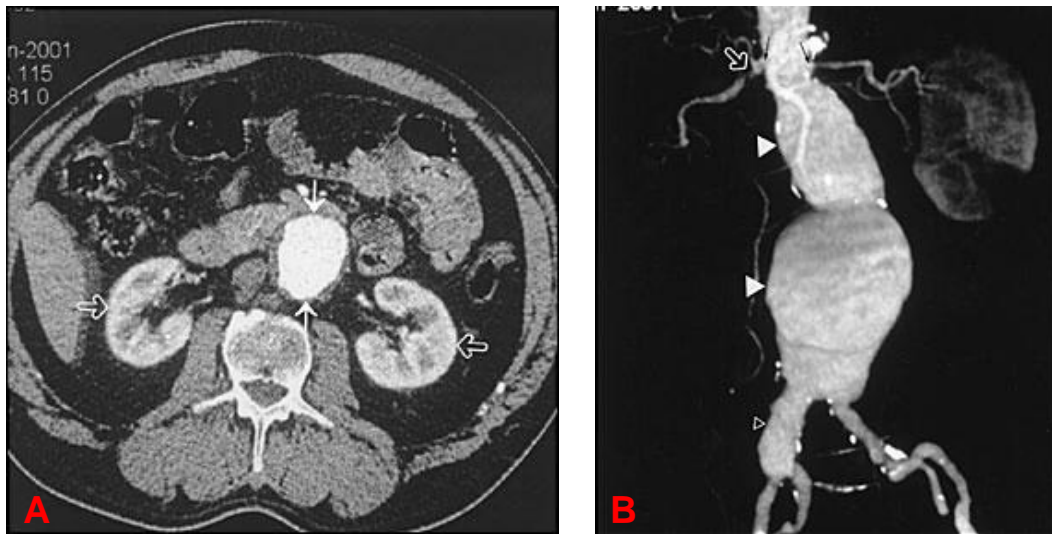


Figure 15 Computed Tomographic Scan and Computerized Tomographic Angiogram of AAA

A: Computed tomographic scan of the abdominal aorta at the level of the kidneys (outline arrows) showing an aneurysm (arrows) with a small amount of mural thrombus along the posterior and lateral walls. **B:** Reconstructed computerized tomographic angiogram scan in the frontal projection demonstrating the bilobed infrarenal aortic aneurysm (arrow heads). The renal arteries are well visualized (small arrows), revealing a severe stenosis of the proximal right renal artery (outline arrow) and the proximity of the aneurysm neck to the renal arteries. The relationship of the aneurysm to the iliac arteries and the aneurysmal dilatation of the right common iliac artery (outline arrow head) are also well displayed. CTA is accomplished by combining all of the axial slices to produce a three-dimensional reconstructed image of the AAA which can be rotated into any plane that best demonstrates the relevant anatomy (www.aafp.org).

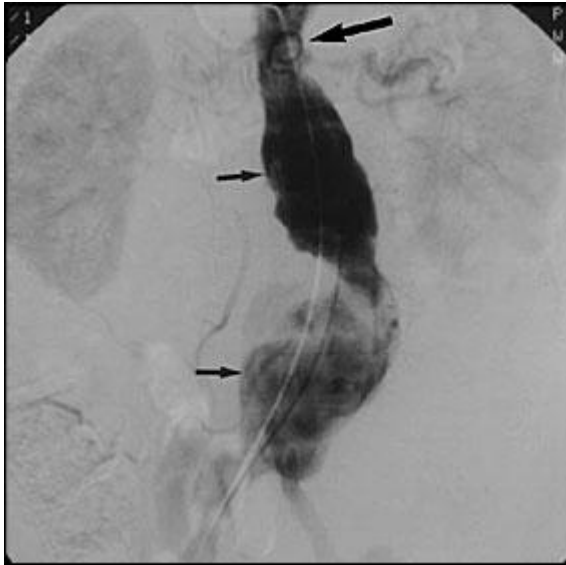


Figure 16 Aortogram of AAA

Early arterial phase of a posteroanterior abdominal aortogram using iodinated contrast. medium to demonstrate a bilobed aneurysm (arrows) originating just below the renal arteries (www.aafp.org).



Figure 17 Magnetic Resonance Angiogram of AAA

MRA demonstrating an abdominal aneurysm below the level of the kidneys in the infrarenal aorta (www.radiopaedia.org).

2.4.2.1 Open Surgical Aneurysm Repair

Open repair of an AAA is a major operation involving an incision from just below the breastbone to the top of the pubic bone to allow the surgeon to clamp the aorta and replace the dilated segment of the vessel with an aortic graft which can be sewn or 'stented' in place. The graft is sutured to the aorta connecting one end of the aorta at the site of the aneurysm to the other end of the aorta. The graft is made of a strong, durable, man-made plastic material such as Dacron (textile polyester synthetic graft) or polytetrafluoroethylene (PTFE) a non-textile synthetic graft. Subsequently the aortic clamps are removed to restore bloodflow once again. Following surgery complications that may occur include risk of haemorrhage, cardiac complications and ischaemia to the lower limbs and kidneys during or after the operation.

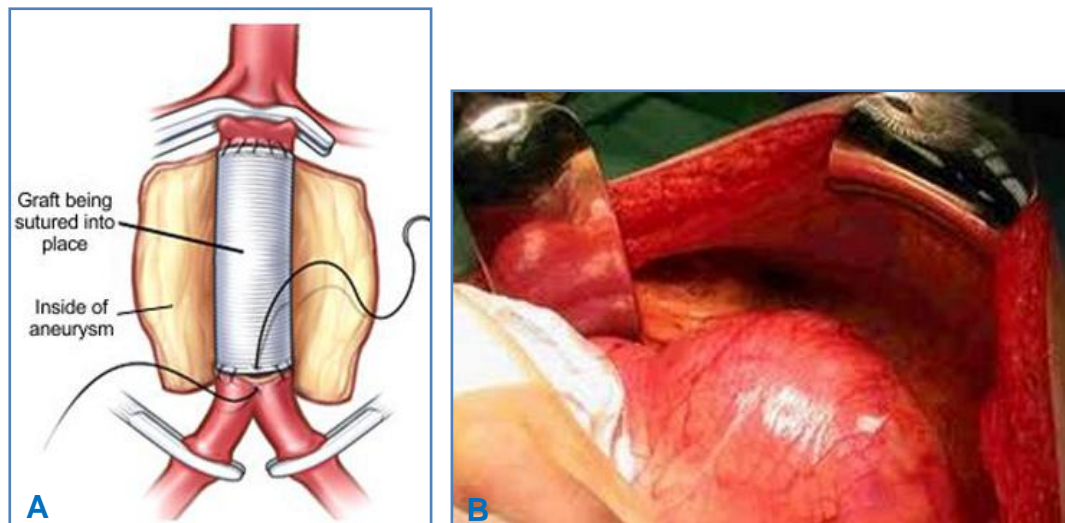


Figure 18. Diagram and Photograph Taken During Open Surgery for AAA

A: Diagram illustrating how the weakened segment of aorta is replaced with the artificial graft. **B:** Photograph taken during open surgical AAA intervention showing a large aneurysm prior to surgical repair (www.edinburghsurgeon.com, www.urmc.rochester.edu).

2.4.2.2 Endovascular aneurysm repair (EVAR)

EVAR was first pioneered in the early 1990s and is now widely used for the treatment of AAA. EVAR is performed by inserting a stent graft via the femoral artery (using x-ray images) for guidance into the lumen of the aneurysmal portion of the aorta. The endovascular stent graft consists of a graft that provides a conduit for blood flow and a stent that anchors the graft to the aorta and provides structural support for the graft material. The aim is to divert blood flow into the graft in order to bypass the aneurysm. The advantages of endovascular repair over open repair is that it is less invasive than open surgery and has a lower mortality rate and length of post-operative care in hospital is reduced. Disadvantages include the need for life-long follow-up imaging and the long-term durability of graft material is yet to be proven.

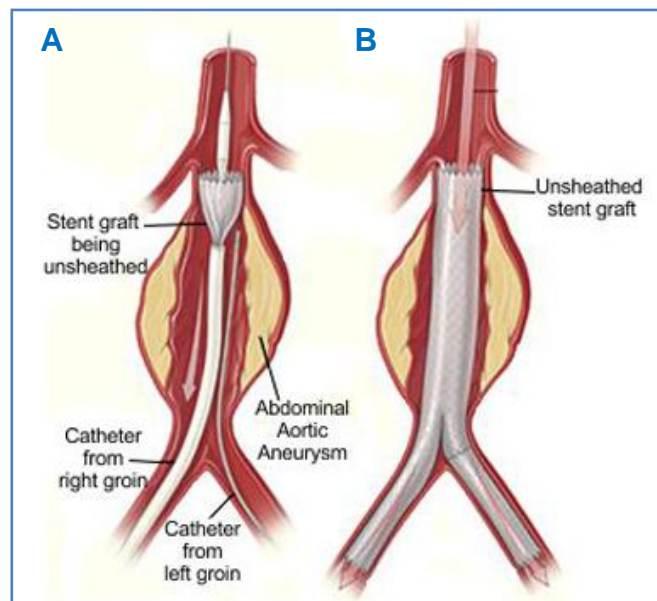


Figure 19. Diagram of Endovascular Aneurysm Repair (EVAR)

Diagram illustrating **A**: A catheter wire inserted via the common femoral artery and the stent graft passed up over the catheter wire into the aorta. **B**: The stent graft after it has been deployed to line and reinforce the aorta (www.edinburghsurgeon.com).

2.5 Medical Treatment of AAA

Patients diagnosed with a small aneurysm are given advice on diet and life style but additionally if they are hypertensive they will be prescribed medication such as angiotensin-converting enzyme (ACE) inhibitor to lower blood pressure which is a high risk factor for AAA. In addition, if they are diagnosed with high cholesterol they will also be prescribed statins (a group of cholesterol lowering medicines) as having a high level of LDL cholesterol is potentially dangerous as it can lead to a hardening and narrowing of the arteries and is also a risk factor for AAA. Statins reduce LDL by blocking the synthesis of cholesterol in the liver cells thereby lowering blood cholesterol levels.

2.6 Pharmacological research for Medical Treatment of AAA

Existing treatment of AAA involves routine screening for early detection of aneurysms, surveillance of small and medium sized AAAs and surgical intervention for larger aneurysms. Currently there are no proven pharmaceutical treatments for preventing the expansion and rupture of AAA, although many scientists are carrying out studies and clinical trials involving potential targets against AAA.

2.6.1 Therapeutic Targets for Treatment of AAA

2.6.1.1 Pro-inflammatory Mediators

Reactive oxygen species play a role in AAA possibly as pro-inflammatory mediators. Two animal studies found α -Tocopherol (vitaminE), a lipid-soluble antioxidant, was found to significantly attenuate the formation of AAA

(Nakahashi *et al.*, 2002, Gavrilu *et al.*, 2002). On the contrary a large randomized study reported that long-term supplementation with vitamin E did not reduce the rate of AAA rupture (Tornwall *et al.*, 2001).

2.6.1.2 17 β -Estradiol

Female reproductive hormones, such as estrogen may play protective roles against the development of AAA. Rats treated with 17 β -estradiol exhibited less macrophage infiltrate, lower MMP-9 levels and developed smaller aneurysms after elastase infusion compared to controls (Ailawadi *et al.*, 2004). Martin-McNulty *et al.*, (2003) also found in an Ang II-induced AAA model, treatment with 17 β -estradiol reduced expression of MCP-1, activity of NF- κ B, and aneurysm size.

2.6.1.3 Inhibitors of Renin-Angiotensin System (RAS)

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension, congestive heart failure and other cardiovascular disorders. The ACE inhibitors captopril, lisinopril, and enalapril all prevented the development of AAA created by elastase infusion in rats (Liao *et al.*, 2001). Fujiwara *et al.*, (2008) reported that treatment with Valsartan (an angiotensin II type 1 (AT1) receptor antagonist) prevented AAA development and infiltration of macrophages and suppressed NF- κ B activation and MMP-9 expression in elastase-induced AAA in rats. In contrast, another study found AT1 receptor blockers did not prevent AAA rupture (Hackman *et al.*, 2006) and furthermore Sweeting *et al.*, (2010) reported an association with increased AAA dilation.

2.6.1.4 Statins

The group of cholesterol-lowering drugs (3-hydroxy-3-methylglutaryl coenzyme A)(HMG-CoA) reductase inhibitors more commonly known as statins have been suggested as a possible therapy for AAAs based on their pleiotropic actions, independent of their lipid lowering effects (Takemoto and Liao, 2001, Schonbec. and Libby, 2004). Steinmetz *et al.*, (2005) found that simvastatin suppressed aneurysm formation in elastase-infused C57BL/6 wildtype and hypercholesterolemic *ApoE*^{-/-} mice and in elastase-induced AAA in rats where it also reduced levels of MMP-9 and NF- κ B (Kalyanasundaram *et al.*, 2006). Other studies suggested treatment with atorvastatin prevented AAA development, macrophage infiltration and suppression of MMP-9 and MCP-1(Shiraya *et al.*, 2009, Yoshimura and Aoki., 2012). Dunne *et al.*, (2014) reviewed the clinical evidence regarding the role of statins in the medical management of small AAAs and refuted the claim that statins attenuate AAA growth and that further studies with stringent identification, verification of statin usage and a standardised method of estimating AAA growth rates were required.

In contrast to this, research by Yoshimura *et al.*, (2015) indicated that statins preferentially inhibit the Rac1/NF- κ B pathway to suppress MMP-9 and chemokine secretion in human AAA and could attenuate AAA progression. Further studies by Piechota-Polanczyk *et al.*, (2015) found statin treatment in patients with AAAs may influence the concentration of proteases and their inhibitors (TIMPs) in aneurysmal wall tissue and intraluminal thrombi (LTs). Interestingly, Pafili *et al.*, (2015) looked at the effect of pharmacological agents used for the treatment of patients with diabetes mellitus on AAA formation and results showed statins, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, fenofibrate, antibiotics and some hypoglycemic agents are now been suggested to have a potential modest protection from AAAs.

2.6.1.5 Mast Cell Stabilizer

Mast cells are an inflammatory type of cell found in the walls of human AAA which, when activated, leads to the release of pro-inflammatory cytokines such as IL-6 and IFN- γ . In elastase-induced AAA in mice, disodium cromoglycate (DSCG) a mast cell stabilizer, significantly reduced AAA expansion, preserved the elastic lamina, decreased mast cell and macrophage infiltration, reduced IFN- γ , IL-6 and MMP activity (Sun *et al.*, 2007). Tsuruda *et al.*, (2008) using a different mast cell stabilizer known as tranilast, stated that, in calcium chloride-induced rodent models of AAA, tranilast attenuated AAA formation. Despite the interest in these drugs no clinical trial results are yet available to fully understand how effectively they might reduce AAA expansion.

More recently, Sillesen *et al.*, (2015) undertook a randomised clinical trial to investigate whether the mast cell inhibitor, pemirolast, could retard the growth of medium-sized AAAs but there was no significant difference in growth between patients receiving the placebo and those receiving pemirolast and they concluded that treatment with pemirolast did not retard the growth of medium-sized AAAs.

2.6.1.6 Intracellular Signaling Pathways

Signaling pathways may be effective in treating experimental AAA, including the Rho/Rhokinase, NF- κ B, and JNK pathways. Treatment with fasudil, a Rhokinase inhibitor resulted in reduction of both the incidence and severity of Ang II-induced AAA in apolipoprotein E-deficient mice in addition to inhibiting VSMC apoptosis and proteolysis by MMP-2 and 9 (Wang *et al.*, 2005). NF- κ B inhibition by pyrrolidine dithiocarbamate (PDTTC) suppressed development of elastase-induced AAA in mice (Parodi *et al.*, 2005). Pharmacological inhibition of c-Jun N terminal kinase (JNK) with SP600125, a specific JNK inhibitor, has been reported to prevent or regress AAA in CaCl₂-induced

mouse AAA and AngII-induced ApoE^{-/-} mouse AAA models suppressing MMP activity and migration of inflammatory cells (Yoshimura *et al.*, 2005).

Vorkapic *et al.*, (2016) investigated the importance of tyrosine kinase pathways in the development of aneurysms using Imatinib, a selective inhibitor of several tyrosine kinases, in a mouse model of AAA. Treatment with Imatinib decreased aortic diameter and vessel wall thickness indicating tyrosine kinase inhibitors may be useful in the treatment of pathological vascular inflammation and remodelling in AAA.

2.6.1.7 Enzymes for ECM Metabolism

MMPs are well known for their role in the progression of AAA and therefore are possible targets for pharmacological intervention. Treatment with doxycycline, a tetracycline derivative reduced MMP-9 production and caused a significant reduction in the incidence of AAA induced by elastase infusion in rats (Petrinec *et al.*, 1996). Since then many studies using doxycycline have been carried out under a variety of different experimental conditions that have supported Petrinec's research (Curci *et al.*, 1998, Huffman *et al.*, 2000, Prall *et al.*, 2002, Manning *et al.*, 2003, Kaito *et al.*, 2003, Sho *et al.*, 2004, Bartoli *et al.*, 2006, Vinh *et al.*, 2008). A number of studies into the effect of doxycycline on human AAA have also been carried out. Preoperative treatment with doxycycline was found to decrease MMP-9 expression in aneurysm tissue (Curci *et al.*, 2000). Doxycycline was also found to slow down AAA expansion in comparison to patients treated with a placebo during a randomized trial (Mosorin *et al.*, 2001). Another clinical trial demonstrated the safety of doxycycline and its ability to reduce MMP-9 plasma levels (Baxter *et al.*, 2002). Lindeman *et al.*, (2009) also reported that doxycycline inhibited infiltration of neutrophils and cytotoxic T cells in the walls of human AAA, thus reducing inflammatory effects.

Systemic treatments of MMP inhibitors have shown effectiveness in animal models, but often did not translate to clinical success either because of the low doses used or due to systemic side effects of MMP inhibitors. Nosoudi *et al.*, (2015) proposed a targeted nanoparticle (NP) - based delivery of MMP inhibitors at low doses to the AAA site for preventing expansion of aneurysms in patients without systemic side effects. Intravenous injections of elastin antibody-conjugated BB-94 (MMP inhibitor batimastat) - loaded NPs targeted to the site of aneurysms in a rat model of AAA resulted in inhibited MMP activity, elastin degradation, calcification and aneurysmal development in the rat aorta.

Baxter *et al.*, (2016) are currently enrolling patients into their Non-Invasive Treatment of Abdominal Aortic Aneurysm Clinical Trial (N-TA³CT) which is a Phase IIb randomized, placebo-controlled clinical trial testing doxycycline to determine if it reduces the growth of small AAAs and systemic markers of inflammation over a 24 month time period. They predict the outcome will demonstrate at least a 40% reduction in aneurysm growth rate.

2.6.1.8 Anti-inflammatory agents

Prostaglandins act as a class of biological mediators in the inflammatory process, and are converted from arachidonic acid by cyclooxygenase (COX). The expression of COX-2 but not COX-1 is upregulated in human AAA tissue (Holmes *et al.*, 1997). King *et al.*, (2006) using the selective COX-2 inhibitor celecoxib found it decreased the incidence and severity of AAA formation in Ang II-induced ApoE^{-/-}-mouse AAA models, whereas selective COX-1 inhibition had no effect on AAA formation. Additionally, the incidence of Ang II-induced AAA was reduced in COX-2-deficient mice in comparison to wild-type mice (Gitlin *et al.*, 2007).

Inflammation has an important role in AAA progression and several studies have attempted to attenuate aneurysm formation by targeting inflammatory

cells such as macrophages. Iida *et al.*, (2013) demonstrated the ability of the peptide CXCL4-CCL5 heterodimer inhibitor MKEY to both suppress experimental AAA initiation and stabilize existing aneurysms in murine models through the impaired infiltration of mural monocytes/macrophages.

Yan *et al.*, (2016) investigated the effects of zinc which is known to have an anti-inflammatory action (Bao *et al.*, 2010) on AAA progression and its related molecular mechanisms in a rat model of AAA. The results showed that zinc supplementation significantly suppressed the CaCl₂-induced expansion of the abdominal aortic diameter, preserved aortic medial elastin fibres, attenuated macrophage and lymphocyte infiltration and reduced MMP-2 and MMP-9 production. This study also demonstrated that treatment with zinc could prevent the development of rat experimental AAA by induction of A20 (a zing-finger transactivating factor) - mediated inhibition of the NF-κB canonical signalling pathway. Kortekaas *et al.*, (2014) evaluated the anti-inflammatory potential of ACE (angiotensin-converting-enzyme) inhibitors (independent of their blood pressure lowering effect) in AAA patients who were treated with ramipril 2-4 weeks preceding surgery. Post-surgical analysis of AAA tissues showed ACE inhibition quenched multiple aspects of vascular inflammation in AAA but this did not translate into reduced aneurysm growth. An earlier study by Takai *et al.*, (2013) had also looked at the effect of ACE inhibitors against elastase-induced AAA progression in mice and concluded MMP-9 inhibition by imidapril might contribute to the attenuation of AAA progression in AT1 receptor-knockout mice.

Chapter 3. General Methodology

3. General Methodology

3.1 Cell Culture

3.1.2 *Materials List*

- HASMCs (TCS Cellworks and Caltag Buckingham, UK).
- Dulbecco's Modified Eagles Medium (DMEM) Invitrogen, Paisley, UK)
- Foetal calf serum (FCS) (GlobePharm LTD. UK).
- Penicillin/Streptomycin (Invitrogen. Paisley, UK)
- Phosphate Buffered Saline (Invitrogen. Paisley, UK)
- Accutase (Innovative Cell Technologies Inc.).
- L-Glutamine (Sigma Aldrich. Dorset, UK).
- Smooth Muscle Cell Complete Medium (Caltag. Buckingham, UK)
- Glass cover slips (VWR International Ltd. Dorset, UK).
- T25, T75, T150 filter-cap flask and 6/12 well plates (Triple Red Ltd. Oxfordshire, UK).
- Lab-Tek Chamber slides (Nalgene Nunc International).

3.1.3 *Cell Culture Protocol*

The primary HASMC culture was seeded into T25 flasks and routinely grown in DMEM supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin or Smooth Muscle Cell Complete Medium which was replaced every two to three days. The cells were kept in 5% CO₂, 95% air at 37°C in a humidified Jencons Millenium

incubator (Jencons Ltd. Bedfordshire, UK). Upon reaching confluence (100% monolayer covering flask base) the cells were split 1:5 into T75 flasks. HASMCS were washed in PBS then dissociated from the flasks using 2mls Accutase /T25 and incubated at 37°C for approx 2-5 minutes. DMEM was added to inactivate the Accutase and the cells were brought out of suspension by centrifugation at 1600rpm for 5 minutes. The supernatant was removed and the pellet resuspended in fresh DMEM. For hypoxia studies cells were passaged into 6-well plates or for cell staining experiments grown on sterilised 13mm diameter glass coverslips in 6/12-well plates or chamber slides.



Figure 20 Laminar Flow Cabinet

All cell culture work was carried out under aseptic conditions inside a laminar flow cabinet which was switched on 10 minutes prior to use to allow air flow to reach safe levels and was thoroughly sprayed with 70% alcohol before using.

3.1.4 HASMC Cell Storage and Resurrection of Cells

Cell stocks were routinely stored in liquid nitrogen. Confluent T25-flasks were harvested using Accutase solution, following which the cell pellet was then resuspended in a freeze-down medium, consisting of 50% FCS, 40% growth medium and 10% DMSO. 1ml volumes of freeze-down medium were used to resuspend the pellet from each T25 flask of cells. Cell suspensions were stored in NUNC Cryo-tubes and frozen to -70°C at -1°C per min in the Cryo 1°C Freezing Container and then placed into liquid nitrogen storage.

Cells were resurrected rapidly by placing NUNC Cryo-tubes under warm running water. Cells were washed once by centrifugation of the cell suspension at 1600rpm for 5mins and the subsequent pellet resuspended in normal DMEM-based medium.

3.1.5 Trypan Blue Exclusion Cell Viability Counts

The Trypan Blue exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as Trypan blue which cannot permeate viable cells and appear clear under the light microscope, dead or compromised cells take up the Trypan blue dye and appear blue in colour.

3.1.6 Materials List

- Accutase (TCS Cellworks LTD. Buckinghamshire, UK),
- Trypan Blue (Sigma Aldrich Company Ltd. Dorset, UK).

3.1.7 Trypan Blue Exclusion Cell Count Protocol

Cells grown in 12-well plates or T25-flasks were dissociated by incubation with 500µl or 2000µl Accutase, respectively, per well/flask at 37°C, 5%CO₂ for 5-10 minutes. The cell suspension was mixed 1:1 with Trypan blue and cells were counted using a Neubauer double rhodium coated counting chamber (Fisher Scientific UK Ltd). Approximately 15µl of the cell/Trypan blue mixture was applied to the counting chamber and blue (dead) and clear (viable) cells were counted from five of the nine 1mm² squares on the counting chamber (as indicated in Figure 21). The average number of cells per square was used in the calculation of the number of cells per ml of the starting cell suspension, as shown below:

$$\text{Viable cells/ml} = \text{Mean cells per square} * 2 * 10,000$$

- ***2** (Dilution factor of Trypan blue).
- ***10,000** (Volume correction from volume of 1mm square under cover slip, 10⁻⁴ml to 1ml).

The percentage of viable cells can be calculated after repeating the above calculation for Trypan blue stained cells.

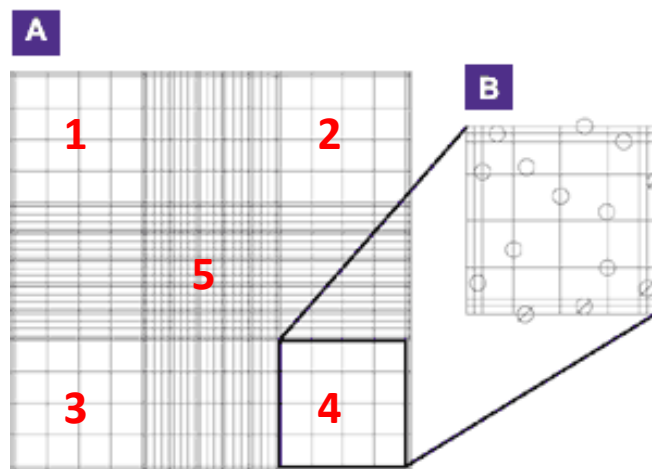


Figure 21 Cell Counts Using a Haemocytometer

The typical grid layout of the haemocytometer, divided into nine 1mm squares. Cell counts were taken from the five numbered squares (as indicated in A). Only cells in contact with two of the four boundaries of each square were counted (as indicated in B).

3.2 Hypoxia Studies

Human aortic smooth muscle cells (HASMCs) were passaged into 6-well plates and grown to sub-confluence under normal conditions in the presence of 10% FCS in DMEM with additions of 2mM L-glutamine, penicillin (10,000 IU/ml) and streptomycin (10,000 IU/ml). The cells were then maintained in serum-free medium under severe hypoxia (5%CO₂, 94%N₂ and 1%O₂) inside a sealed Minimac hypoxia chamber (Figure 22) at 37°C for up to 72 hours. Controls were maintained throughout the 72 hour period in an incubator at 37°C in 95% air, 5%CO₂. Conditioned media and cell lysate samples were collected at the termination of allotted time points and stored at -80° for later analysis. Additionally cells were also grown on 13mm diameter coverslips in 6 or 12-well plates under severe hypoxic conditions

for immunostaining purposes or under normal oxygen levels for comparison or characterisation purposes.



Figure 22 Don Whitley Minimac Hypoxia Chamber

This workstation was used to maintain the HASMCs under severe hypoxic conditions (1% hypoxia) fully incubated at 37 °C. The 'rapid lock' manually-operated portholes allow both access for the operator's arms and the transfer of samples in and out of the chamber with the automatic gas refill system for the sleeves, ensuring the internal atmosphere is not compromised by the transfer of oxygen through the ports to provide a strict hypoxic environment.

The ability to manipulate samples under hypoxic conditions without compromising the chamber atmosphere is important as some proteins, especially those in the HIF-1 family are rapidly degraded once the cells become normoxic again, therefore it is vital to collect the proteins without having to expose the cells to normal oxygen levels.

To further ensure that a strict hypoxic environment was being maintained, a calibrated oxygen sensor (Analox Oxygen Analyser) shown in Figure 23 below was placed inside the hypoxia chamber to monitor oxygen levels at all

times but especially important during the manipulation and removal of samples at allotted time-points.



Figure 23. Analox Oxygen Analyzer (Analox Sensor Technology Ltd, Yorkshire, UK

3.3 Immunostaining

Immunohistochemistry (IHC) is a technique widely used in all areas of research and refers to the process of detecting antigens within cells of a tissue section by the interaction of antibodies tagged with a visible label targeted against and binding specifically to specific antigens. Correct processing of the tissue section allows the ultra structure of the tissue to be maintained prior to IHC which subsequently enables visualisation of the distribution and immunolocalisation of specific cellular antigens within the proper tissue context.

Antibody-mediated antigen detection techniques are separated into direct and indirect methods. For this study the more sensitive indirect method was employed involving an unlabelled primary antibody that binds to the target antigen and a labelled secondary antibody that reacts with the primary antibody. The secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised and results in

signal amplification due to the binding of several secondary antibodies to each primary antibody. IHC target antigens can be detected through fluorescent or chromogenic techniques which involve enzymatic activity resulting in the production of a coloured precipitate. In this case a commercially available kit based on the biotin/avidin system was used in most of the immunostaining procedures. Avidin, a large 68,000 molecular weight glycoprotein can be labelled with alkaline peroxidase and has a very high affinity for the low molecular weight vitamin biotin which can be conjugated to a variety of biological molecules such as antibodies. (Vector Laboratories).

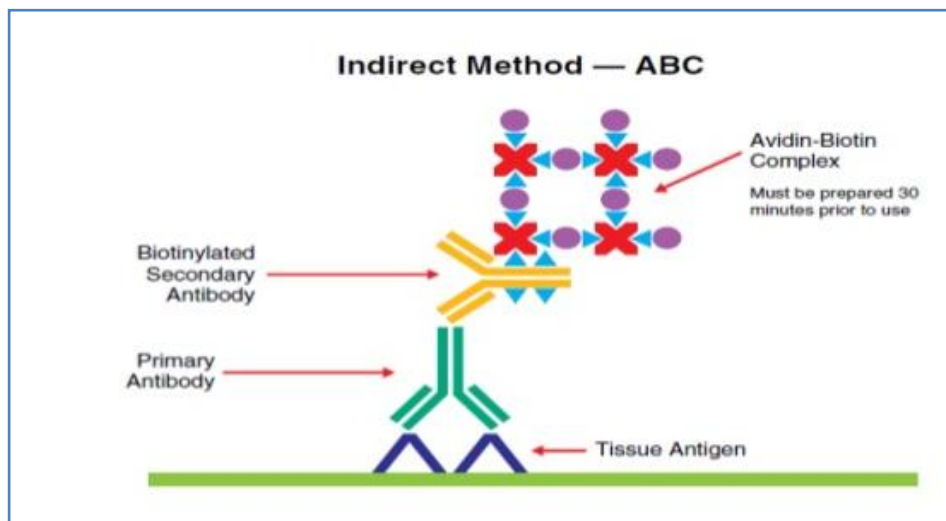


Figure 24 The Avidin-Biotin Complex

An unlabelled primary antibody reacts with its target antigen and a biotinylated secondary antibody which is raised against the IgG of the primary antibody. The biotin molecule is easily conjugated to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin- complex.

3.3.1 Fast Red Staining of HASMCs and Fibroblasts

3.3.2 Materials List

- Phosphate buffered saline ((Invitrogen. Paisley, UK).
- Methanol (VWR International Ltd. Dorset, UK), Acetone VWR International Ltd. Dorset, UK).
- Tween-20 (polyoxyethylene-sorbitan monolaurate) (Sigma Aldrich. Dorset, UK).
- Horse serum (Sigma Aldrich. Dorset, UK).
- Alkaline phosphatase-conjugated avidin biotin complex, antimouse IgG (VECTASTAIN[®] ABC-AP kit (Vector Laboratories Ltd. Peterborough, UK).
- Levamisole Solⁿ (Vector Laboratories Ltd. Peterborough).
- Naphthol AS-MX phosphate/Fast red TR Fast TM (Sigma Aldrich. Dorset, UK).
- Superfrost Plus Microscope Slides (VWR International Ltd. Dorset, UK).
- Aquamount (VWR International Ltd. Dorset, UK).
- Primary and secondary antibodies

3.3.3 Fast Red Staining Protocol

HASMCS and fibroblasts were grown on 13mm diameter coverslips in 6 or 12-well plates for characterisation purposes. Conditioned media was aspirated from the wells, cells were washed with PBS which was subsequently removed and replaced with ice-cold acetone/methanol (50:50) for fixation. After incubation the fixative was aspirated, the cells washed in PBS and then incubated for 30 minutes in blocking buffer (0.5% Tween-20 and 1.5% horse serum in PBS) at room temperature. The blocking buffer was removed and cells incubated in primary antibodies for 2 hours at room temperature. Excess 1^o antibody was removed by washing cells in PBS

three times for five minutes after which the secondary antibody biotinylated horse anti-mouse IgG.(VECTASTAIN® ABC kit) was applied for 30 minutes to one hour at room temperature and subsequently removed by a repeat of the previous wash steps. The cells are then incubated in the ABC reagent solution (prepared according to the manufacturer's instructions) for 30 minutes at room temperature and the previous wash steps repeated. Visualisation of positively stained cells was obtained using Naphthol AS-MX phosphate/Fast red TR Fast during which Levamisole (125mM) can be added to the solution to inhibit any endogenous alkaline phosphatase activity. The reaction was followed under the microscope and when optimal staining was achieved the reaction was stopped by rinsing in running water followed by counterstaining with Mayer's Haematoxylin for approximately two minutes. Finally the coverslips holding the stained cells were removed from the 6 or 12-well plates and placed cell-side down onto glass slides using Aquamount mounting medium and allowed to dry at room temperature. Samples were viewed under a Zeiss Axioscope microscope and images were captured using the Zeiss KS300 program (Carl Zeiss Ltd. Hertfordshire, UK).

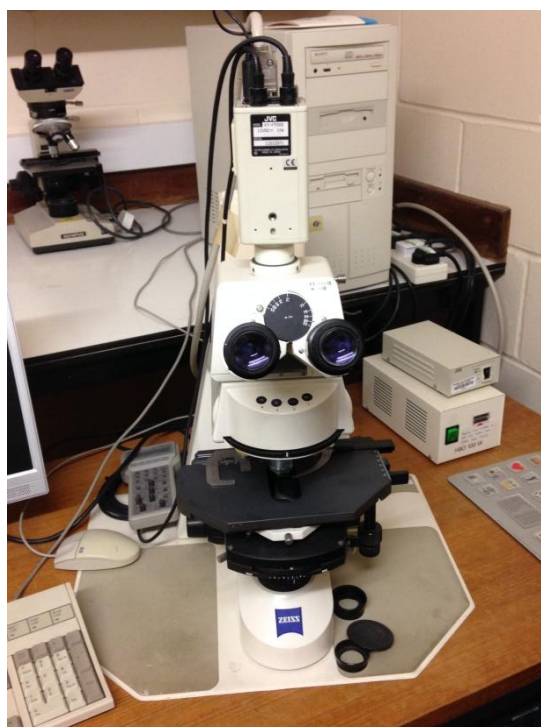


Figure 25 Zeiss Axioscope Fluorescent Microscope



Figure 26 Phase Contrast Microscope

This microscope enabled living cells to be visualised and photographed. Also it allowed daily monitoring of proliferating cells for growth progression and to check if cells are free from infection.

3.4 Bradford Assay

Protein concentration prior to sample analysis was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hertfordshire, UK).

The principle underlying Bradford assay is the binding of the Coomassie Blue G250 dye to proteins which can be utilized to measure the concentration of total protein in a sample. This method actually measures the

presence of the basic amino acid residues, arginine, lysine and histidine which contributes to formation of the protein-dye complex and under acidic conditions results in a color change in the dye from brown to blue (Bradford 1976).

3.4.1 Bradford Assay Protocol

The Biorad microtitre plate protocol was used (96-well plate). Serial dilutions of samples (10 μ l) were placed in triplicate into separate wells. Biorad dye reagent was diluted 1:5 with dH₂O and then filtered through watman paper. 200 μ l of the diluted dye reagent was added to each sample and allowed to incubate for at least 5 minutes at room temperature. Following this, absorbance (OD) was measured spectrophotometrically at 595nm in a plate reader.

Biorad bovine serum albumin (BSA) was used as a standard (1.4mg/ml stock). Doubling dilutions of the BSA were prepared to a concentration of 0.01 μ g/ μ l and 10 μ l of each dilution was added to wells in triplicate. Dye reagent was added as before and absorbances read at 595nm. Prism software was used to create a standard curve from which the unknown protein concentrations in the samples can be extrapolated.

Chapter 4. Analysis of MMP-2 and MMP-9 Levels in HASMCs Exposed to Severe Hypoxia

4. Analysis of MMP-2 and MMP-9 levels in HASMCs exposed to severe hypoxia.

4.1 Introduction

Increased matrix metalloproteinase (MMP) activity is implicated in aneurysm formation through elastin destruction and collagen degradation. MMP-2 is thought to play an important role in the initiation of aneurysm formation and is the dominant gelatinase in early aneurysms, whilst MMP-9 has significant role in later aneurysms (Freestone *et al.*, 1995).

The aorta contains several different cell types such as fibroblasts, smooth muscle cells (SMCs) and endothelial cells but SMCs are considered the principal cell type in the tunica media of the aorta. Crowther *et al.*, (2000) demonstrated SMCs derived from aneurysmal aorta produce increased levels of MMP-2 and are the primary source of MMP-2 in AAA whilst macrophages are the primary source of MMP-9 in aneurysms (Hibbs., 1992). The mechanism for AAA induction has not been fully elucidated but arterial wall hypoxia has been proposed as a candidate for the initiation of aneurysm formation. Animal models of AAA have demonstrated evidence of low oxygen tension within aortic media (Bjornheden *et al.*,1999) which amongst other things may alter smooth muscle cell function resulting in aortic degradation and potential aneurysm formation (Vorp *et al.*, 1996). Furthermore, research has shown that hypoxia exists *in vivo* in aneurysms of patients with intraluminal thrombus, a common feature of AAA which contributes to arterial wall hypoxia and weakening (Vorp *et al.*, 1998, 2001).

Previous research by our Group (Erdozain *et al.*, 2011) demonstrated significant increases in MMP-2 and MMP-9 in HASMC conditioned media after exposure to sequential decreases in oxygen tension (5-1%) over a 96 hour time period. However, cell viability assays (lactate dehydrogenase) carried out on all hypoxic and normoxic samples found NADH production in supernatants from cells exposed to hypoxia for longer than 72 hours, therefore, evaluation of samples at 1% oxygen tension which were beyond

this time-point were not carried out. In light of these results, the aim of this further investigation was to assess HASMC MMP secretion and induction times after exposure to constant severe hypoxia (1%) over a 48 hour time period rather than the previous sequential decreases. Previously, cells were maintained in media containing foetal calf serum (FCS) throughout the period of hypoxia and therefore in these experiments, in order to rule out foetal calf serum as a confounding factor, cells were maintained in serum-free media 24 hours prior to and during exposure to hypoxia.

4.2 Chapter Aims

The hypothesis suggests that levels of MMP-2 and MMP-9 protein will be increased during exposure to severe hypoxia.

The aim of the chapter will be:

1. To culture stocks of HSMCs to generate sufficient cells for the experimental hypoxia study and characterisation.
2. To culture human neonatal fibroblast cells for immunocytochemical characterisation control purposes.
3. To determine whether MMP-2 and MMP-9 has been secreted into the HSMC conditioned media after exposure to prolonged 1% hypoxia or normal oxygen levels using zymography techniques.
4. To determine if there are differences in secreted MMP levels between the hypoxic and normoxic samples.
5. To corroborate the identity of the MMPs present in the culture supernatant by immunoblotting.
6. To perform cell viability assays on HASMCs exposed to severe hypoxia.
7. To carry out inhibition assays to further identify sources of gel proteolysis.
8. To confirm the localisation of MMP-2 and MMP-9 in control and aneurysmal tissue using immunohistochemical techniques.

4.3 HASMC Characterisation

Prior to any experimentation cell lineage of all cultures were confirmed by immunostaining for SMC - specific α -actin (SMA) which is a 42 kDa protein and one of the six isoactins expressed in mammalian cells. SMA is the most abundant of all the isoforms constituting 40% of total cell protein and over 70% of total actin content (Fatigati *et al*, 1984). Additionally HASMCs were probed with antibodies against the 55 kDa intermediate filament protein desmin. Fibroblasts are the primary contaminating cell type in VSMC cultures therefore neonatal skin fibroblasts were also established under exactly the same culture conditions as for HASMCs to use as a comparison and provide negative controls.

4.3.1 Fast Red Staining of HSMCs and Fibroblasts

HASMCS and fibroblasts were grown to subconfluence on 13mm diameter coverslips placed in 6-well plates. After fixation in ice-cold acetone/methanol (50:50) cells were incubated in blocking buffer (0.5% Tween-20 and 1.5% horse serum in PBS) for 30 minutes followed by a 2 hour incubation in the following primary antibody solutions (all supplied by DAKO Ltd. Cambridgeshire, UK) monoclonal mouse anti-human smooth muscle actin (1:100), monoclonal mouse anti- *Aspergillus niger* glucose oxidase (negative control) (1:100), mouse monoclonal anti-prolyl 4-hydroxylase (1:100), mouse monoclonal anti-human desmin (1:50) and monoclonal mouse anti-human smooth muscle myosin heavy chain (1:50). A biotinylated horse anti-mouse IgG secondary antibody was applied for one hour at room temperature followed by incubation for 30 minutes in Vectastain's ABC reagent solution.

Visualisation of positively stained cells was obtained using Napthol AS-MX phosphate/Fast red and the reaction followed under the microscope until a pink/red precipitate appeared. After counterstaining in Mayer's haematoxylin for approximately 2 minutes, cells were mounted cell-side down onto

microscope slides and viewed under a Zeiss Axioscope microscope and images were captured using the Zeiss KS300 program (Carl Zeiss Ltd. Hertfordshire, UK). The full protocol has been described earlier in Chapter 3: General Methodology.

4.3.2 HSMC Characterisation Results

4.3.2.1 Fibroblasts and HASMCs under the Microscope

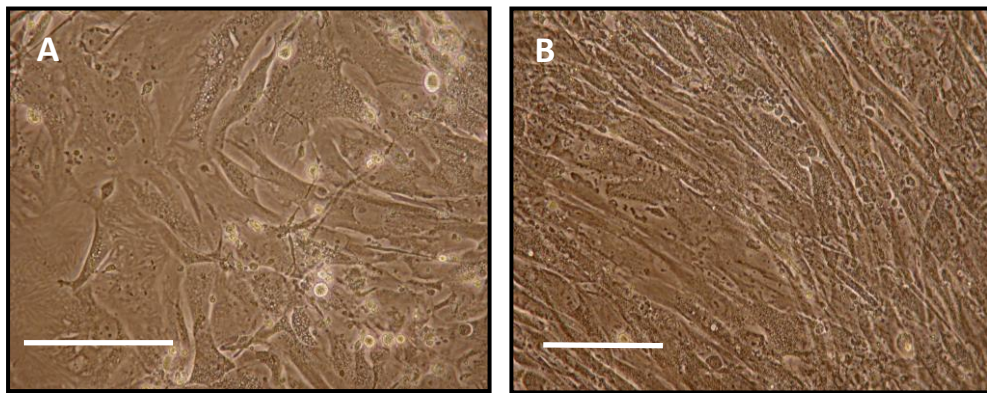


Figure 27 Living Fibroblasts in Cell Culture

Living fibroblasts were viewed under phase contrast microscopy (Nikon Eclipse TS100. Nikon Instruments UK) and images were captured using a Nikon Coolpix digital camera (Nikon UK Ltd. Surrey, UK). A and B show sub-confluent and confluent fibroblasts respectively (Bar = 50 μ m).

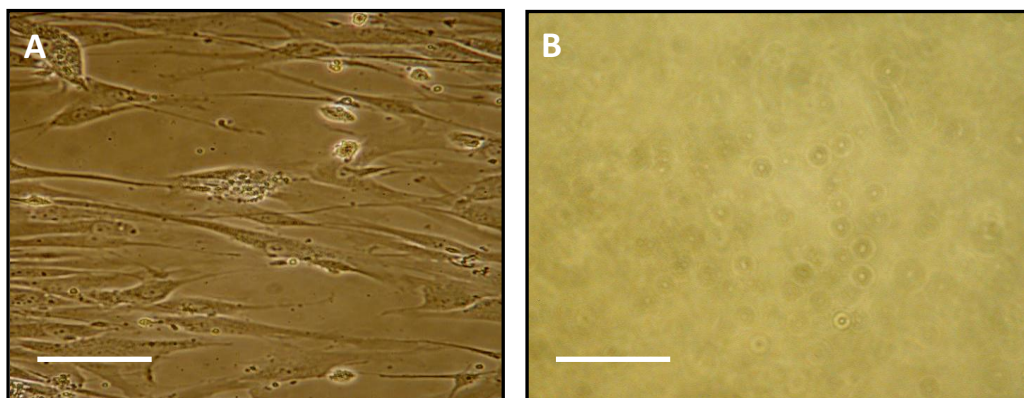


Figure 28 Living HASMCs in Cell Culture

Living HASMCs were viewed under phase contrast microscopy. A. Sub-confluent HASMCs. B. HASMCs after being subjected to Accutase treatment and losing adhesion from the flask ready for subculture respectively (Bar = 50 μ m).

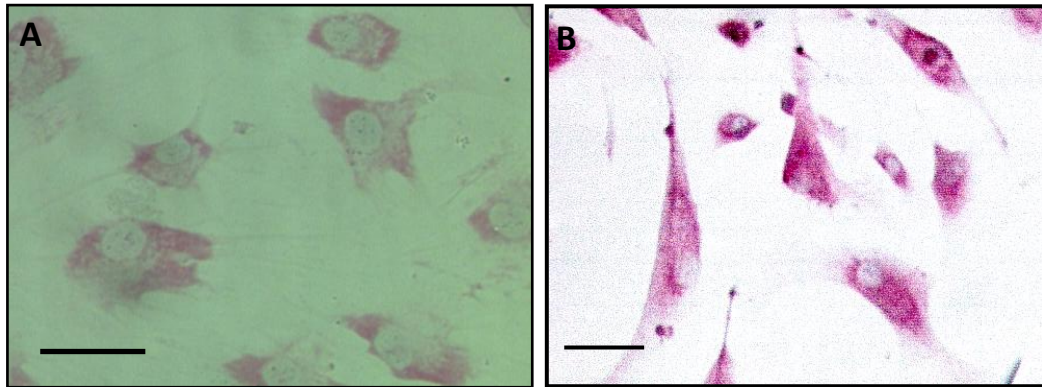


Figure 29 Immunostaining of HASMCs and Fibroblasts

HASMCs (A) and fibroblasts (B) grown on glass coverslips were labelled with anti-human prolyl 4-hydroxylase (fibroblast) primary antibody (1:100 dilution) and biotinylated anti-mouse IgG secondary antibody (1:200 dilution) then visualised using Fast Red. Nuclei were counterstained using Mayers Haematoxylin. (Bar = 25µm).

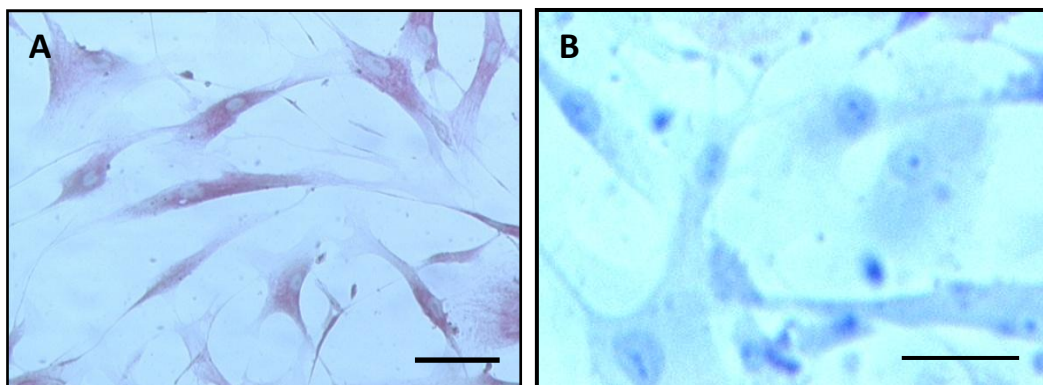


Figure 30 Immunostaining of HASMCs and Fibroblast with Alpha Actin

HASMCs (A) grown on glass coverslips were labelled with anti-human SMC-specific α -actin (SMA) primary antibody 1:100 dilution. Fibroblasts (B) were also probed with SMA for comparison 1:100 dilution A biotinylated anti-mouse IgG secondary antibody (1:200 dilution) was applied to all samples. Nuclei were counterstained using Mayers Haematoxylin. Localisation of α -actin was visualised using Fast Red and viewed under a Zeiss Axioscope microscope, images were captured using the Zeiss KS300 program. (Bar = 25µm).

Under phase contrast microscopy, cultured HASMCs revealed a typical spindle-shaped morphology containing an oval shaped nucleus which grew to form regularly oriented parallel bundles attaining the characteristic smooth muscle “hill and valley” pattern on reaching 100% confluence (Chamley-Campbell *et al.*, 1979) However a few cells exhibited a more spread out flattened morphology which is not unusual in cultured smooth muscle cells (Kocher and Gabbiani,1987).

Fibroblasts generally have a more flattened appearance than smooth muscle cells and contain a large rounder shaped nucleus containing more nucleoli (Chamley-Campbell *et al.*, 1979) Immunocytochemical staining of the HSMCs for α SMA showed a very positive pinkish red cytoplasmic stain at the 1:100 dilution In comparison, the fibroblasts were negative for SMA staining as expected but positive for prolyl 4-hydroxylase.

The cells were also probed for desmin (not shown) but gave negative or very weakly positive results which correlates with the findings of Absher *et al.*, (1989) who noted that although *in vivo* desmin is the intermediate protein expressed, in culture SMCs can very rapidly alter their cytoskeletal protein profile and express vimentin rather than desmin.

All controls slides gave negative results with no staining observed when primary antibodies were omitted or after probing with the *Aspergillus niger* glucose oxidase antibody, as this protein should not be present within mammalian cells.

4.4 Gelatin zymography

4.4.1 Principles of Zymography

Expression of MMP-2 and MMP-9 in conditioned media from HASMCs after exposure to severe hypoxia for up to 48 hours was studied using the technique gelatin zymography.

Zymography is a sensitive method for examining matrix metalloproteinases. Gelatin is the most commonly used substrate and is useful for demonstrating the activity of the gelatinases such as MMP-2 and MMP-9. This technique is a modification of SDS-PAGE (sodium dodecylsulphate polyacrylamide gel), based on the incorporation of an *in situ* enzymatic substrate (gelatin) into the electrophoretic gel at the time of polymerization. Proteolytic activity is reversibly inhibited by SDS during electrophoresis and recovered by incubating the gel in the appropriate buffers delaying proteolysis until the sample proteins have been resolved into bands of concentrated activity. The advantage of this system is the detection of both the proenzyme and active forms of MMPs, which can be distinguished on the basis of molecular weight and is possible because the proenzymes are activated *in situ* as the latent MMP unfolds due to action of SDS. The zymogram is subsequently stained with Coomassie Brilliant Blue where areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme allowing the visualization of gelatin-degrading enzymes.

4.4 2 Gelatin Zymography Protocol

MMPs were separated on a vertical 7.5% polyacrylamide resolving gel containing 1% gelatin and 4% polyacrylamide stacking gel. Electrophoresis was performed at 125 V and allowed to run until the gel-front neared the end of the resolving gel, followed by incubation for 1.5 hours in 2.5% Triton X-100 at room temperature to renature the proteins, with subsequent

incubation at 37°C in development solution (50Mm CaCl₂, 0.5M Tris-HCL pH 7.6) for between 24-48 hours. Thereafter the gels were stained with 0.2% Coomassie Brilliant Blue R-250 for approximately one hour and then destained in methanol, glacial acetic acid and dH₂O (4:1:5, v/v/v) following which the zymogram was left for 48 hours in water to enhance band resolution. Gelatinolytic activity was demonstrated as clear bands against a blue background of stained gelatin. Purified human MMP-2 and MMP-9 protein were used as standards to identify the protein bands. Semiquantitative image analysis was carried out using Scion Imaging Software (version 4.0.2; www.scioncorp.com) to evaluate the relative enzymatic activity in ADU (Arbitrary Density Units)/ml⁻¹mg cellular protein.

4.4.2.1 Dilution of samples

After equally loading and running several gels it became clear that to obtain decent bands at all time-points was not possible. The early time-points required sufficient cell culture supernatant in order to digest the gelatin contained within the polyacrylamide gel and produce bands of lysis, but for the later time points this resulted in very intense bands due to the high levels of MMP-2 enzymatic activity which were also prone to merge into one another making an accurate densitometric analysis very difficult. Additionally loading too much protein can also result in smears or streaking across the polyacrylamide gel. In order to rectify this problem the culture supernatants appertaining to the later time-points were diluted and these dilutions taken into account during the final analysis and calculations. Figure 31 shows an example of this problem and the subsequent solution.

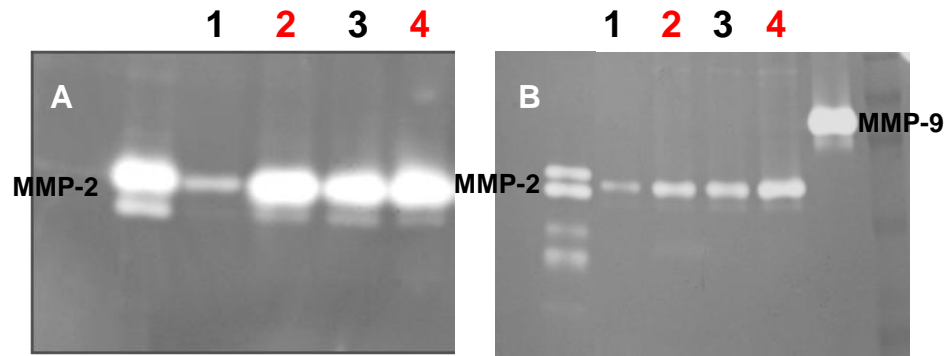


Figure 31 Comparison of Diluted and Undiluted Samples

Slides A and B show bands of lysis from HAMC culture supernatant samples at time-points: 30 hours (lanes 1 and 2) and 48 hours (3 and 4). Lanes containing hypoxic samples are annotated in red and normoxic samples annotated in black.

Slide A: Demonstrates how difficult it is to visually detect the difference between the undiluted samples in lanes 2 to 4.

Slide B: Shows the same samples after dilution which produced much clearer separate bands of lysis making density analysis far more accurate as there is more contrast for the imaging programme to detect and no merging at the outer regions of lysis between samples.

4.4.2.2 Results of Gelatin Zymography

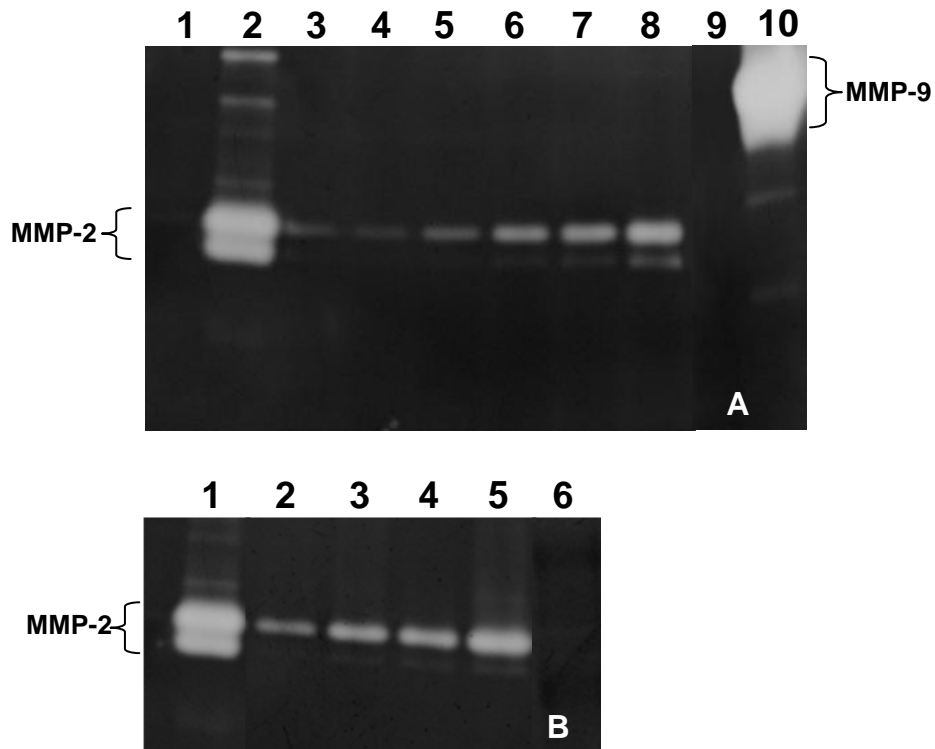


Figure 32 Representative Zymography Gels Showing Analysis of MMP-2 and MM-9 Levels in HASMC Supernatant.

Culture supernatants from HASMCs exposed to 1% hypoxia or normoxia for 48 hours were equally loaded onto 7.5% polyacrylamide gels containing 1% gelatin. Electrophoresis was performed at 125V after which the proteins were renatured in 2.5% Triton-X solution followed by a 24 hour incubation in development solution and subsequent staining with 0.2% Coomassie Blue R-250 and destaining in methanol, glacial acetic acid and dH₂O (4:1:5, v/v/v). Purified human MMP-2 and 9 standards (Chemicon Europe Ltd. UK) served as positive controls and sample buffer as a negative control.

Slide A: 1: Negative control (sample buffer) 2: Positive control (Purified human MMP-2) 3: 1hrN 4: 1hrH 5: 3hrsN 6:3hrsH 7: 6hrsN 8:6hrsH 9: RSM10: Positive control (Purified human MMP-2).

Slide B: MMP-2 enzymatic digestion of the gels gave very intense bands at the latter time-points (30 hrs, 48 hrs) therefore for more accurate analysis to the culture supernatant relating to these time-points was diluted before loading and then adjusted for in the final calculations. 1: Positive control (Purified human MMP-2) 2: 30hrsN 3:30 hrsH 4: 48hrsN 5: 48hrs H 6: RSM. (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

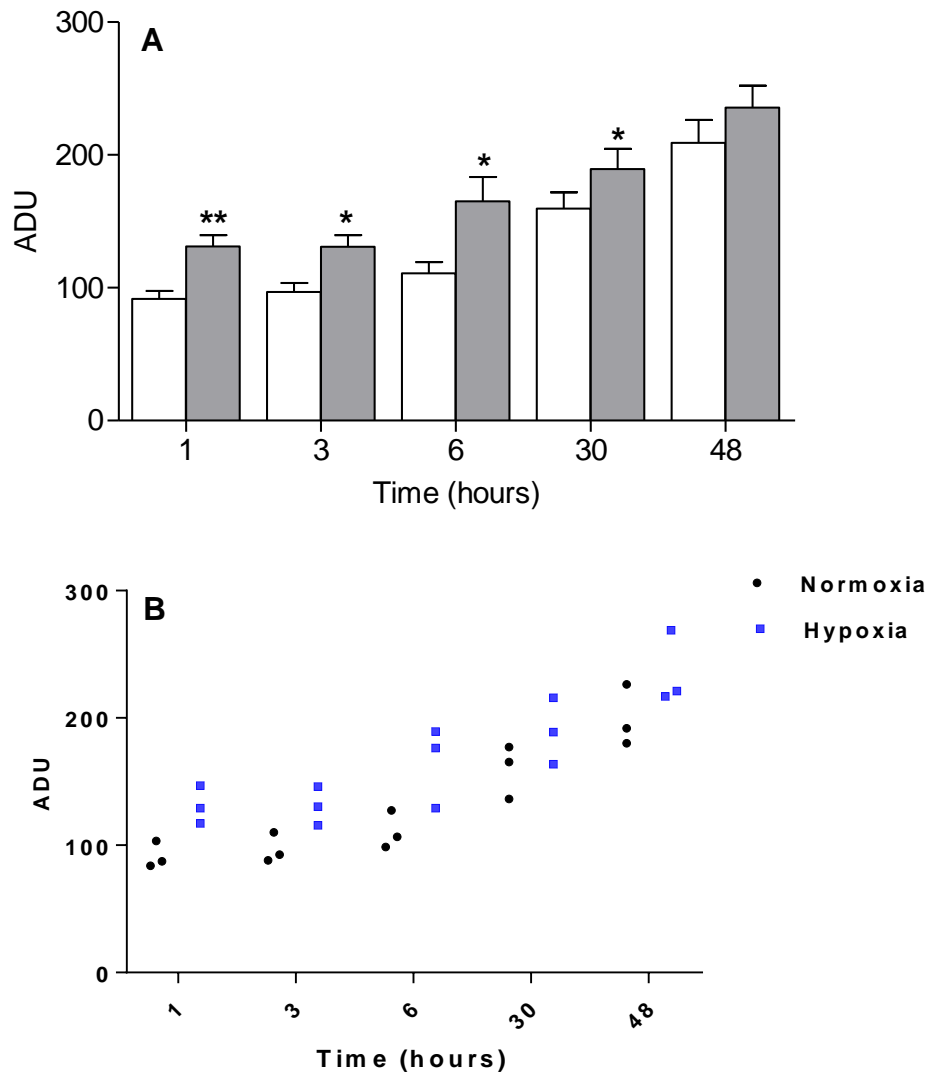


Figure 33 Analysis of MMP-2 Activity in HASMC Culture Supernatant

A: Bar graph and B: Scatter plot showing levels of MMP-2 in HASMC conditioned media after exposure to hypoxia (1%) or normoxia for 48hrs assessed by densitometric analysis. Hypoxia (filled bars) compared to normoxia (open bars). Results expressed as ADU (Arbitrary Density Units)/mg ml⁻¹ cellular protein. Data expressed as mean ± SEM of triplicate values. Results are from three independent experiments repeated in triplicate. n = 3. Statistical analysis was performed using Student's t-tests and statistical significance was indicated by p values < 0.05.

Results of gelatin zymography evaluation revealed that hypoxia for 1 hour led to a significant increase in secreted pro and active MMP-2 species (p < 0.01). Significant increases were also noted at 3 hours (p < 0.05), at 6 hrs (p < 0.05) and at 30 hrs (p < 0.05). Analysis at 48 hours showed sustained

elevations, but no statistical significance was found. MMP-9 was not detected in any of the HASMC conditioned media samples.

4.5 Assessment of HASMC Lysate Samples for MMP-9

Lysate from HASMCs exposed to 1% hypoxia or normoxia was assessed using gelatin zymography to determine if any MMP-9 was present inside the cells as secreted MMP-9 was not detected in HASMC conditioned media. However, in our previous study (Erdozain *et al.*, 2011) MMP-9 was detected in conditioned media from HASMCs exposed to decreasing levels of hypoxia over a 72 hour time period (Erdozain *et al.*, 2011). A maximum of 7 μ g of protein per well was loaded onto the gels which were incubated in development buffer for 48 hours rather than the usual 24 hours to give the optimum chance of detecting any MMP-9 present in the HASMC lysate samples.

4.5.1 Results of Assessment for MMP-9 in HASMC Lysate

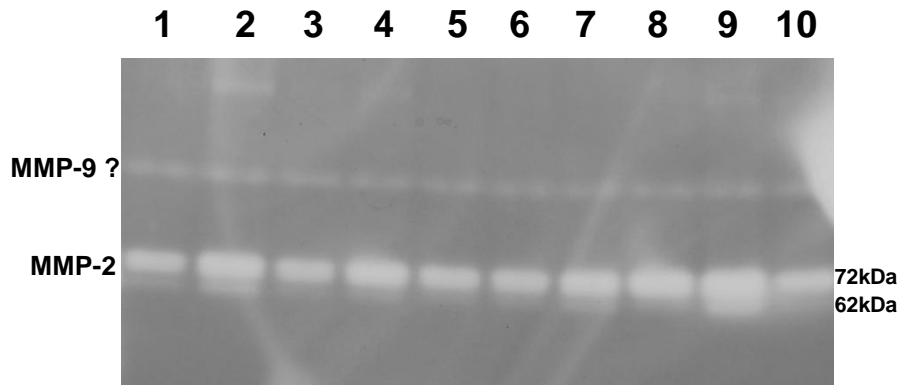


Figure 34 Analysis of MMPs in HASMC Cell Lysate

Lysate from HASMCs exposed to hypoxia or normoxia for 48 hours was overloaded onto a 7.5% gel and assessed using gelatin zymography. Incubation was carried out over a two day time period in order to maximise enzymatic activity. 1: 1hr N 2: 1hr H 3: 3hrs N 4:3hrs H 5:6hrs N 6: 6hrs H 7: 30hrs N 8: 30hrs H 9: 48hrs N 10: 48hrs H. H (HASMCs exposed to hypoxia) N (HASMCs cultured under normoxic conditions to serve as controls).

Bands of a higher molecular weight than MMP-2 can be seen on the above gel which could be consistent with MMP-9. In order try and identify these bands the gel was repeated and the sample size decreased to reduce the streaking apparent on the above gel. Additionally the incubation period was reduced and MMP-2 and MMP-9 positive controls included along with a molecular weight ladder.

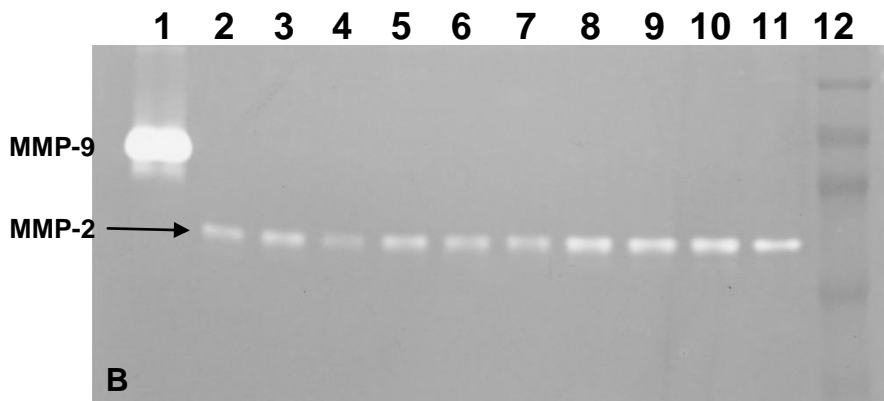
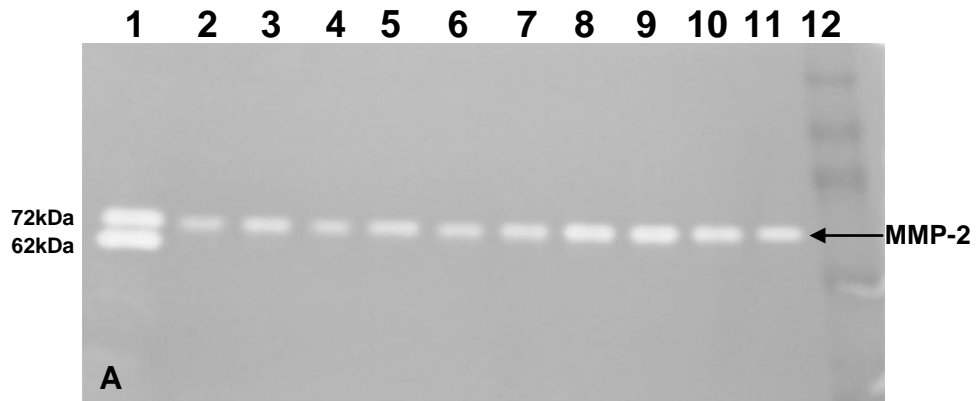


Figure 35 Repeat HASMC Lysate Analysis for MMP-9

Lysate (3 μ g) from HASMCs exposed to hypoxia or normoxia for 48 hours was loaded onto a 7.5% gel and assessed using gelatin zymography. Incubation was carried out overnight.

Slide A: 1: MMP-2 positive control 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM.
Slide B: 1: MMP-9 positive control 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM. H (HASMCs exposed to hypoxia) N (HASMCs cultured under normoxic conditions to serve as controls).

After reducing the protein concentration from 7µg down to 3µg MMP-9 was undetected in both normoxic and hypoxic lysate samples in both gels shown Figure 35. Therefore these results make the bands seen on the gel in Figure 34 seem less likely to be MMP-9, unless of course MMP-9 concentrations within the samples were so small that only larger sample volumes would allow detection.

4.5.2 Zymographic Control Gels

During our earlier study Erdozain *et al.*, (2011) had maintained the cells in media containing 10% FCS prior to and during the period of decreasing hypoxia levels. FCS is known to effect the expression of some proteins therefore controls maintained in 10% FCS media were set up for comparison purposes to rule out foetal calf serum as a potential confounding factor. All other experiments during this study used cells maintained in serum-free media 24 – 48 hrs prior to the starting time-point.

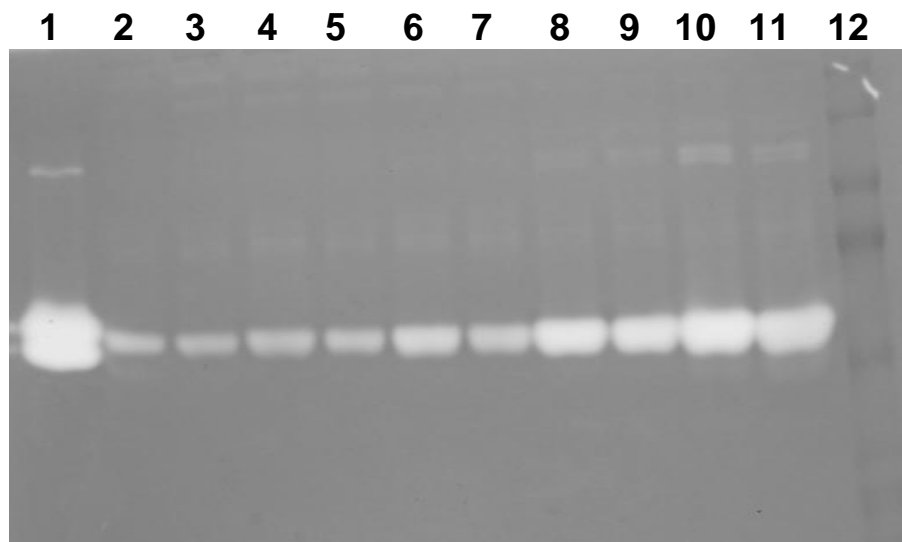


Figure 36 10% FCS Control Gel

Levels of MMP-2 in culture supernatants containing 10% FCS from HASMCs exposed to hypoxia or normoxia for 48 hours and assessed using gelatin zymography. Purified human MMP-2 was used as a standard to identify the bands. 1: MMP-2 positive control 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM. (N: normoxia, H: hypoxia).

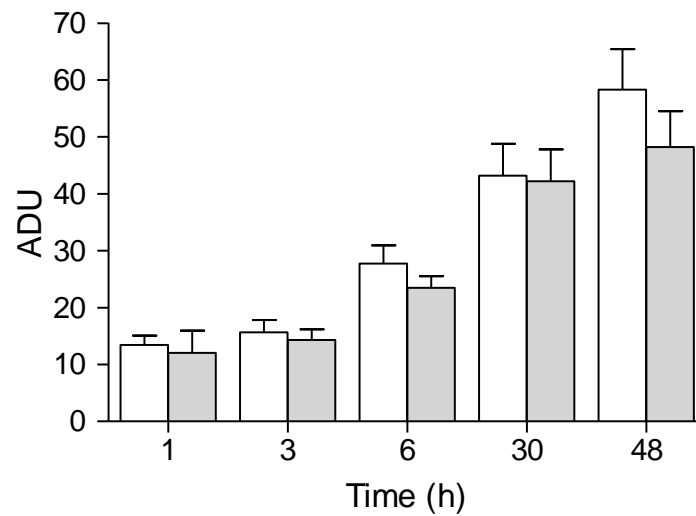


Figure 37 Levels of MMP-2 in 10% FCS Control Gel

Levels of MMP-2 in conditioned media containing 10% FCS from HASMCs exposed to 1% hypoxia (filled bars) or normoxia (open bars) for 48 hours. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Results are from one experiment repeated in triplicate. n = 1.

A further control zymography gel (Figure 38) was set up with only 2 lanes, containing freshly prepared medium including 10% FCS (Lane 1) and conditioned media with 10% FCS (Lane 2). This was to determine if MMP-2 was present in the medium containing FCS, any MMP-2 present in this sample must originate from the FCS. MMP-2 is expected to be present in the conditioned medium sample containing FCS as this supernatant was removed from wells containing HASMCs.

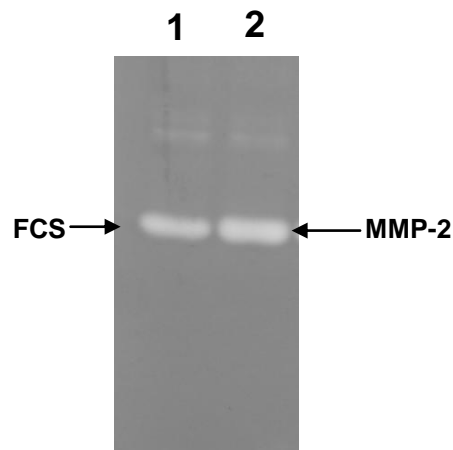


Figure 38 Control Gel Demonstrating MMP-2 in FCS

Zymographic analysis of MMP-2 levels in FCS. Lane 1 was loaded with 20 μ l of freshly prepared medium including 10% FCS and Lane 2 loaded with 20 μ l of HASMC conditioned medium also containing 10% FCS and incubated overnight at 37°C. The band in Lane 2 corresponds to MMP-2 as confirmed by the molecular weight marker (not shown) and Lane 1 also shows a significant band corresponding to MMP-2. The wells were equally loaded. The results seem to indicate that FCS does contain significant amounts of MMP-2 as the medium to which it was added was not conditioned by HASMCs and therefore the FCS must be the source of MMP-2 seen in Lane 1. Although a negative control was not included this time, previously, in all earlier zymographic analysis, freshly prepared medium not containing FCS has been run on a gel and has never produced a band of lysis.

The earlier results of gelatin zymography evaluation shown in Section 4.4.2.2 Figures 32 and 33, revealed that hypoxia for 1 hour led to a significant increase in secreted pro and active MMP-2 species ($p < 0.01$). Significant increases were also noted at 3 hours ($p < 0.05$), at 6 hours ($p < 0.05$) and at 30 hours ($p < 0.05$). Analysis at 48 hours showed sustained elevations but statistically were not significant. In the control samples the elevations for MMP-2 within the HASMC culture supernatant were probably due to constitutive expression of MMP-2 accumulating in the conditioned media, especially in the latter time-points where nutrient depletion induced stress might increase this expression.

Freestone *et al.*, (1995) demonstrated that MMP-2 was the dominant elastolytic enzyme in the initiation of small aneurysms and findings in this study have shown that 1% hypoxia can upregulate MMP-2 activity in HASMCs which is also consistent with other research findings. In 2006 Miyoshi *et al.*, (2006) reported that hypoxia upregulated MMP expression in hepatoma cells during cancer studies and Ben -Yosef *et al.*, (2002) found that prolonged hypoxia of endothelial cells appeared to enhance MMP-2 production and secretion.

Although it is well documented that MMP-9 is implicated in AAA, in particular as the diameter of the aneurysm increases (Freestone *et al.*, 1995), and also has been demonstrated in AAA tissue. During the present study, my results showed secretion of MMP-9 was not detected in normoxic or hypoxic samples after the 48 hour exposure to 1% oxygen. This data appears to correlate with earlier findings by our Group (Erdozain *et al.*, 2011) where a significant increase in MMP-9 was only seen at 2% hypoxia after 72 hours of decreasing O₂ levels suggesting the driving force behind MMP-9 release is a sustained sequential decrease in O₂ over time and which also seems more comparable to the chronic hypoxic conditions found within the aneurysm. Furthermore Thompson *et al.*, (1995) identified macrophages as the primary source of MMP-9 in human AAA tissue, therefore in my study where they were obviously absent, may account for the lack of MMP-9 within the conditioned media but does not discount the possible effect of hypoxia on MMP-9 secretion within the real environment of an aneurysm. Additionally, a variety of tumour cell lines were examined by Himelstein and Koch, (1998) for induction of MMP-9 and MMP-2 under hypoxic conditions. Hypoxia did upregulate MMP-9 in one alveolar rhabdomyosarcoma cell line but they were unable to demonstrate a consistent hypoxia-mediated increase in MMP-9 suggesting that MMP-9 expression may not directly be affected by exposure to hypoxia *in vitro*.

Section 4.5.2 described control zymograms (Figures 36 and 37) which had been set up to assess MMP-2 levels in conditioned medium containing 10% FCS from HASMCs which had been subjected to 48 hours of experimental 1% hypoxia. During an earlier study by our Group (Erdozain *et al.*, 2011), the

cells were maintained in medium containing 10% FCS prior to and during a 96 hour period of decreasing hypoxia levels. FCS is known to effect the expression of many genes and may also lead to unwanted stimulation of the cells during experimentation. Therefore, controls maintained in 10% FCS media were set up for comparison purposes to rule out foetal calf serum as a potential confounding factor. FCS is routinely used as a supplement to basal growth medium in culturing most cells and is vital to enable *in vitro* proliferation and healthy maintenance of cells. Bovine serum albumin is a major component of FCS but it also provides a wide variety of other components including hormones, attachment factors, carrier proteins, amino acids, sugars, lipids and importantly, numerous growth factors.

During the culturing of HASMCs prior to hypoxia exposure, 10% FCS was routinely added to the culture medium as it provides optimal conditions for growth. However, 24 hours prior to the start of any hypoxia study, FCS was withdrawn and the cells maintained in serum-free media during the 48 hour experimental period apart from the aforementioned controls which did contain 10% FCS. 'Serum starvation' is commonly used in experiments as the poorly defined complex and variable composition of FCS represents an important and undesirable confounding factor while performing bioassays, therefore elimination of serum from culture medium provides more reproducible experimental conditions (Pirkmajer and Chibalin, 2011). Additionally it is common to serum starve the cells to enable cell synchronization and ensure they enter quiescence (non-dividing state) to better represent physiological conditions found *in vivo*.

Results from these control samples demonstrated unusual findings as the normoxic HASMC conditioned media samples containing FCS contained higher levels of MMP-2 protein at all time-points in comparison to the hypoxic HASMC conditioned media samples (Figures 36 and 37). These control results were the complete reverse of results found in all HASMC conditioned media samples where cells had been kept in serum-free media prior and during exposure to hypoxia. The HASMCs were grown to and used at sub-confluence for the start of all hypoxia studies. One explanation for

these opposing results is firstly, the cells in the control samples containing FCS would still have had the capacity to proliferate in comparison to the cells maintained in serum-free media with the resulting effects of serum starvation, as explained earlier. Secondly and importantly, in several other areas of research it has been demonstrated that proliferation of cultured cells under hypoxic conditions can be reduced. Ray *et al.* (2008) studied oxygen regulation of human arterial SMC proliferation and survival, demonstrating that in HASMCs incubation at 1% O₂ reduced cell proliferation, delayed G1/S interphase transition and increased apoptotic cell death. Similarly, Cogo *et al.*, (2003) looked at the effects of hypoxia on rat airway SMC proliferation and showed that exposure to 1% O₂ for 72 hours decreased cell proliferation by up to 21%. More recently, Mamede *et al.*, (2013) looked at the effects of 2% hypoxia in a human hormone-independent prostate cancer cell line (PC3) and their results showed a decrease of 64%-68% in cell proliferation in hypoxia in comparison to cells kept under normoxic conditions. Therefore, it seems probable that all the HASMCs maintained in FCS-containing medium continued to proliferate, but proliferation of cells kept under hypoxic conditions was significantly reduced in comparison to those maintained under normoxia leading to significantly lower cell numbers in hypoxic FCS-containing conditioned media samples. Lower cell numbers in hypoxic samples could explain why MMP-2 levels were reduced, even at pO₂ levels where MMP-2 levels are generally increased, in comparison to the much higher cell numbers of the normoxic samples which were able to constitutively secrete MMP-2.

Additionally and of relevance here, some genes contain one or more serum response elements (SREs) in their promoter regions which are activated by serum response factors (SRFs). Zhe *et al.*, (2003) determined that in human and mouse lung fibroblasts *MMP-2* and *MMP-14* were among the genes modulated by SRF, and this could explain increased MMP-2 protein levels in these HASMC samples maintained in medium-containing FCS. FCS can modulate gene expression, either positively or negatively, therefore it interesting to find that *MMP-2* expression may have been altered in response to FCS and modified in the two different experimental conditions

(hypoxia and normoxia) to increase *MMP-2* gene expression under normoxia and down-regulate *MMP-2* during hypoxia.

4.6 Inhibitory Profile of MMPs

To further confirm that results obtained in Figure 32 were due to the effects of MMP-2 and not from the participation of other proteolytic enzymes such as serine, cysteine and aspartic acid proteases, the inhibitory profile of the gelatinases was assessed by performing the normal gelatin zymography protocol but with the addition of the following inhibitors to the reaction buffer prior to incubation. 10mM of the calcium chelator ethylenediaminetetraacetic acid (EDTA), 2mM of the zinc chelator 1,10-phenanthroline, 1mM of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), 1 μ M leupeptin (serine /cysteine inhibitor) and 1 μ M pepstatin A (aspartic acid inhibitor). MMPs are zinc and calcium dependent proteases therefore one would expect that the metal chelating inhibitors EDTA and 1,10-phenanthroline should abolish all MMP activity resulting in the absence of bands from treated gels.

4.6.1 Materials List

All materials were obtained from Sigma Aldrich, Dorset, UK. unless stated otherwise.

- Ethylenediaminetetraacetic acid (EDTA).
- 1,10 phenanthroline.
- Leupeptin.
- Phenylmethylsulfonyl fluoride (PMSF).
- Pepstatin A

4.6.2 Inhibition Assay Results

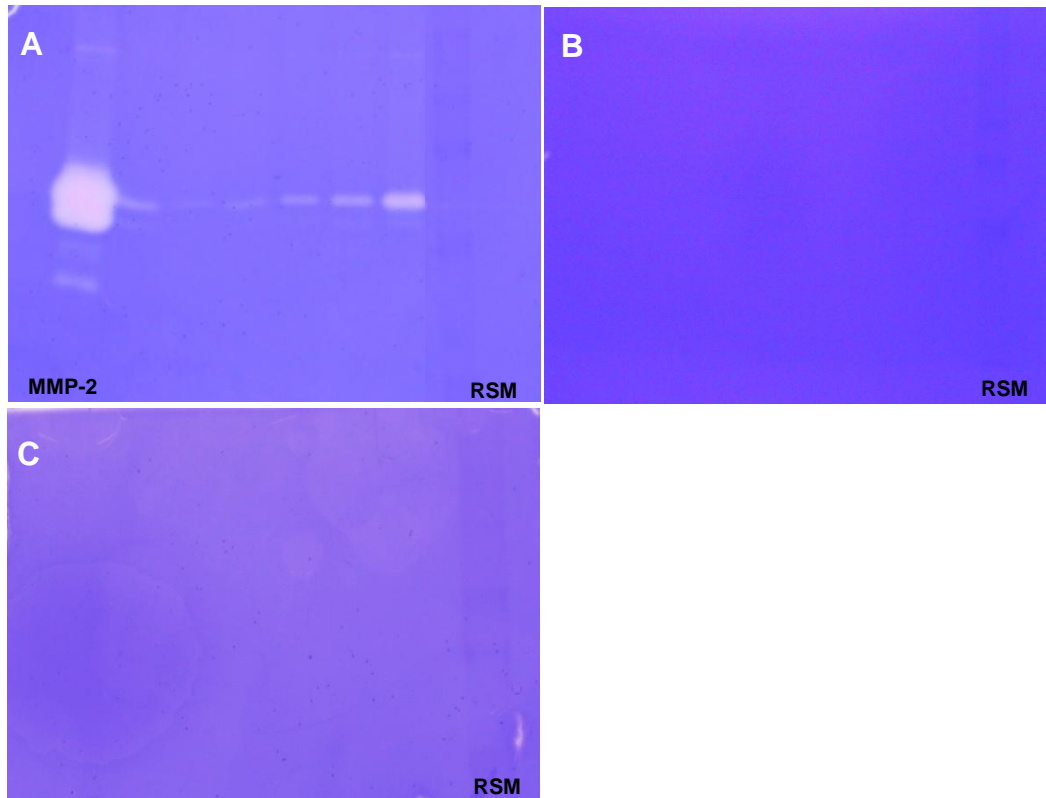


Figure 39 Inhibition of MMP-2 Gelatin Zymography Gels

Conditioned media from HASMCs exposed to 1% hypoxia or normoxia were assessed using the normal zymography protocol but with the addition of inhibitors to the reaction buffer. Positive control (MMP-2 standard) and RSM(Rainbow size marker) were applied.

Slide A: Zymogram after incubation with 1mM PMSF, 1 μ M leupeptin and 1 μ M pepstatin A.

Slide B: After incubation with 10mM EDTA.

Slide C: Incubated with 2mM 1, 10-phenanthroline.

Inhibition assays performed to verify the identity of MMP-2 demonstrate that gelatinase activity was completely ablated by calcium chelation (EDTA) in

Slide B and zinc chelation (1, 10 phenanthroline) in Slide C by chelating and removing the calcium and zinc ions essential for MMP activity thus preventing bands of lysis appearing on the gels. The inhibitors of serine, cysteine and aspartic acid proteases had no effect on gelatinase activity in Slide A and therefore bands of lysis were present on this gel. This means it is reasonable to assume that it is indeed MMP-2 responsible for these bands and not due to the aforementioned proteases which would have been inhibited by the PMSF, leupeptin and pepstatin A if present.

4.7 Expression of MMP-2 protein

MMP-2 immunoblot analysis was carried out to further corroborate that MMP-2 protein was present in the HASMC culture supernatant and verify the increases in enzymatic activity seen in the hypoxic HASMC samples in comparison to normoxic samples was due to MMP-2 activity and not other proteases.

4.7.1 Materials list

- ECL™ Western Blot Detection Reagents (G.E. Healthcare)
- ECL™ Advance Western Blot Detection Reagents (G.E. Healthcare)
- Hybond™-C extra nitrocellulose (Amersham Biosciences UK Ltd. Buckinghamshire, UK).
- Kodak BioMax XAR Film (VWR International Ltd. Dorset, UK).
- Polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma Aldrich Company Ltd. Dorset, UK).
- Mouse monoclonal anti-human MMP-2 (8B4) (Abcam Plc., Cambridge, UK).
- Polyclonal horseradish peroxidase-conjugated goat anti-mouse IgG (Dako UK Ltd).

4.7.2 Western and Dot Blot

The immunodetection of MMP-2 protein was assessed using the Western Blot and Dot Blot techniques. The Western Blot protocol has been described in detail in Chapter 7, section 7.3.2. All materials and recipes for sample buffer, running buffer, blotting buffer, washing buffer, blocking buffer, the stacking gel and resolving gel are shown in Appendix 1.

4.7.2.1 Levels of MMP-2 Assessed by Western Blot

Samples standardised for protein (7µg) were loaded onto 7.5% SDS PAGE gels, then transferred onto nitrocellulose membranes, blocked with 4% non-fat milk and probed with mouse monoclonal anti-human MMP-2(1:200) overnight at 4°C. Unbound primary antibody was removed in washing buffer and then replaced with a polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10000) (Dako UK Ltd., Ely, UK) prepared in blocking buffer for 1 – 2 hours.

Proteins were visualised using an Amersham ECL Advance™ Western blotting detection kit (GE Healthcare Life Sciences, Buckinghamshire, UK) instead of the basic ECL kit which proved not to be sensitive enough to detect the amount of MMP-2 present in the samples. When using ECL Advance it was necessary to increase the dilution of secondary antibody from 1:200 to 1:10000 and increase the length of time the membrane was immersed in washing buffer in order to reduce the background signal. Also, exposure of the nitrocellulose membrane to x-ray film in order to measure the chemiluminescent signal was reduced considerably.

4.7.2.2 Results of HASMC MMP-2 Protein Levels

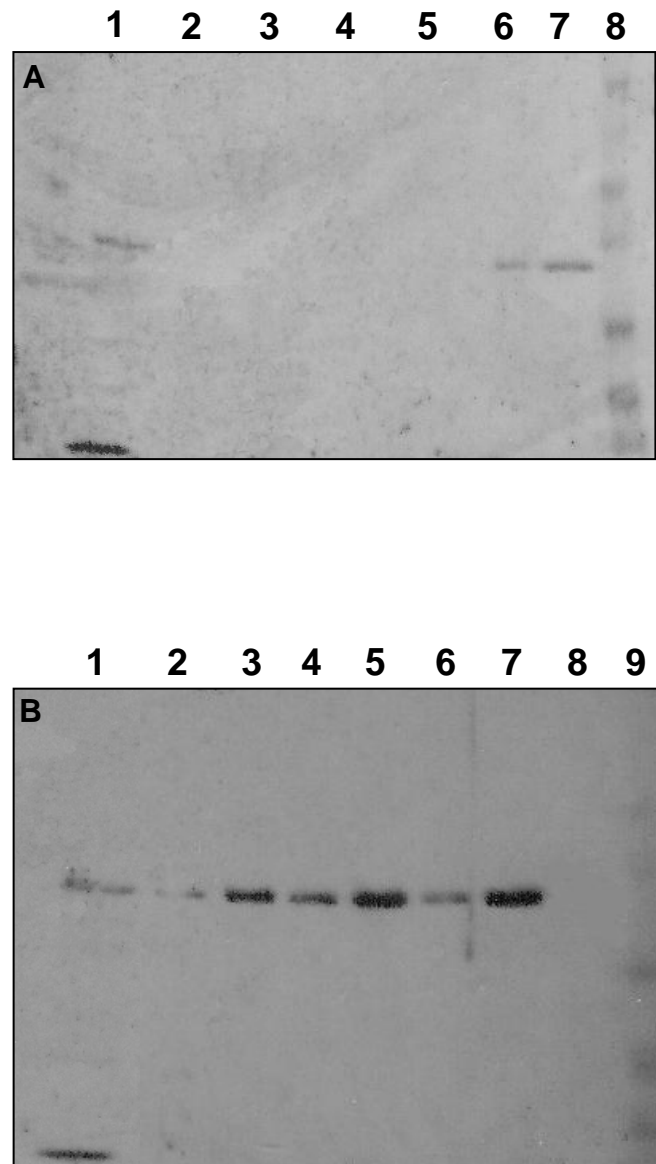


Figure 40 Levels of MMP-2 Protein

Levels of MMP-2 protein in culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were assessed by immunoblotting. Samples were equally loaded and subsequently probed with monoclonal mouse anti-MMP-2 antibody (1:200) and a HRP-conjugated goat anti-mouse immunoglobulin secondary antibody (1:10000)

Slide A: 1: Purified MMP-2 2: Negative (dH₂O) 3: Negative (Lysis buffer) 4:1hr N 5:1hr H 6: 3hrs N 7: 3hrs H 8: RSM.

Slide B: 1: Purified MMP-2 2: 6hrs N 3: 6hrs H 4:30hrs N 5:30hrs H 6: 48hrs N 7: 48hrs H 8: Negative 9: RSM. N (Normoxia) H (Hypoxia).

It was relatively easy to detect the enzymatic activity of MMP-2 using the zymography even if MMP-2 levels were low as this technique is very sensitive, but to detect the protein using immunoblotting was more problematic especially at the earlier time-points. Therefore, the first two earlier time-points were analysed using the dot blot method as well so that increased volumes of conditioned media could be analysed in conjunction with the more sensitive ECL Advance to allow visualisation of MMP-2 protein.

4.7.2.3 The Dot Blot Method

The Dot Blot technique is a simplification of the Western Blot method for detecting and identifying proteins. The main difference between the two techniques is the method of protein transfer onto the nitrocellulose as in a Dot Blot the proteins are not first separated by electrophoresis but instead are applied as dots directly onto the membrane through the circular template of the Dot Blot apparatus (Jencons Ltd. Bedfordshire, UK) which is attached to a vacuum pump. The advantage of this method is that it is significantly quicker and proteins can be concentrated onto one small area but has the disadvantage of being unable to identify the molecular weight of the protein. To aid the transfer of protein onto the nitrocellulose membrane the blotting paper and membrane were soaked in transfer buffer prior to assembling the dot blot apparatus. After transfer of proteins onto the nitrocellulose membrane the membrane was blocked for approximately one hour in 4% non-fat milk at room temperature followed by overnight 4°C incubation in mouse monoclonal anti-human MMP-2 primary antibody (1:250) (Abcam Plc., Cambridge, UK) followed by a two hour incubation in polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:200) (Dako UK Ltd., Ely, UK) at room temperature. See Western Blot protocol (Chapter 7, section 7.3.2) for the visualisation of bound protein.

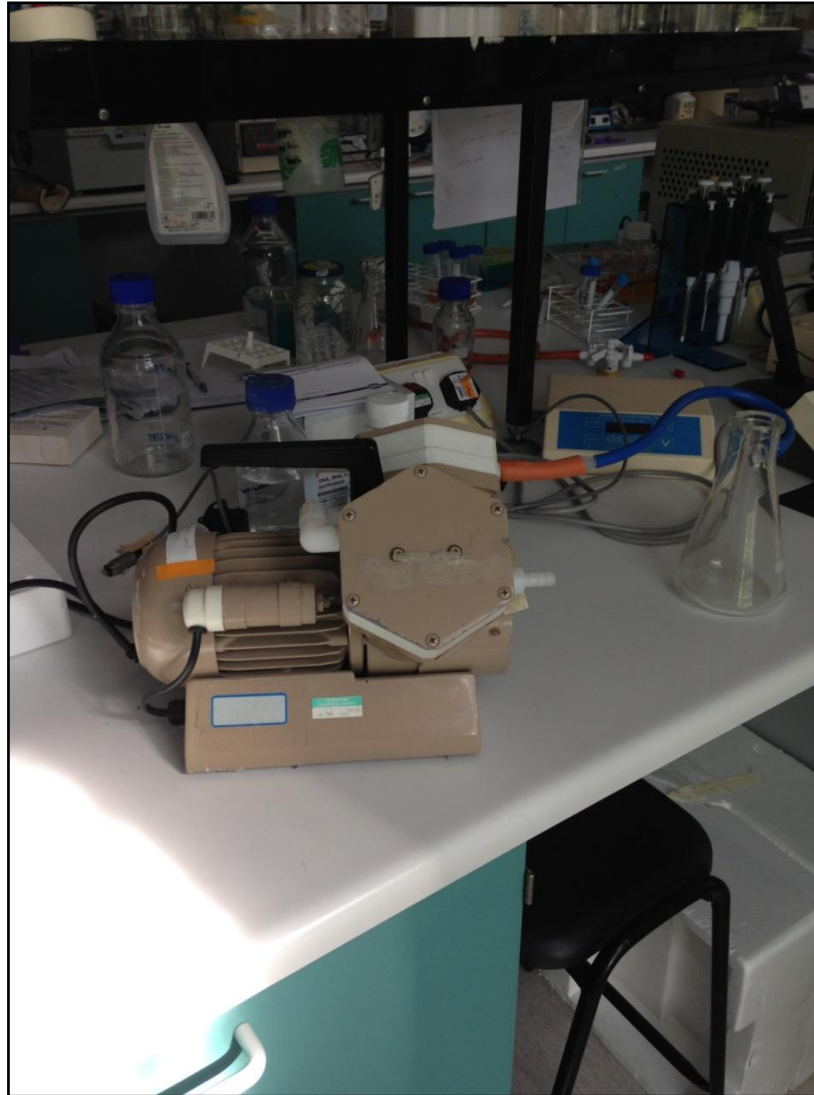


Figure 41 Dot Blot Vacuum Pump

4.7.2.4 Results of MMP-2 Protein Levels Assessed by Dot Blotting

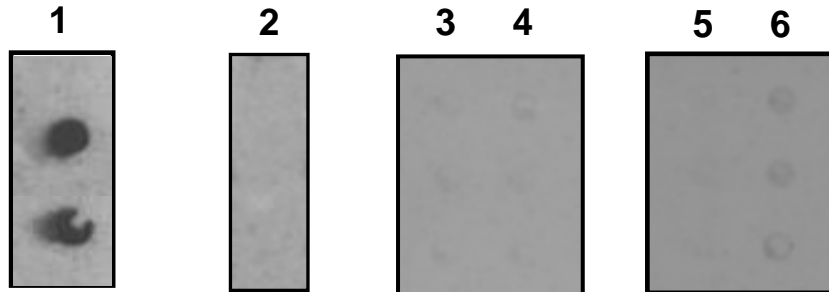


Figure 42 MMP-2 Levels at 1 hr and 3 hr Time-points

Levels of MMP-2 protein in culture supernatants at the 1 hr and 3 hr time-points from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were assessed by the dot blot method. Samples were equally loaded in triplicate and subsequently probed with monoclonal mouse anti-MMP-2 antibody (1:250) and a HRP-conjugated goat anti-mouse immunoglobulin secondary antibody (1:200). Proteins were visualised using standard ECL reagent.

Lane 1: Purified MMP-2, Lane 2: Negative control (Lysis buffer), Lane 3: 1hr N Lane 4: 1hr H Lane 5: 3hrs N Lane 6: 3hrs H (N: Normoxia, H: Hypoxia).

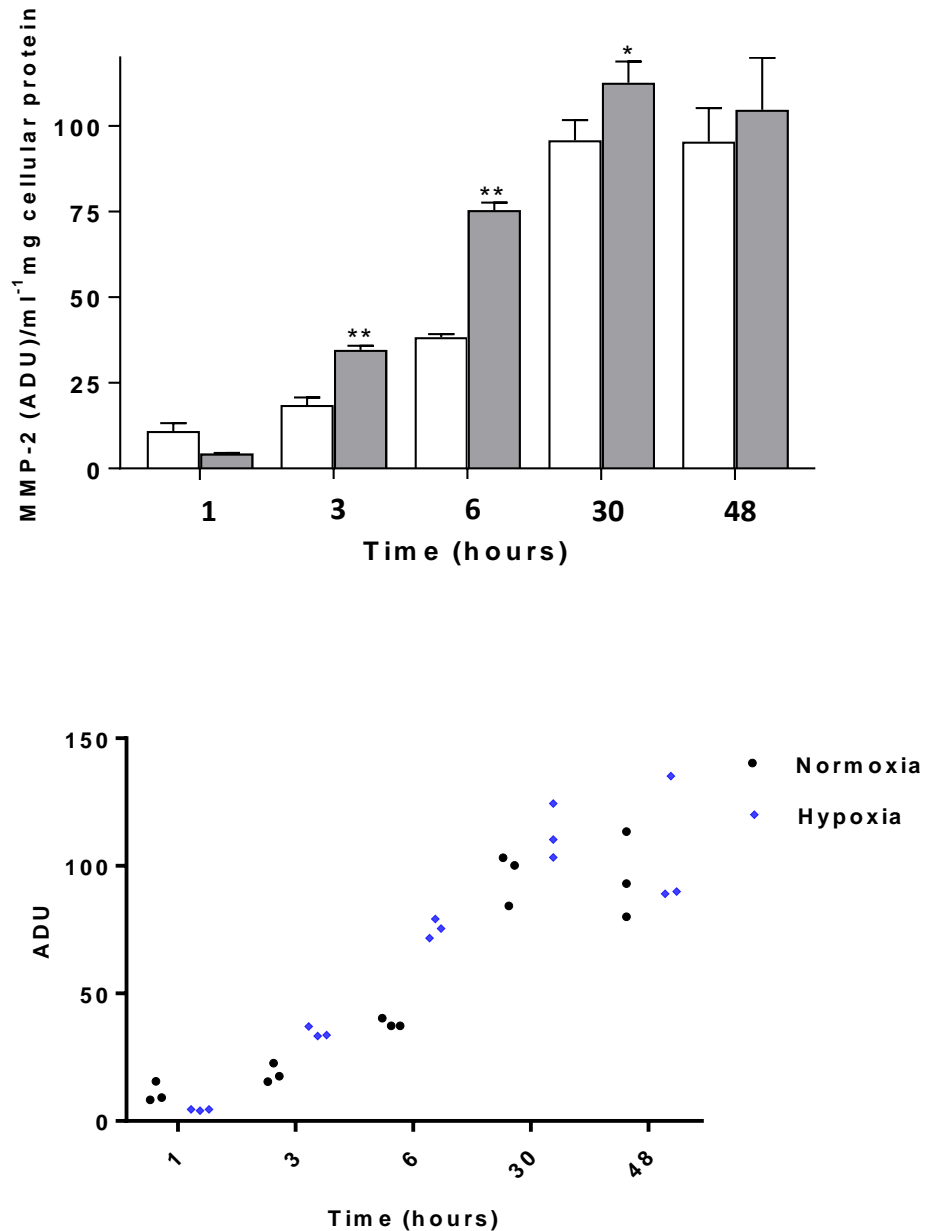


Figure 43 Bar and Scatter Graphs Showing Analysis of MMP-2 Protein Levels in HASMC Supernatant

Levels of MMP-2 protein in HASMC conditioned media after exposure to hypoxia 1% (filled bars) or normoxia (open bars) for 48hrs and assessed by immunoblotting followed by densitometric analysis. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values.* $p < 0.05$; ** $p < 0.01$. Results are from three independent experiments repeated in triplicate. $n = 3$. Statistical analysis was performed using Student's t-tests and statistical significance was indicated by p values < 0.05 .

MMP-2 immunoblot analysis corroborated that MMP-2 protein was present in the HASMC culture supernatant and that there were differences in MMP-2 protein levels between the hypoxic and normoxic samples. At the 1-hour time-point there was no significant difference in levels of MMP-2 protein between the hypoxic and normoxic samples, but after 3, 6 and 30 hours, a significant rise ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) in protein levels was evident. The 48-hour time-point also showed an increase in MMP-2 protein but this was not statistically significant.

4.7.3 Comparison of Total Protein for Hypoxic and Normoxic Samples

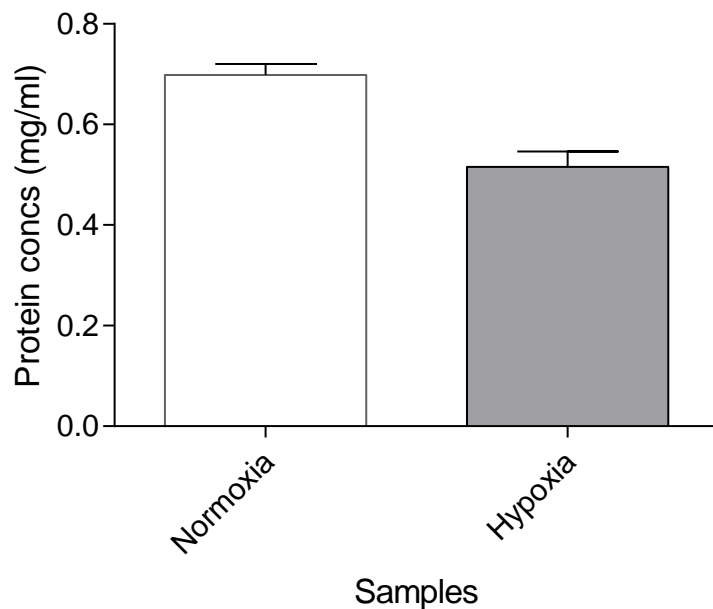


Figure 44 Protein Content of HASMC Whole Cell Lysate from Normoxic and Hypoxic Samples

On completion of the hypoxia study the total protein content of hypoxia and normoxia samples was assessed by Bradford assay. Analysis of the averaged results showed higher protein levels in the normoxic samples in comparison to hypoxic samples. This was not surprising as during hypoxia you might expect none essential protein transcription to be switched off in order to cope with hypoxic stress and nutrient depletion.

4.8 Lactate Dehydrogenase Assay (Marker of Cell Damage)

This assay was carried out in order to ensure that any gelatinase activity present in the HASMC conditioned media was due to secretion from the cells rather than released from compromised cells damaged by prolonged hypoxia or a treatment that was cytotoxic. The cytoplasmic enzyme lactate dehydrogenase (LDH) is present within all mammalian cells. The normal plasma membrane is impermeable to LDH but damage to the cell membrane results in a change in membrane permeability and subsequent leakage of LDH into the extracellular fluid (Rae, 1977). *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. Any release of LDH into culture supernatant correlates with the amount of cell death and membrane damage providing an accurate measure of any cellular toxicity induced by hypoxia or a cytotoxic treatment and can be used to assess cell viability. This assay is based upon the ability of LDH to catalyze the chemical reaction below:



4.8.1 Materials List

All items were obtained from Sigma Aldrich Company Ltd. Dorset, UK unless stated otherwise.

- Allopurinol
- Nicotinamide adenine dinucleotide (NAD⁺)
- Sodium Lactate Diphenyliodonium Chloride (DPI) (ICN Biochemical Inc. OH, USA)

4.8.2 Protocol for Lactate Dehydrogenase (LDH) Assay

Conditioned media or lysate was assayed for LDH activity with 1.5mM sodium lactate, 1mM NAD⁺, 1μM diphenyliodonium chloride (DPI), 10μM allopurinol and phosphate buffered saline (PBS) which was added to a cuvette and allowed to incubate at 37°C in the spectrophotometer for 4-5 minutes.

The generation of NADH was followed by measuring the absorbance change at wavelength 340nm over time using a Hitachi U-2010 spectrophotometer. The change in NADH concentration was calculated using Beers Law and the extinction coefficient of NADH $6.22 \times 10^3 \text{ }^{-1}\text{cm}^{-1}$. Cell lysate served as a positive control, PBS and time zero samples served as negative controls.

4.8.3 LDH Cell Viability Results

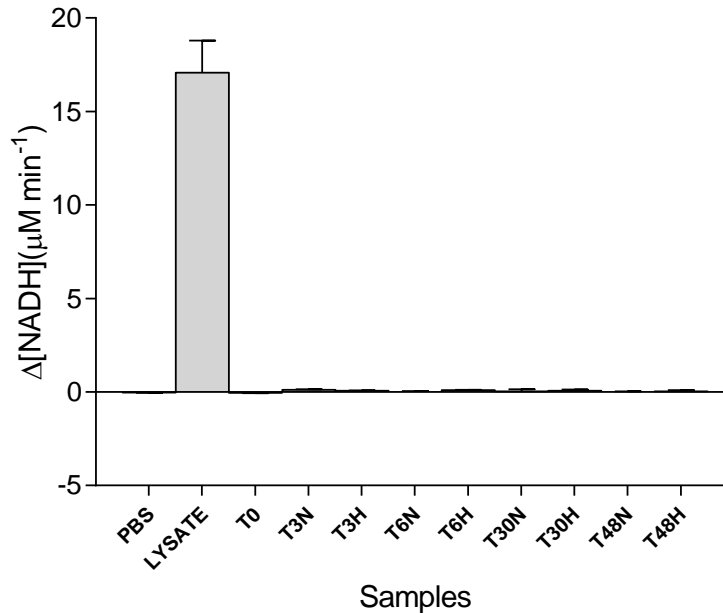


Figure 45 Analysis of HASMC Cell Viability After Exposure to 1% Hypoxia

1.5mM sodium lactate, 1mM NAD^+ , 1 μM diphenyliodonium chloride (DPI), 10 μM allopurinol and phosphate buffered saline (PBS) was added to sample in the cuvette, incubated at 37°C and the generation of NADH was followed by measuring the absorbance change at wavelength 340nm over time. Cell lysate served as a positive control, PBS and time zero samples served as negative controls.

As anticipated the test samples all demonstrated negative values showing that no LDH was present in the conditioned media samples ensuring that the severe hypoxia had not destroyed the cell membranes allowing MMP protein to be released by any other method other than secretion. The positive control cell lysate presented high positive values as also expected as LDH would have been released at the time of lysis. Negative controls also presented negative values.

4.9 Distribution of MMP-2 in AAA

4.9.1 Immunohistochemistry

With local ethics approval, AAA tissue was obtained from consented patients undergoing elective surgical repair. Sections were taken from the proximal, distal and body of aneurysm tissue from 5 consenting patients removed during surgery. Normal tissue free from signs of aortic disease was obtained from the aortic cuff of 5 x renal transplant donors for control purposes. (n = 10).



Figure 46 Section of Aneurysmal Sac

4.9.1.1 Paraffin Wax Embedded Sections

At the time of retrieval tissue samples were immediately fixed in 0.9% NaCl (10%v/v) histological formaldehyde for approximately 48 hours and subsequently processed using a Shandon Hypercentre II firowax-embedding station (Shandon Inc., Pennsylvania, USA).



Figure 47 Shandon Hypercentre Wax-embedding Station used to process Harvested Tissue

4.9.1.2 Cutting and Mounting Sections

After processing the paraffin blocks were trimmed back to an optimal cutting surface and then 5 μm sections were cut using a sledge microtome. The 5 μm thickness was chosen as the optimal size as thicker slices did not produce such good detail after immunostaining. Next the sections were placed in a 40°C water bath to allow them to expand and flatten out before being floated onto superfrost microscope slides (being careful to avoid air bubbles getting under the tissue section) and then allowed to air dry. The sections were baked overnight at 60°C prior to deparaffinization and staining.



Figure 48 Sledge Microtome used to Cut Paraffin-embedded Sections

4.9.1.3 Deparaffinization and Rehydration of Paraffin Embedded Sections

The sections were dewaxed in two changes of xylene for 5 minutes before being put through a graded series of ethanol. To complete the rehydration process the sections were immersed twice in dH₂O for 5 minutes each before a final rinse in PBS.

4.9.1.4 Immunohistochemistry

4.9.1.5 Materials List

- Mouse monoclonal anti-human MMP-2 (Abcam, Cambridge, UK).
- Alkaline phosphatase-conjugated Avidinbiotin complex, anti-mouse IgG (ABC) kit (Vector Laboratories, Vectastain® ABC-AP reagent, AK-5001, Peterborough, UK).
- Mouse monoclonal IgG1 negative control (DAKO)
- Naphthol AS-MX phosphate/Fast red TR Fast™ (Sigma-Aldrich Ltd., Dorset, UK).
- Levamisole (Vector Laboratories, SP-5000).

4.9.1.6 Immunohistochemistry Protocol

Tissue sections were incubated for 20 minutes with diluted normal blocking serum originating from the species in which the secondary antibody was made (horse serum). Excess serum was removed from the sections before an overnight incubation at 4°C in the primary antibody diluted in blocking solution. Unbound primary antibody was removed by washing for 5 mins in 1x PBS followed by a 30 minute to 1 hour incubation at room temperature in biotinylated anti-mouse IgG secondary antibody diluted in blocking solution. Slides were then washed for 5 minutes in 1x PBS before being incubated for 30 minutes at room temperature with VECTASTAIN® ABC-AP reagent which was prepared according to the manufacturer's instructions 30 minutes prior to use. The wash steps were repeated proceeded by a 30 minute incubation in alkaline phosphate substrate solution with the addition of Levamisole to inhibit any endogenous alkaline phosphatase activity. The reaction was followed under a light microscope until the desired stain intensity had developed, after which the reaction was stopped by bringing the slides to tap water. Sections were then counterstained with Mayers

Haematoxylin for approximately 1 – 2 minutes and given a final rinse in running tap water before coverslips were mounted onto the slides using Aquamount mounting medium. Slides were viewed under a Zeiss Axioskop 2 microscope and images captured using the Zeiss KS300 program. (Carl Zeiss Ltd. Hertfordshire, UK).

4.9.1.7 Results of MMP-2 Immunohistochemistry

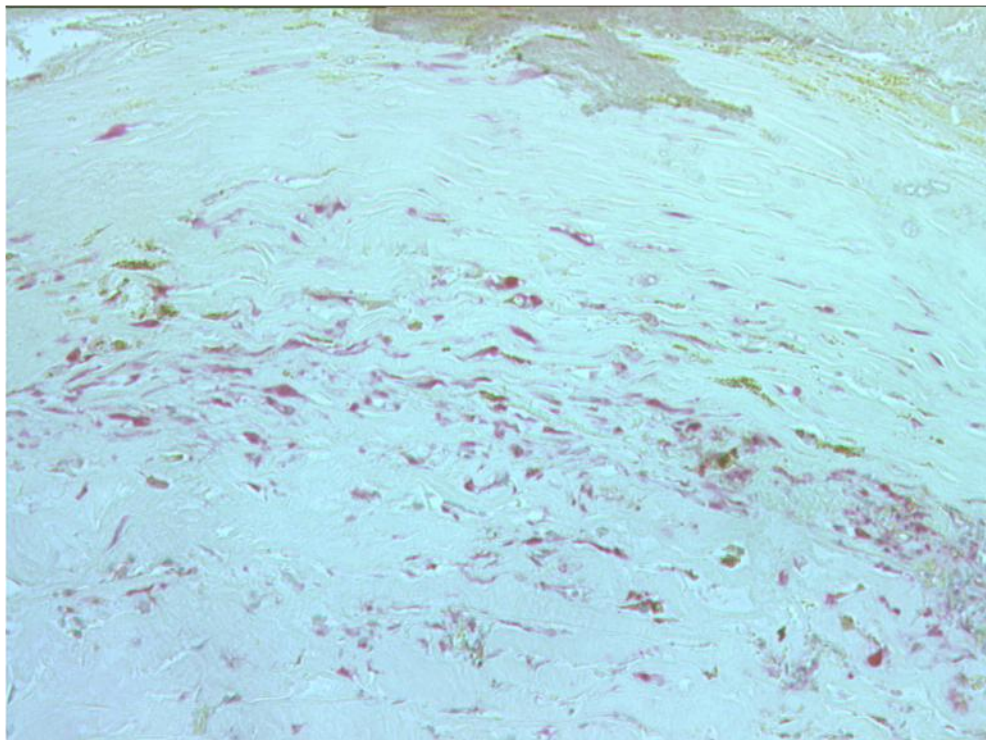


Figure 49 Photomicrograph Showing the Distribution of MMP-2 (red) in Human AAA Tissue (x63)

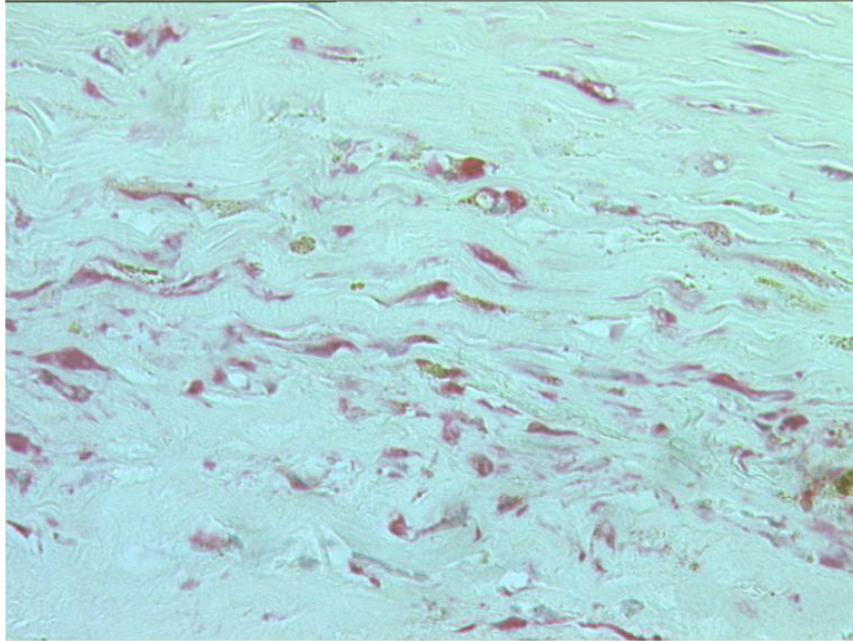


Figure 50 Photomicrograph Showing the Distribution of MMP-2 (red) in Human AAA Tissue (x 126) (Figure 49 at Higher Magnification)

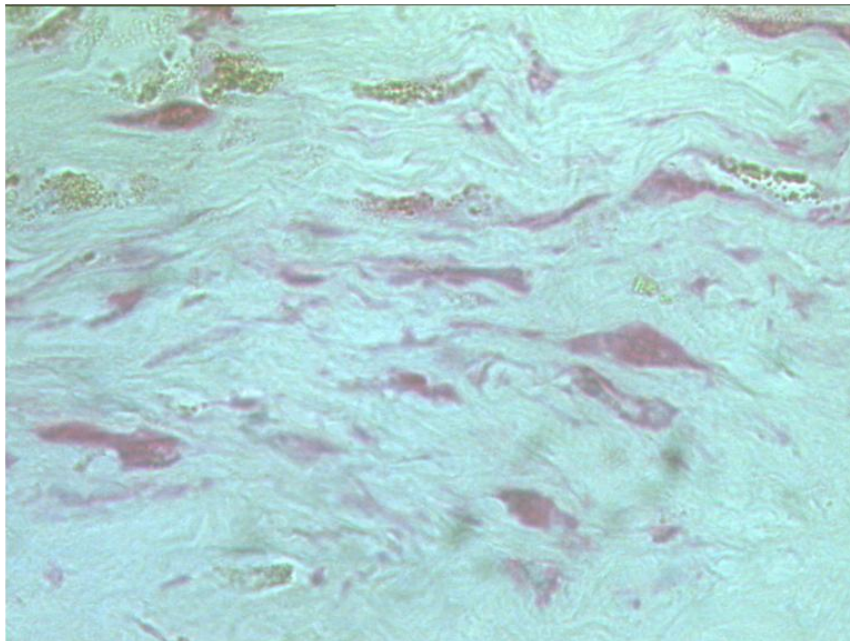


Figure 51 Photomicrograph Showing the Distribution of MMP-2 (red) in Human AAA Tissue (x252) (Figure 49 at Higher Magnification)

Sections of human aneurysmal tissue were probed with mouse anti-human MMP-2 (1:200) followed by a biotinylated horse anti-mouse secondary antibody (1:200) and counterstained with Mayer's haematoxylin. Figures 49 – 51 demonstrate the immuno-localisation of MMP-2 within the body region of the aneurysm taken from the same field of view on the same slide at different magnifications. (n=5)

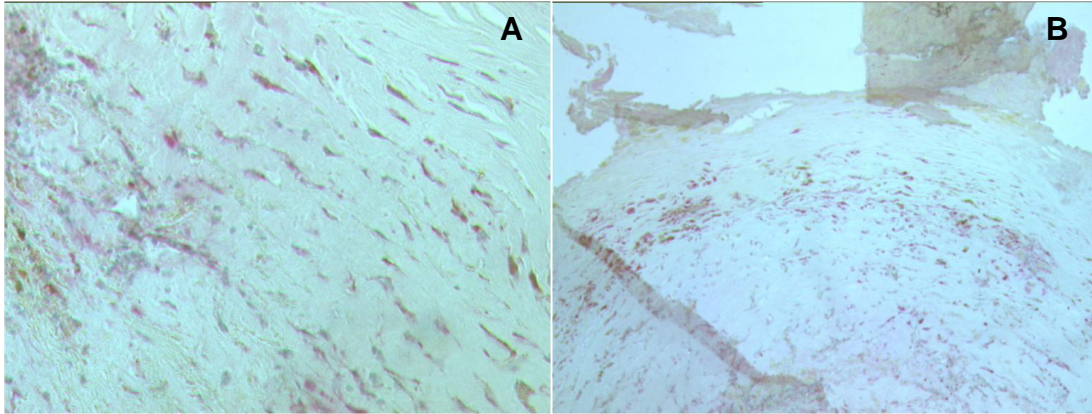


Figure 52 Photomicrograph Showing the Distribution of MMP-2 in Human AAA Tissue A: (x126) B: (x 31.5)

Sections of human aneurysmal tissue were probed with mouse anti-human MMP-2 (1:200) followed by a biotinylated horse anti-mouse secondary antibody (1:200) and counterstained with Mayers haematoxylin. Slide A: shows localisation of MMP-2 to the medial smooth muscle cells and the inflammatory infiltrate seen on the left of the section from within the body region of the aneurysm. Slide B: demonstrates the difficulties presented when trying to cut sections of aneurysmal aorta as the tissue is very friable or brittle and tears very easily. A typical fold in the tissue is also apparent in this section which can sometimes present false positivity. (n = 5)

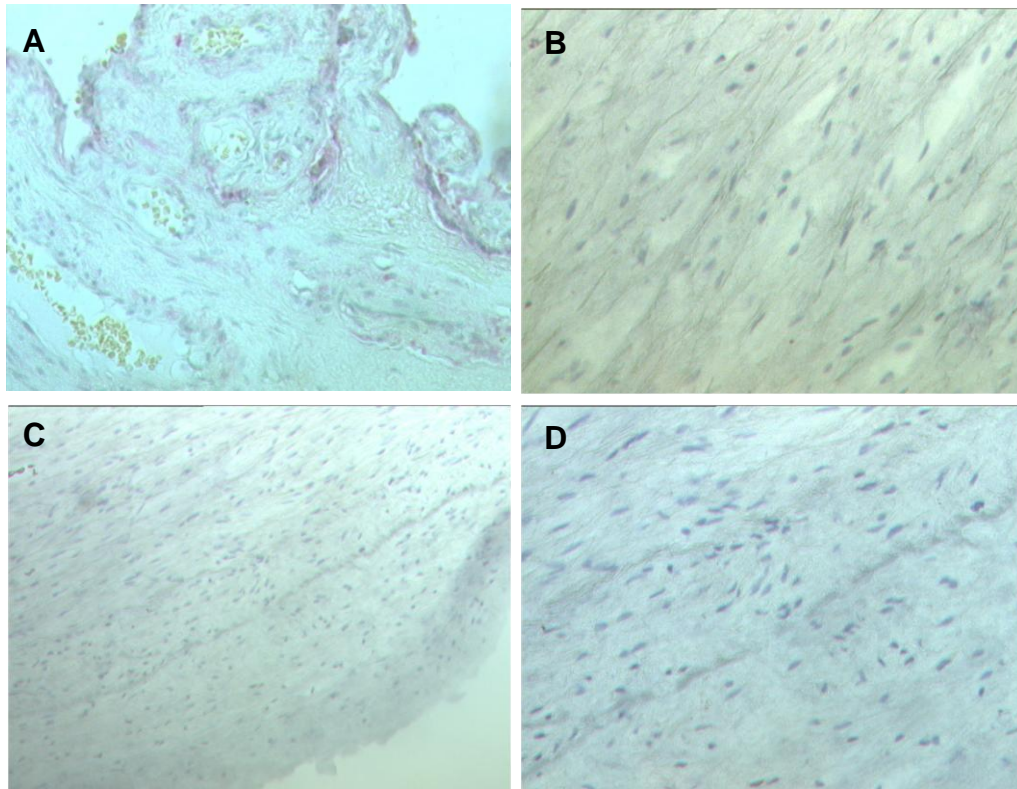


Figure 53 Photomicrograph of Control Sections of Human Aorta

Sections of human placenta and normal aortic tissue were used as positive and negative controls respectively. The placental positive control was probed with mouse anti-human MMP-2 (1:200) x126 (A) and control aortic tissues were probed with monoclonal mouse IgG1 directed against *Aspergillus niger* glucose oxidase (1:200) x126 (B) or mouse anti-human MMP-2 (1:200) x63 (C) and x126 (D) all followed by a biotinylated horse anti-mouse secondary antibody (1:200) and counterstained with Mayers haematoxylin. (n = 5)

All control aortic sections stained negatively for *Aspergillus niger* glucose oxidase (an enzyme not present in mammalian cells) or if the primary antibody was omitted. Control or 'normal aortic' tissue was obtained from consenting donors who had no sign of aneurysmal disease.

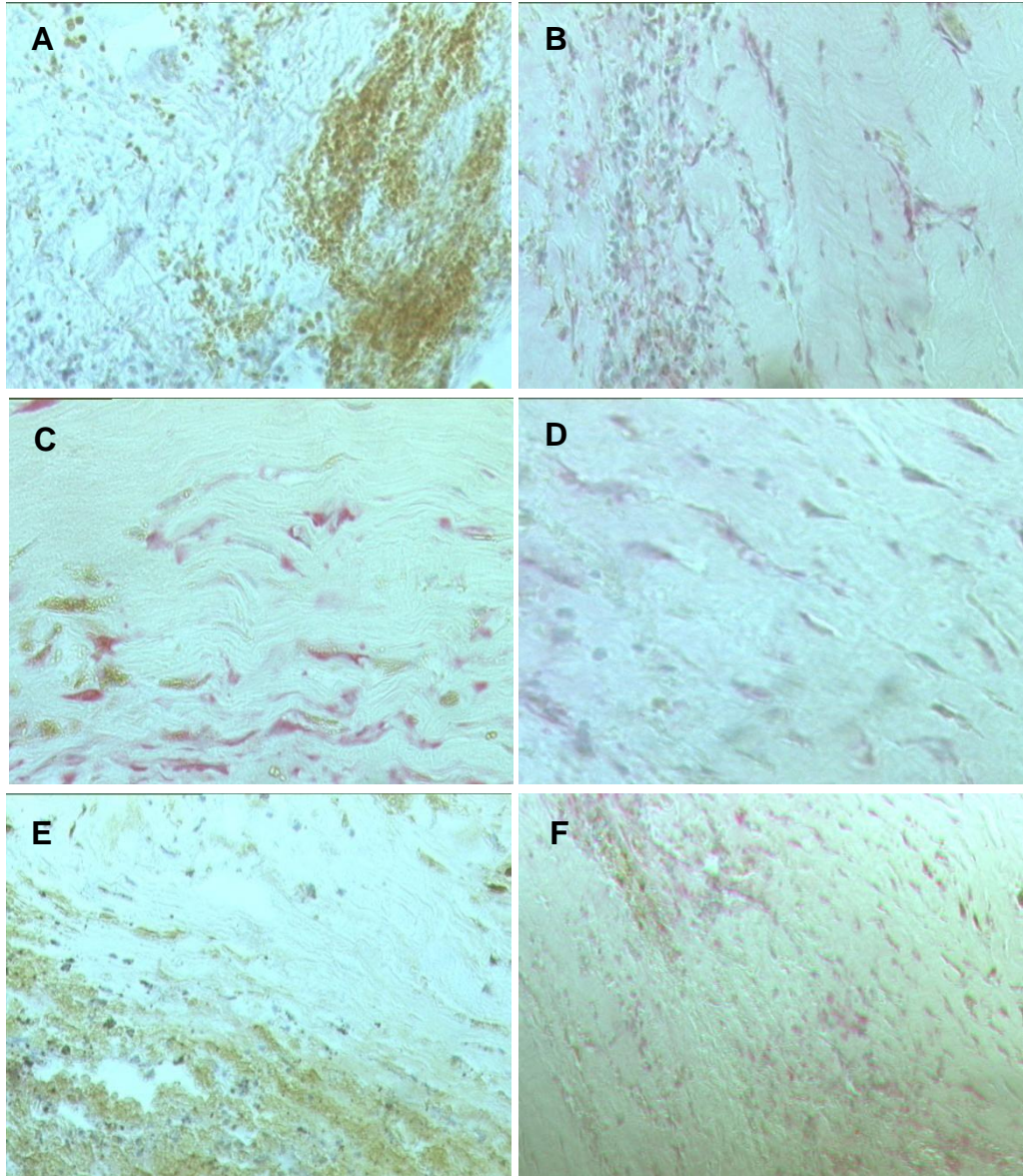


Figure 54 Photomicrographs Showing Localisation of MMP-2 (shown in red) in AAA Tissue

Sections were probed with mouse anti-human MMP-2 (1:200) followed by a biotinylated horse anti-mouse secondary antibody (1:200) and counterstained with Mayers haematoxylin. The distribution of MMP-2 can be seen within the medial regions of aneurysmal aortae. Slide A: (x126) shows inflammatory infiltrate and hemosiderin in the body region of the aneurysm. Slide B: (x126) and D: (x252) shows medial localisation of MMP-2 in the distal region of the aneurysm at different magnifications. Slide C: (x126) and E: (x 126) both demonstrate loss of medial SMCs and overall disorganization of medial elastin structure. Slide F: (x63) demonstrates medial MMP-2 distribution within the proximal region of the aneurysm. (n = 5)

Figures 49 - 52 and Figure 54 demonstrate the presence of MMP-2 in AAA tissue in comparison to the control aortic tissues which were free of aneurysmal disease. MMP-2 immunopositivity was observed in the vascular smooth muscle cells (VSMCs) within the region of *Tunica media* and the inflammatory infiltrate of the AAA sections. These findings correspond with work carried out by earlier researchers who determined that aneurysmal aortic medial SMCs were the primary source of MMP-2 in AAA (Crowther *et al.*, 1996, 2000., Patel *et al.*, 1996). All the aneurysmal sections studied appeared to show decreased medial SMCs which would be consistent with SMC apoptosis a common feature of AAA.

4.9.2 Haematoxylin and Eosin (H and E) Staining of Normal and AAA Sections

H and E staining is one of the most commonly used in histology, Eosin is an acidic dye that is negatively charged and it stains basic or acidophilic structures red or pink such as the cytoplasm of a cell. Haematoxylin can be considered as a basic dye and it stains acidic or basophilic structures a purplish blue and therefore will stain the cell nucleus blue.

4.9.2.1 Tissue Preparation and H and E Stain

4.9.2.2 Materials List

All materials were from VWR Int. Ltd, Dorset, UK unless stated otherwise.

- DePeX Mounting Medium
- Iso-pentane
- Superfrost Plus Microscope Slides
- Tissue-Tek (Sakura. Berkshire, UK)
- Harris Haematoxlin, Eosin (Sigma Aldrich Company Ltd. Dorset, UK).

4.9.2.3 Tissue Preparation Protocol

A beaker of iso-pentane was chilled in liquid nitrogen. Tissue-Tek was spotted onto a cork disk and was submerged in the iso-pentane for approximately 5 minutes after which time the Tissue Tek was sufficiently hardened. The cork disk was removed and a tissue sample, approximately 0.5cm², was pressed into the set Tissue-Tek. The sample was then covered in Tissue-Tek and was incubated in the chilled iso-pentane for about 5mins, or until all Tissue-Tek was solidified. Following this preparation, the sample mounted onto the cork-disk was stored at -70°C for several hours to ensure the sample was completely frozen.

Using a Cryostat (Bright Instruments Company Ltd, Huntingdon, UK) the frozen samples were sectioned into 5µm slices which were immediately placed onto microscope slides. Sections were then stained with Haematoxylin and Eosin using the protocol given in Appendix I. Samples were viewed under a Zeiss Axioscope microscope and images were captured using the Zeiss KS300 program (Carl Zeiss Ltd. Hertfordshire, UK).



Figure 55 Cryostat Used for Cutting Frozen Sections of Tissue

4.9.2.4 Haematoxylin and Eosin Results

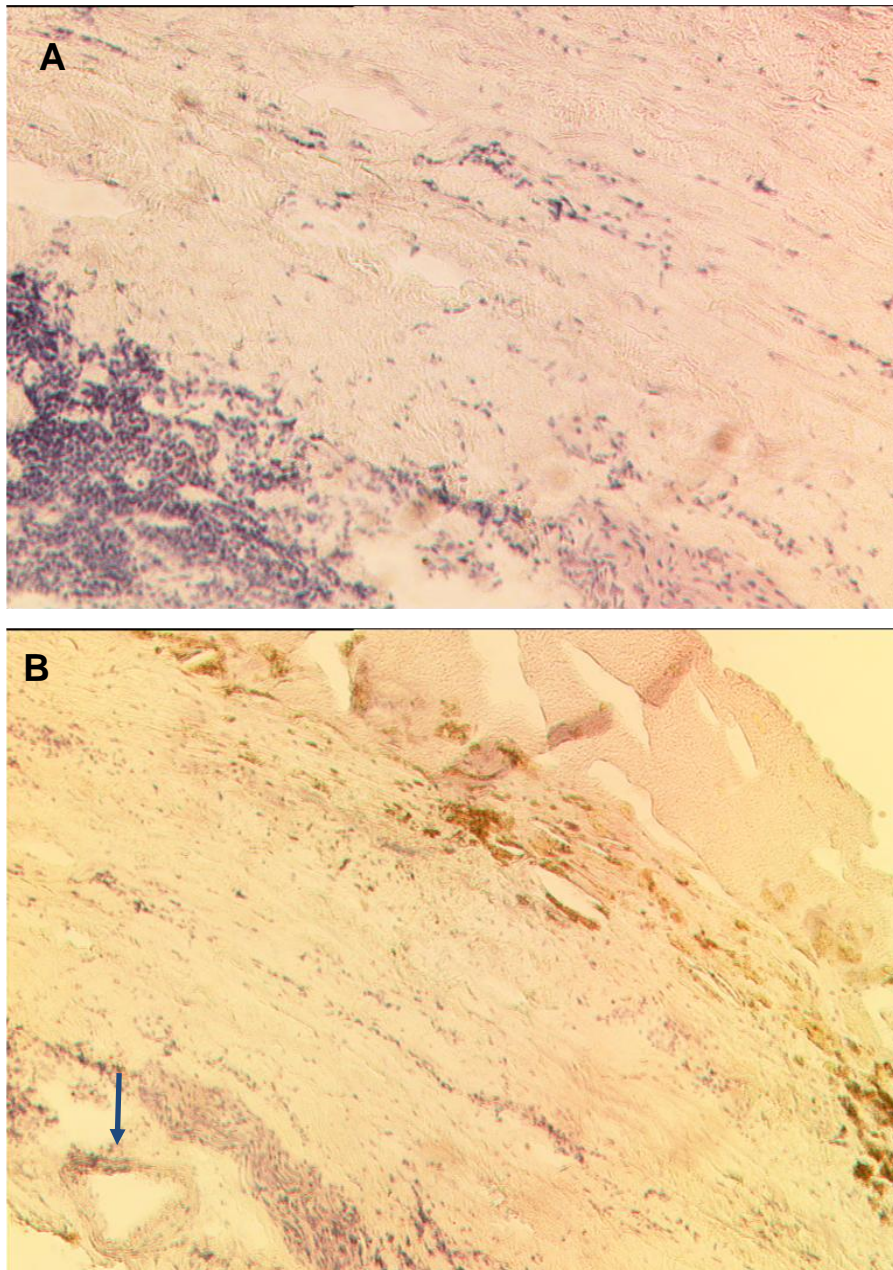


Figure 56 Photomicrographs of the AAA body Region Stained with Haematoxylin and Eosin

Slide A: (x 63) H and E staining of the body region of the aneurysm shows dense inflammatory infiltrate within the medial region and reduced VSMC.

Slide B: (x 31.5) Inflammatory infiltrate is also present in this section, additionally hemosiderin can be seen at the outer edge of the media as a yellowish brown pigment. A *vasa vasorum* can be seen at the bottom left the slide marked with a blue arrow.

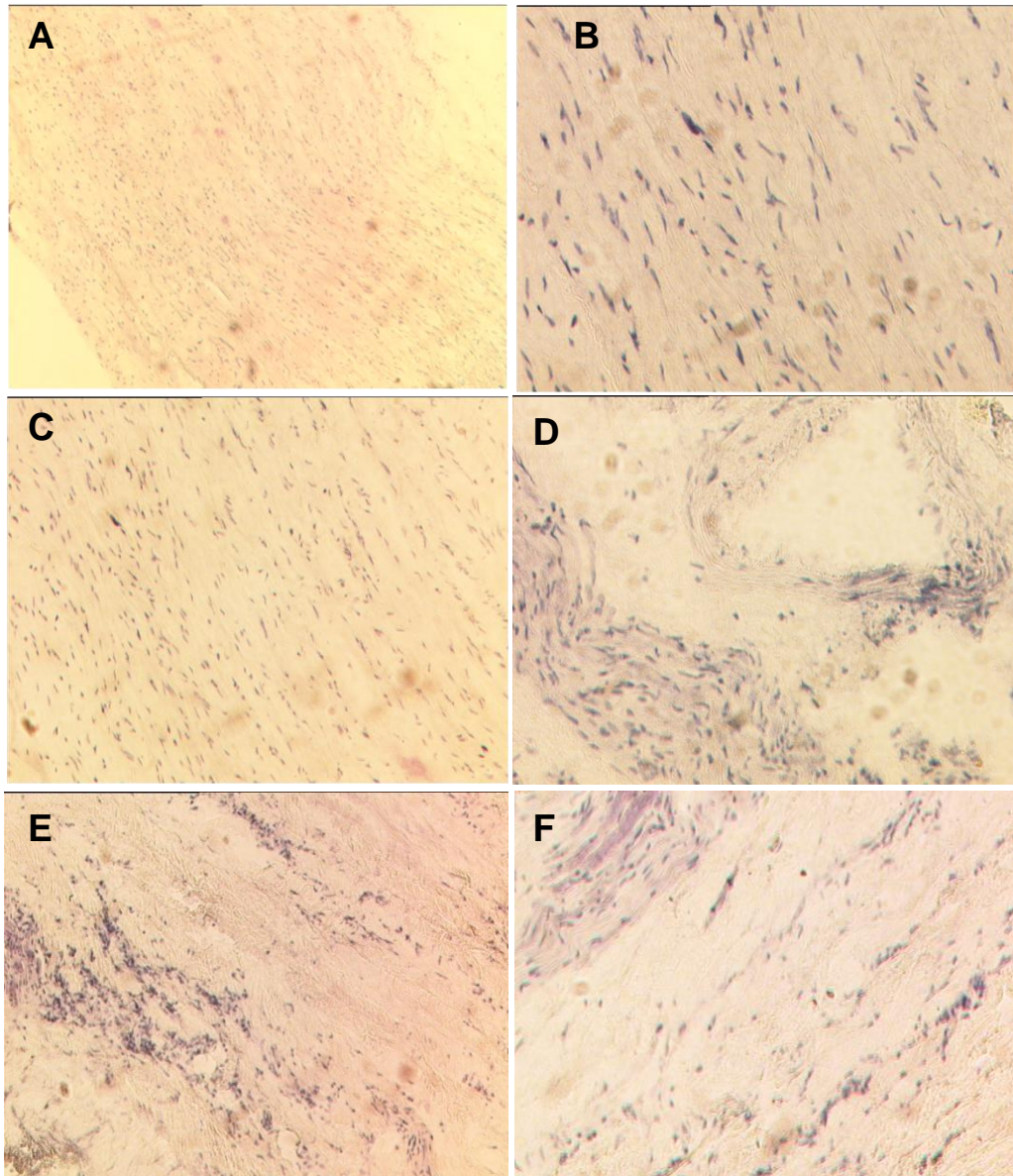


Figure 57 Photomicrograph of Control Aortic and AAA Tissues Stained with Haematoxylin and Eosin

A range of control or 'normal' aortic tissue was stained using haematoxylin and eosin for comparison with AAA tissue. A: (x 31.5) B: (x 126) C: (x 63). Additionally AAA tissue from different regions of the aneurysm is shown demonstrating the structural disorganisation and loss of SMC. D: (x 126) AAA body, E: (x 63) proximal AAA, F: (x 63) distal AAA.

Chapter 5. Investigation into the Potential Expression of MMP-7 in HASMCs Exposed to Severe Hypoxia.

5. Investigation into the Potential Expression of MMP-7 in HASMCs Exposed to Severe Hypoxia.

5.1 Introduction

MMP-7 known also as Matrilysin was first described by Sellers and Woessner (1988) in postpartum rat uterus. Distinguished by its low molecular mass (28 kDa) and its lack of a C-terminal domain, it is the smallest member of the MMP-family. It is located on chromosome 11 q22 and like the other members of the MMP family it is a zinc and calcium-dependent endopeptidase and is secreted as a zymogen requiring activation brought about by cleavage of the propeptide (Thompson and Parks, 1996). Once activated, MMP-7 has a broad proteolytic activity against a variety of extracellular matrix substrates, including gelatin, collagens, proteoglycans, elastin, laminin, fibronectin and casein (Woessner and Taplin, 1988, Miyazaki *et al.*, 1990, Wilson and Matrisian, 1996). In addition, MMP-7 has several other functions including proMMP-2 and proMMP-9 activation, regulation of bioactive molecules controlling inflammation, cell proliferation, apoptosis, angiogenesis and is thought to play an important role in the early steps of tumour progression (Li *et al.*, 2006).

Research has demonstrated increased levels of MMP-7 in the aneurysm wall of AAA, seemingly expressed by cells within the aortic wall such as SMCs (Fontaine *et al.*, 2002, Kazi *et al.*, 2005) and suggesting that MMP-7 may play a late role in aneurysm progression through promotion of continued cellular attrition within the aortic wall (Ikonomidis *et al.*, 2007). Furthermore Burke *et al.*, (2003) identified a putative HRE containing HIF-1 binding sequences within the promoter of the MMP-7 gene which also could mark it as a candidate for hypoxic induction.

In our earlier study (Erdozain *et al.*, 2011), Multiplex PCR analysis revealed significant transcriptional up-regulation of MMP-7 mRNA (p 0.001) from

HASMC lysate after the HASMCs in culture were exposed to hypoxia for 48 hours in comparison to control samples.

Therefore, since MMP-7 has been implicated in tumour metastasis and inflammation, has broad substrate specificity and is expressed in SMC under hypoxic conditions, MMP-7 may be a controlling factor in aneurysm development and progression. It was deemed important to determine whether the significant increases seen in MMP-7 mRNA also translated at the MMP-7 protein level after exposing HASMCs to severe hypoxia (1%) for 48 hours.

5.2 Heparin-enhanced Gelatin Zymography

In contrast to the gelatinases (MMP-2 and MMP-2) MMP-7 is difficult to detect at low levels in conventional casein or gelatin zymography and therefore the technique heparin-enhanced gelatin zymography described by Yu and Woessner (2000) was employed to assess any expression of MMP-7. The addition of heparin to samples enables MMP-7 to be detected at much lower levels, possibly by inducing a conformational change that increases activity, facilitating refolding, reducing autolysis or by helping anchor the enzyme in the gel during the overnight incubation (Yu and Woessner., 2001). Casein is often used for MMP-7 but gelatin can also be used, as it too is a substrate of matrilysin.

Vertical 12% SDS-polyacrylamide gels were copolymerized with 1mg/ml of pig type A gelatin (Sigma Aldrich Co. Ltd. Dorset, UK) overlaid with water-saturated propan-1-ol (Fisher Scientific UK Ltd. Leicestershire, UK) to obtain a level gel surface and allowed to set at room temperature for approximately 30 minutes. Upon setting, the water-saturated propan-1-ol was rinsed off with distilled water and a 4% polyacrylamide stacking gel was applied on top of the resolving gel into which the gel combs were inserted to create wells.

A final concentration of 0.3mg/ml heparin (porcine intestinal mucosa from Sigma) was added to the HASMC samples in non-reducing buffer prior to loading onto the gels. For heparin to produce its enhancing effect it is necessary for the heparin to run to the same region of the gel as the enzyme, which fortunately for MMP-7, movement of heparin and enzyme is almost equal ((Yu and Woessner, 2001).

10µl of a heat-denatured Rainbow size marker (Fisher Scientific UK Ltd. Leicestershire, UK) was used as a molecular weight marker. Human placental homogenate was used as a positive control. Non-reducing loading buffer or lysis buffer provided negative controls. Electrophoresis was carried out in electrophoresis/running buffer at the slightly higher voltage of 150V which is crucial for heparin-enhanced SDS-Page and allowed to run until the dye-front neared the bottom of the gel. The gels then underwent three thirty minute washes in 2.5% Triton X-100 renaturing solution before incubation at 37°C in Zymogram development solution (50mM Tris, pH 7.6, 5mM Ca Cl₂). Finally the zymograms were stained with Coomassie Brilliant Blue R-250 solution and destained with 40% methanol/10% glacial acetic acid. A complete list of protocols and reagents used can be found in Appendix 1.

5.2.1 Troubleshooting heparin-enhanced gelatin zymography

In order to maximise MMP-7 detection in the HASMC samples it was necessary to fill gel wells to their maximum volume presenting an unusual problem in that the samples had a tendency to 'float' back out of the well and diffuse into the running buffer resulting in some loss of sample. This had not happened with other samples without heparin therefore it seemed that the heparin was causing this anomaly.

To rectify this problem, the best solution was to increase the concentration of glycerol in the non-reducing buffer whenever heparin was included in the samples. This solved the issue by increasing the density of the sample and

allowing maximal loading of the wells. Incubation times can be very important when using zymography in order to achieve the best possible results, therefore incubation at 37°C was increased from overnight to forty eight hours to allow as much enzymatic digestion as possible, due to the difficulties in detection of MMP-7.

5.2.2 Results of MMP-7 Heparin-enhanced Gelatin Zymography

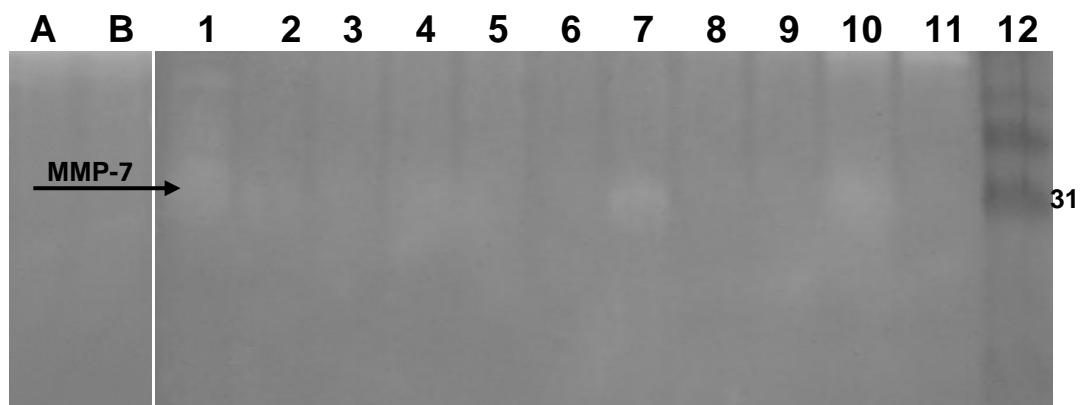


Figure 58 Representative Zymography Gel of MMP-7 Analysis by Heparin-enhanced Zymography

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using heparin enhanced gelatin zymography (heparin at a final concentration of 0.3mg/ml was included in the sample buffer).

A: Negative (lysis buffer) B: Negative (KPO₄ buffer in MilliQ water) 1: Positive control (human placenta homogenized in potassium phosphate KPO₄ plus inhibitors) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11:48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

5.2.3 MMP-7 Increased Sample Volume Analysis and Results

Initially it appeared as though some MMP-7 bands of lysis may be present on the polyacrylamide gel as these 'bands' corresponded the molecular weight of pro MMP-7 (approximately 30 kDa) but because the movement of heparin and MMP-7 is almost equal on the gel the presence of heparin in the samples appeared to be causing the results to be ambiguous.

To try to rectify this problem, larger size combs for the gel plates were used thereby significantly increasing the HASMC conditioned media sample volumes applied.

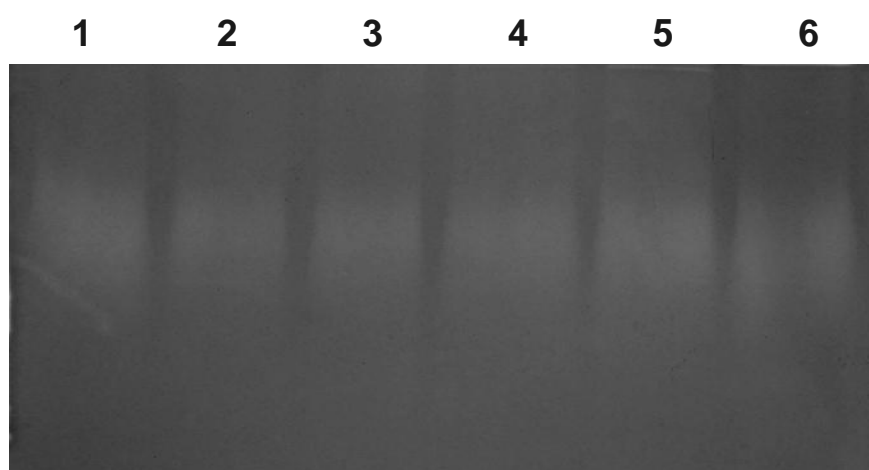


Figure 59 Representative Zymography Gel of MMP-7 Analysis by Heparin-enhanced Zymography (Increased Sample Volume) Gel 1

Significantly increased volumes (30 μ l) of culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using heparin enhanced gelatin zymography (heparin at a final concentration of 0.3mg/ml was included in the sample buffer) 1: 1hr N 2: 1hr H 3: 3hr N 4: 3hr H 5: 6hr N 6: 6hr H (N: Normoxia, H: Hypoxia).

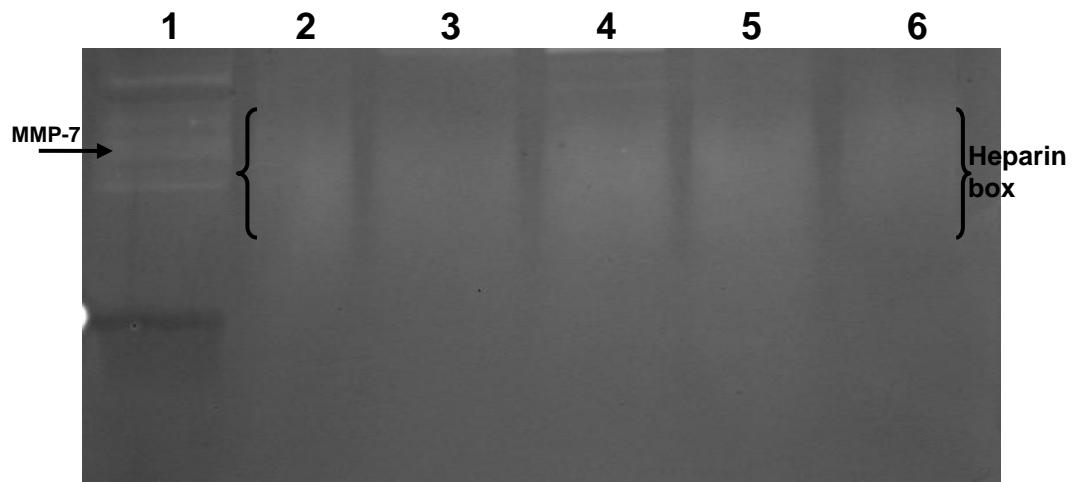


Figure 60 Representative Zymography Gel Analysis of MMP-7 using Heparin-enhanced Zymography (Increased Sample Volume) Gel 2

Significantly increased volumes (30 μ l) of culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using heparin enhanced gelatin zymography (heparin at a final concentration of 0.3mg/ml was included in the sample buffer). 1: Positive control (human placenta homogenized in potassium phosphate KPO₄ plus inhibitors) 2: 30hrsN 3: 30hrsH 4: 48hrsN 5:48hrsH 6: Negative control: sample buffer plus 0.3mg/ml heparin (N: Normoxia, H: Hypoxia).

The heparin enhanced zymography failed to find any MMP-7 present in the HASMC conditioned media despite the sample volume being significantly increased to improve the chance of detecting low levels of the enzyme. Bands of opacity were present on the gels corresponding to the molecular weight of MMP-7 but this appears to be an anomaly caused by addition of heparin to the samples as it was also present in the negative control in Lane 6 of Gel 2 (Figure 60) which obviously did not contain any sample but contained the same concentration of heparin as the conditioned media samples. The heparin box is annotated in Figure 60 showing the region on the gel where the heparin is located.

As a final control the heparin-enhanced zymography was repeated with the same HASMC conditioned medium and the gels subsequently incubated in the MMP inhibitor 10mM EDTA which was added to the zymogram development solution prior to incubation at 37°C.

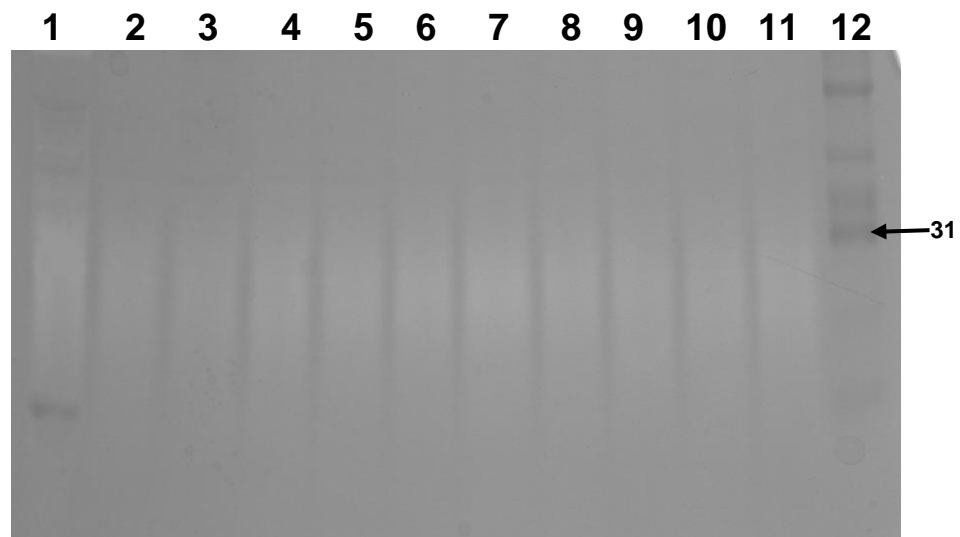


Figure 61 Representative Zymography Gel of MMP-7 Analysis (Inhibition Assay)

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using heparin enhanced gelatin zymography (heparin at a final concentration of 0.3mg/ml was included in the sample buffer) After electrophoresis gels were incubated in 10mM EDTA which was added to the reaction buffer. 1: Positive control (human placenta homogenized in KPO_4 buffer plus inhibitors) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

5.2.4 Analysis of HASMC Lysate for MMP-7 using Heparin enhanced Zymography

Finally, although results had shown that no detectable MMP-7 had been secreted into the HASMC supernatant during the hypoxic period, further heparin-enhanced zymography was carried on HASMC total cell lysate after

exposure to 1% hypoxia for 48 hours to assess if any detectable MMP-7 was present inside the cells. Cells were lysed using ice-cold lysis buffer composed of 0.5% Nonidet, 100mM NaCl, (Sigma Aldrich Co. Ltd. Dorset, UK), (10mM Tris HCL (pH 7.5)(Fisher Scientific UK Ltd. Leicestershire, UK).

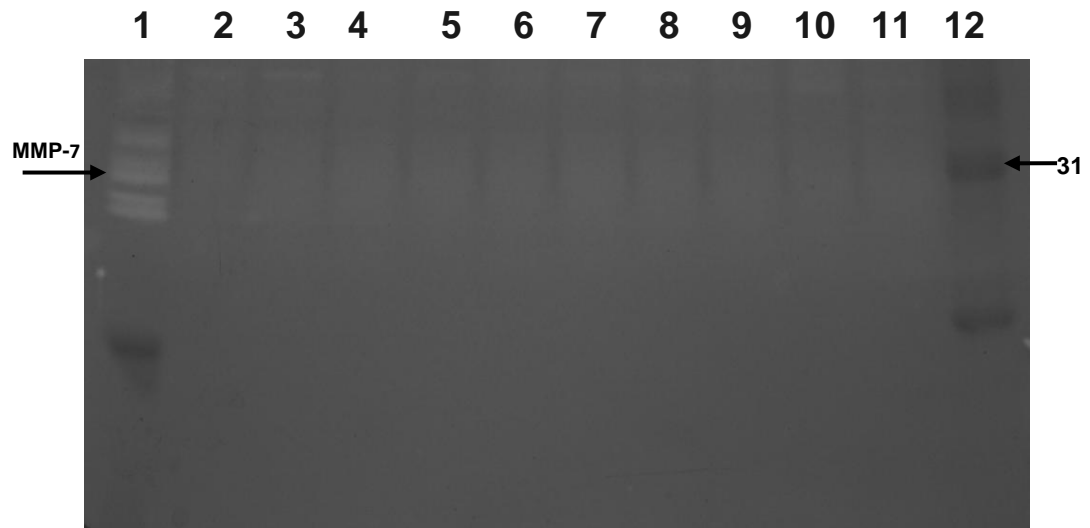


Figure 62 Representative Zymography Gel Analysis of HASMC Cell Lysate for MMP-7

Total cell lysate samples(7 μ g protein) from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were loaded onto 12% gels and analyzed using heparin enhanced gelatin zymography (heparin at a final concentration of 0.3mg/ml was included in the sample buffer).

1:Positive control (human placenta homogenized in KPO₄ buffer plus inhibitors) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

5.2.5 Omission of Heparin from HASMC Lysate Samples

The gel shown overleaf in Figure 63 was prepared exactly as before except that the 0.3mg/ml heparin was omitted from the HASMC total cell samples

as a control to ensure that no bands of lysis were present on the gel apart from the positive control.

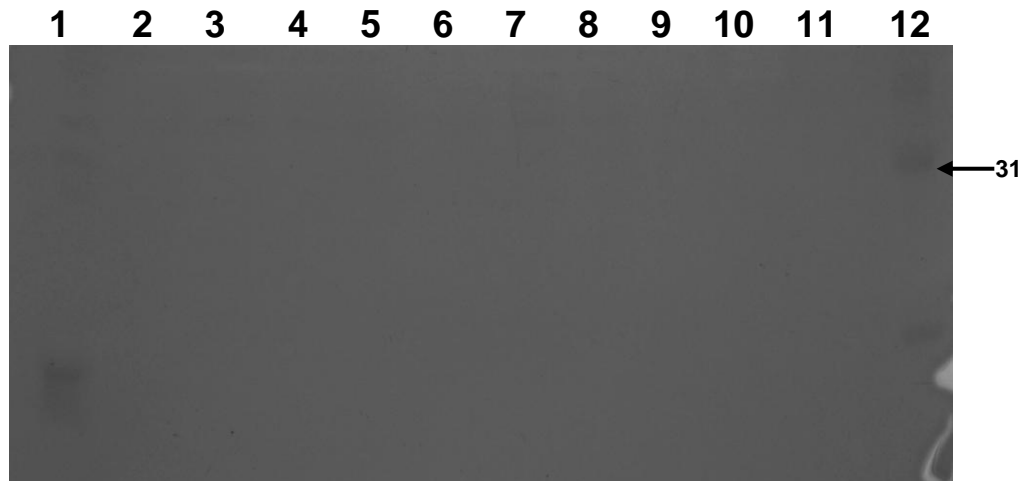


Figure 63 Analysis of HASMC Lysate Samples (Minus Heparin) for MMP-7

Total cell lysate samples minus heparin (7 μ g protein) from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were loaded onto 12% gels and analyzed using gelatin zymography.

1: Positive control (human placenta homogenized in KPO₄ buffer plus inhibitors) 2: 1h N 3: 1hr H 4: 3hrs N 5: 3hrs H 6: 6hrs N 7: 6hrs H 8: 30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

This gel confirms that no bands of MMP-7 lysis are present on the gel. The positive control sample which like all the other samples did not contain heparin is also not showing clear bands of lysis unlike in Figure 62 where bands are clearly evident, which seems to support research which proposes that heparin noticeably increases detection of MMP-7.

**Chapter 6: Analysis of Tissue
Inhibitor of Metalloproteinases
(TIMPs) -1 and 2 levels in HASMCs
exposed to severe hypoxia.**

6. Analysis of TIMP -1 and TIMP- 2 levels in HASMCs exposed to severe hypoxia.

6.1 Introduction

Matrix metalloproteinases (MMPS) are known elastolytic mediators of abdominal aortic aneurysm degeneration, and their activity is tightly regulated by the presence of tissue inhibitors of MMPs (TIMPs). They play an important role in maintaining the integrity of the healthy aorta and imbalances in this system may be instrumental in compromising the arterial wall integrity leading to pathological events such as AAA. TIMP-1 is a 28kDa secreted polypeptide that is capable of inhibiting most MMPs, whereas TIMP-2 (24kDa) is predominantly active against MMP-2. Often the production of TIMPs is elevated by the same stimuli that promote MMP expression (Thompson *et al.*, 2002), therefore, if it is established that hypoxia is a factor in increasing expression of MMPs it would seem prudent to analyse TIMP-1 and TIMP-2 levels in HASMC conditioned media from cells exposed to severe hypoxia (1%) over a 48 time period. Furthermore our earlier study (Erdozain *et al.*, 2011) found significant transcriptional up-regulation of TIMP-1 mRNA using PCR analysis in HASMC lysate after exposing SMCs to hypoxia for up to 48 hours.

Another important feature of TIMP-2 is its role in the activation of proMMP-2, which forms a tight complex with TIMP-2 through the hemopexin domain interactions with the non-inhibitory C-terminal domain of the inhibitor allowing proMMP-2 activation by MT1-MMP on the cell surface (Itoh and Seiki., 2006). If hypoxia is found to increase or decrease TIMP-1 or TIMP-2 levels this obviously would have consequences on the production of some MMPs especially MMP-2.

A technique known as reverse zymography will be used to evaluate the HASMC conditioned media samples.

6.2 Reverse zymography

Reverse zymography is an electrophoretic technique described by Oliver *et al.*, (1997) which is very similar to regular zymography but is used to identify TIMP inhibitory activity within acrylamide gels. Reverse zymography is much more sensitive than Western blot analysis and useful where it may be difficult to acquire antibodies sensitive enough to detect small quantities of TIMPs (Snoek-van Beurden and Von den Hoff, 2005). Additionally it has the benefit of being able to visualize multiple inhibitors simultaneously (Oliver *et al.*, 1997).

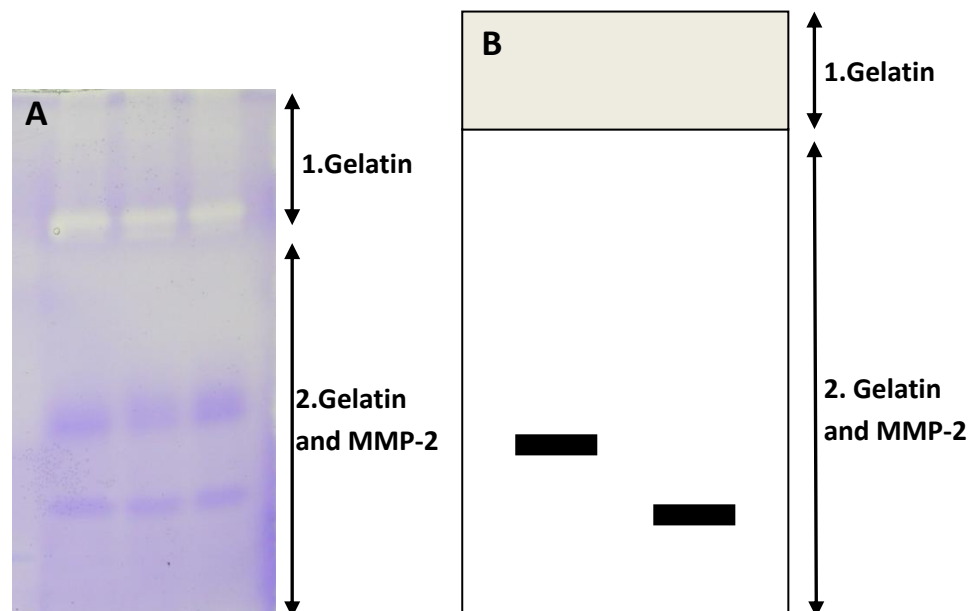


Figure 64 Overview of Reverse Zymography

A: Shows an example of reverse zymography demonstrating dark blue bands representing areas of TIMP inhibition.

B: A diagrammatic overview of a reverse zymogram with incorporated gelatin and purified MMP-2. Black bands represent TIMP activity. Section 1 contains only gelatin and section 2 contains MMP-2 and digested gelatin. (Adapted from Hawkes *et al.*, 2001).

6.2.1 Reverse Zymography Protocol

Vertical 12% SDS-polyacrylamide gels were copolymerized with 1mg/ml of pig type A gelatin (Sigma Aldrich Co. Ltd. Dorset, UK) and also an MMP (usually MMP-2) was incorporated into the gel which was overlaid with water-saturated propan-1-ol (to obtain a level gel surface) and allowed to set at room temperature for approximately 30 minutes. Upon setting, the water-saturated propan-1-ol was rinsed off with distilled water and a 4% polyacrylamide stacking gel was applied on top of the resolving gel into which the gel combs were inserted to create wells. The samples were run at constant voltage of 125 V for approximately 1 hr until the dye front approached the end of the resolving gel, followed by incubation for 1.5 hours in 2.5% Triton X-100 at room temperature to renature the proteins with subsequent incubation at 37°C in development solution (50Mm CaCl₂, 0.5M Tris-HCL pH 7.6) for between 24-48 hours. Thereafter the gels were stained with 0.2% Coomassie Brilliant Blue R-250 for approximately one hour and then destained in methanol, glacial acetic acid and dH₂O (4:1:5, v/v/v). During incubation, the MMP-2 only digests the gelatin in areas where TIMPs are absent therefore, after staining the gel will be colorless except for the TIMP bands. In these bands, the TIMPs will inhibit MMP-2 activity by the formation of a 1:1 complex, and the gelatin will not be digested (Staskus *et al.* 1991, Riley *et al.*, 1999) .This results in blue TIMP bands against a colourless background (Hawkes *et al.*, 2001). The SDS in the gel dissociates any TIMPs bound to MMPs, and thus the total amount of TIMPs in the sample is detected (Riley *et al.*, 1999). A complete list of recipes and materials used can be found in Appendix 1.

6.2 2 Trouble-shooting reverse zymography

Optimization of the gelatin and gelatinase concentration is required because this can dramatically increase the sensitivity of the reverse zymogram. Initially it seemed more likely that using the highest concentration of MMP with a lower concentration of gelatin would give the optimum results.

However after testing a combination of different concentrations of gelatinase and gelatin, suprisingly it seemed that using a much lower concentration of gelatinase in combination with a higher concentration of gelatin incorporated into the gel gave the best results. Also another important factor in achieving good results was the length of the incubation period at 37°C. The optimum incubation period appeared to be around 17 hours. Gels left for significantly longer periods were colourless and devoid of bands.

6.2.3 Results of TIMP-1 and TIMP-2 Reverse Zymography

Levels of TIMP-1 and TIMP-2 in culture supernatants from HASMCs exposed to 1% hypoxia or normoxia for 48 hours were analysed using reverse zymography. 160ng of purified MMP-2 protein (Chemicon Europe Ltd., Chandlers Ford, UK) was incorporated into the polyacrylamide gel at the time of copolymerization and run under non-reducing conditions. TIMP-2 protein (Abcam, Cambridge, UK) at a concentration of 70ng served as a positive control.

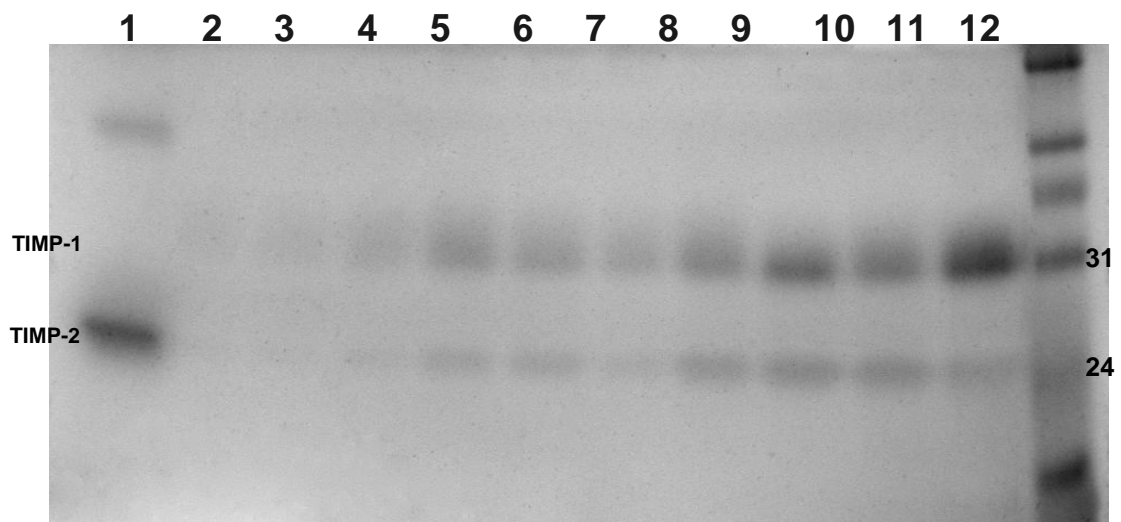


Figure 65 Representative Reverse Zymogram Showing Bands of TIMP Inhibition

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using reverse zymography, incorporating 160ng of purified MMP-2 protein plus 1% gelatin into the gels. 1: Positive control (70ng of purified TIMP-2 protein) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

Bands of TIMP inhibition visualised as dark bands against a lighter background were apparent on the reverse zymogram (Figure 65) with lower bands corresponding to the TIMP-2 positive control and the upper bands seeming to fall within the molecular weight range of TIMP-1.

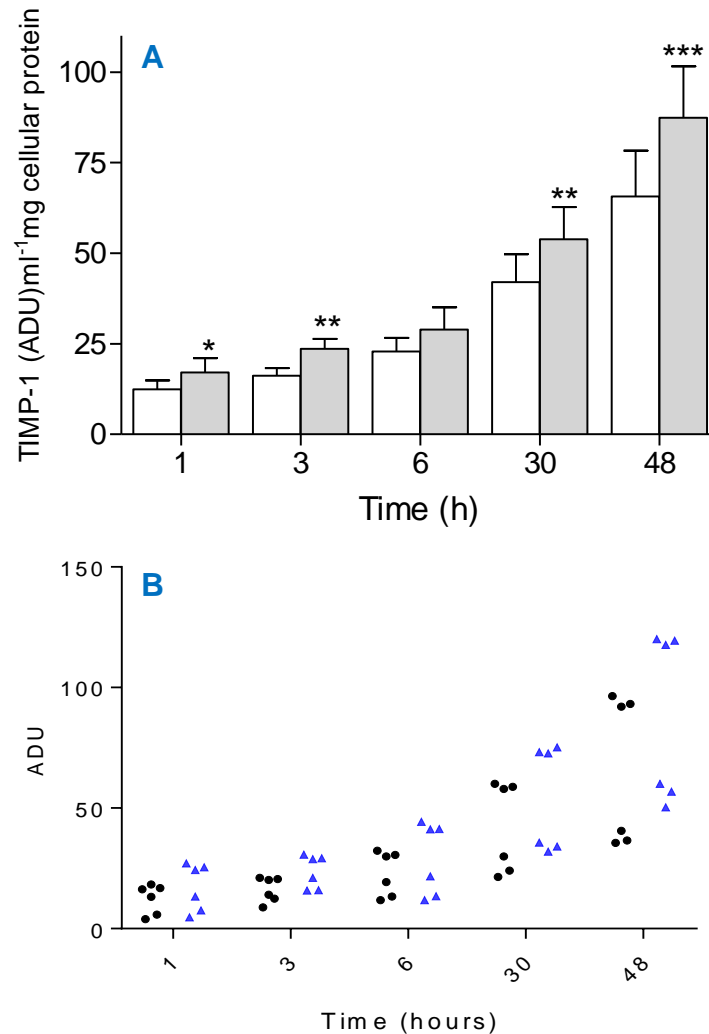


Figure 66 Bar and Scatter Graphs Demonstrating Levels of TIMP-1 in HASMC Supernatant

Analysis of TIMP-1 protein in culture supernatants from HASMCs exposed to 1% hypoxia for 48 hrs. Results expressed as ADU per unit standardised total protein. Data expressed as means \pm SEM of 6 replicate values. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. Results are from three independent experiments with 6 replicates. $n = 3$. Statistical analysis was performed using Student's t-tests.

A: Normoxia (open bars), 1% hypoxia (grey filled bars).

B: Scatter plot : Normoxia (black circles), 1% hypoxia (blue triangles).

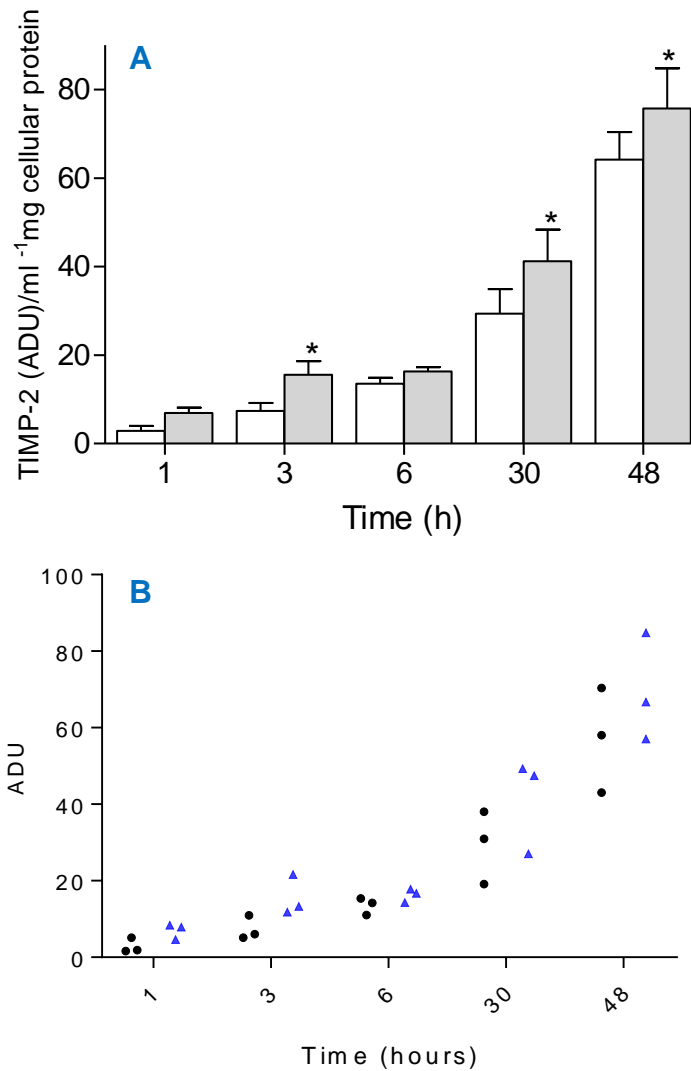


Figure 67 Bar and Scatter Graphs Showing Levels of TIMP-2 in Conditioned Media

Reverse zymographic analysis of TIMP-2 protein in culture supernatants from HASMCs exposed to 1% hypoxia (filled bars) or normoxia (open bars) for 48 h. Results expressed as arbitrary densityunits (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. * $p < 0.05$. Results are from three independent experiments with 3 replicates. $n = 3$. Statistical analysis was performed using Students t-tests.

A: Normoxia (open bars), 1% hypoxia (grey filled bars).

B: Scatter plot : Normoxia (black circles), 1% hypoxia (blue triangles).

Density analysis of the inhibition bands using the Scion Corporation Imaging programme established that in HASMC conditioned media, several time-points demonstrated significant differences in levels of TIMP-1 and TIMP-2 protein between the hypoxic and normoxic control samples.

In Figure 66, significant elevations were seen in TIMP-1 inhibition in hypoxic samples in comparison to control samples at nearly all the time-points, at 1 hour (* $p < 0.05$), 3 hour (** < 0.01) and 30 hours (** < 0.01) and 48 hours (** $p < 0.001$). The 6 hour time-point showed evidence of increased inhibition for the hypoxic samples when compared with normoxia, although no statistical significance was noted.

Interestingly TIMP-1 levels appeared higher than TIMP-2. In Figure 67 there were significant increases seen in TIMP-2 inhibition in hypoxic samples at the 3 hour, 30 hour and 48 hour time-points (* $p < 0.05$) but at the 1 hour and 6 hour time-points, although inhibition levels were higher in hypoxic samples compared to normoxic controls they were not statistically significant.

6.2 4 MMP-9 Reverse Zymography

For comparison purposes and because TIMP-1 is the specific inhibitor of MMP-9, further reverse zymography was carried out incorporating MMP-9 protein instead of MMP-2 into the gels. The procedure was carried as before except that the concentration of purified MMP-9 (Chemicon Europe Ltd., Chandlers Ford, UK) included in the gel at the time of polymerization was reduced to 60ng as earlier optimisation of this assay had proved to give better results at this gelatinase concentration.

6.2.5 Results of TIMP-1 and TIMP-2 Levels in HASMC Supernatant using Reverse Zymography Incorporating MMP-9

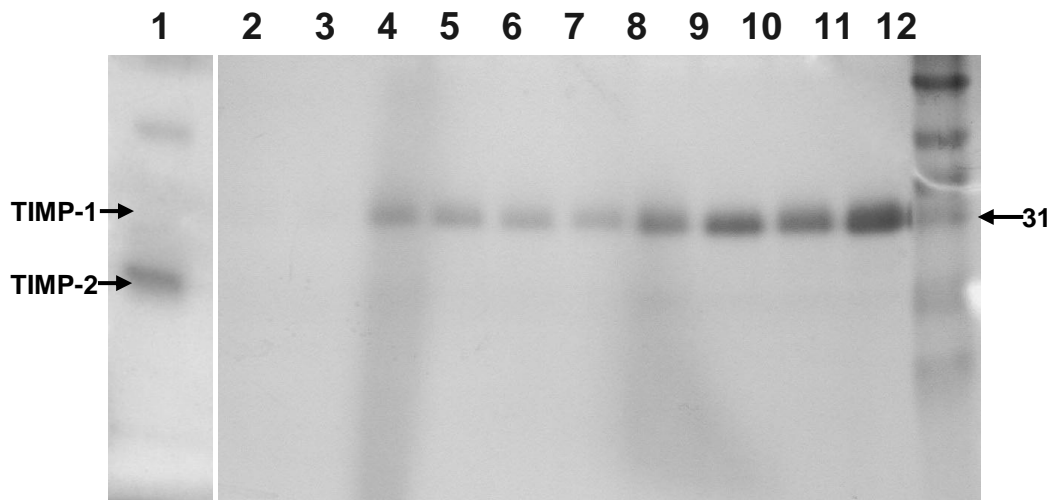


Figure 68 Representative Reverse Zymogram (Incorporating MMP-9) Showing Analysis of TIMP Levels in HASMC Supernatant

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using reverse zymography by incorporating 60ng purified MMP-9 protein into the gels. Purified TIMP-2 protein was used as a positive control. 1: Positive control (70ng of purified TIMP-2 protein) 2: 1h rN 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: 48hrs N 11: 48hrs H 12: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

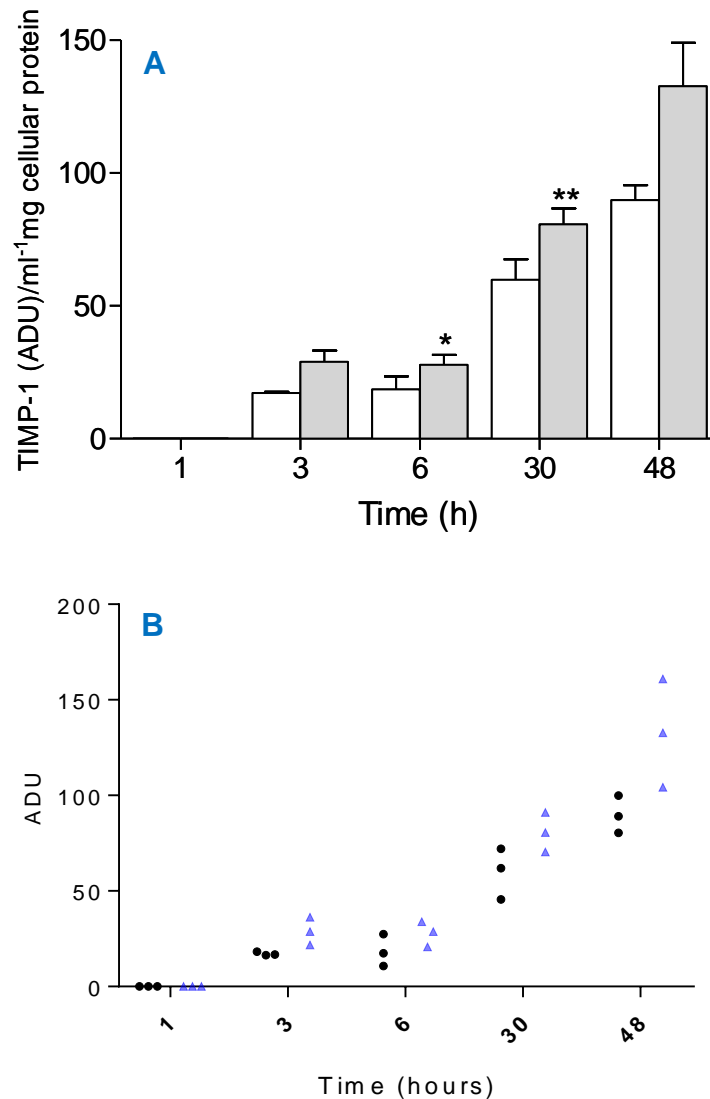


Figure 69 Bar and Scatter Graphs Showing Levels of TIMP-1 in HASMC Conditioned Media

MMP-9 Reverse zymographic analysis of TIMP-1 protein in culture supernatants from HASMCs exposed to 1% hypoxia for 48 hours. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. * $p < 0.05$; ** $p < 0.01$. Results are from three independent experiments with 3 replicates. $n = 3$. Statistical analysis was performed using Student's t-tests.

A: Normoxia (open bars), 1% hypoxia (grey filled bars).

B: Scatter plot : Normoxia (black circles), 1% hypoxia (blue triangles).

Bands of TIMP inhibition were seen on the gel in Figure 68 which corresponded to being within the molecular weight range of TIMP-1. There were significant increases seen in TIMP-1 inhibition in hypoxic samples at the 6 hour time-point (* $p < 0.05$) and 30 hour time-point (** < 0.01). At the 1 hour and 48 hour time-points, although inhibition levels were higher in hypoxic samples compared to normoxic controls they were not statistically significant.

As a contrast to the reverse zymography performed using MMP-2 included in the gel, TIMP-2 inhibition was not detected on the gels incorporating MMP-9. This could be explained as TIMP-2 is more selective and inhibits MMP-2 whereas TIMP-1 has the ability to inhibit most MMPs.

6.2.6 Reverse Zymography Control Gels

To ensure that it was TIMP inhibition responsible for the bands observed on the reverse zymograms, control gels were run at the same time. Hawkes *et al.*, (2001) suggested that a parallel regular SDS-PAGE gel with identical samples is run under identical conditions at the same time. Bands that appear in the reverse zymogram but are not visible in the regular SDS gel after staining with Coomassie Blue must be TIMPS.

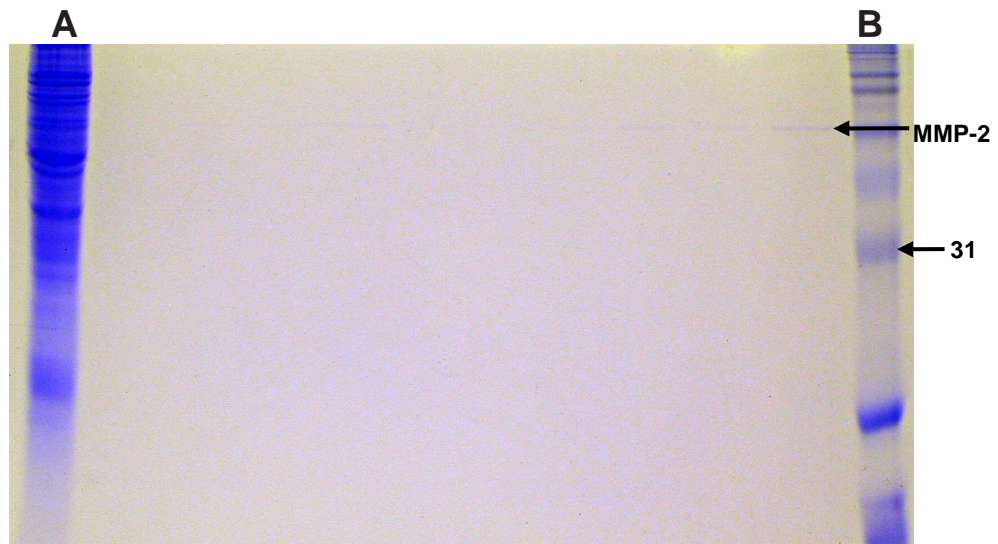


Figure 70 Parallel Control SDS Page Gel 1

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto a regular 12% SDS Page gel (without MMP-2 or 9 incorporated into the gel) and stained using Coomassie Blue. **A:** Lysate from MDA MB 231 cells was used as a positive control **B:** (RSM: Rainbow size marker)

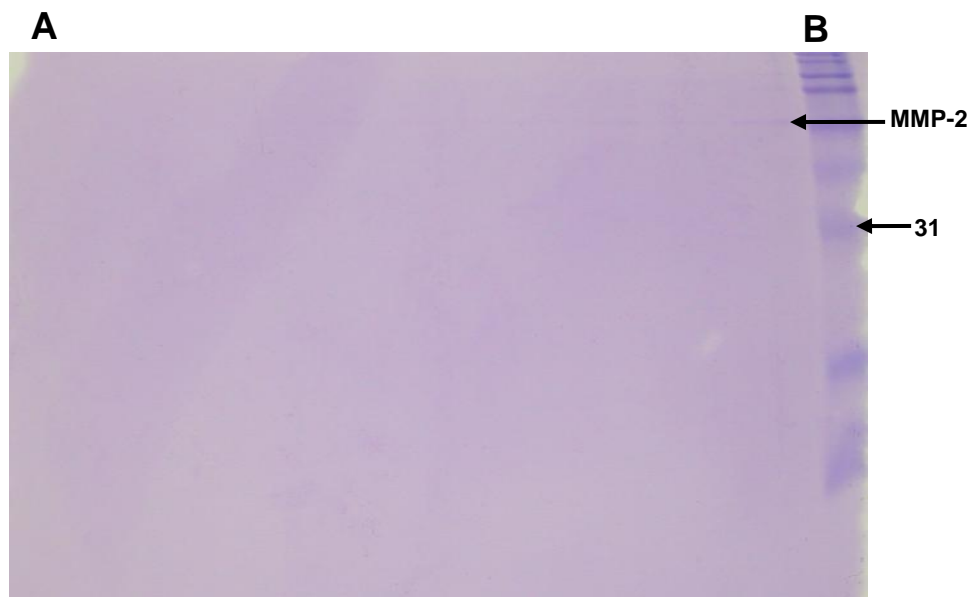


Figure 71 Parallel Control SDS Page Gel 2

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto a regular 12% SDS Page gel (without MMP-2 or 9 incorporated into the gel) and stained using Coomassie Blue. **A:** 70 ng purified TIMP-2 protein was used a positive control. **B:** (RSM: Rainbow size marker).

As described by Hawkes *et al.*, (2001) all the TIMP bands were absent from the two control regular SDS Page gels (Figure 70 and 71) including the positive control Lane A on Gel 2, Figure 71 which contained 70ng of purified TIMP protein and had previously given a distinct band on the reverse zymograms in Figure 65 and Figure 68.

To further verify the bands as TIMPs, control gels were incubated overnight in the presence of 1,10-phenanthroline (10mM) which interferes with development of inhibitor activity in reverse zymography and will inhibit any TIMP bands. Identical samples of HASMC conditioned media were run as normal reverse zymograms containing 1% gelatin. One gel contained 160ng purified MMP-2 and the second 60ng of purified MMP-9 and were treated exactly as the earlier reverse zymograms in except that 10mM 1,10-phenanthroline was added to the incubation buffer.



Figure 72 Control Reverse Zymogram Containing MMP-2 and Incubated in 10mM 1,10-Phenanthroline

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using reverse zymography by incorporating 160ng purified MMP-2 protein into the gels. Gels were incubated overnight in 10mM 1,10-phenanthroline. **A:** 140 ng purified TIMP-2 protein was used a positive control. **B:** (RSM Rainbow size marker).



Figure 73 Control Reverse Zymogram Containing MMP-9 and Incubated in 10mM 1,10-Phenanthroline

Culture supernatants from HASMCs exposed to hypoxia (1%) or normoxia for 48hrs were equally loaded onto 12% gels and analyzed using reverse zymography by incorporating purified 60ng MMP-9 protein into the gels. Gels were incubated overnight in 10mM 1,10- phenanthroline. **A:** 140ng purified TIMP-2 protein was used a positive control. **B:** (RSM: Rainbow size marker).

The parallel gels in Figure 70 and Figure 71 as expected, did not exhibit any bands apart from faint bands at the very top of the gel which were most likely to be MMP-2 and certainly not within the molecular weight range of any of the TIMP family.

Additionally, the gels in Figure 72 and Figure 73 which had been incubated overnight in 10mM 1,10- phenanthroline were negative for bands of TIMP inhibition. Any TIMPS present in the HASMC conditioned media had been inhibited as expected, including the positive control lanes where the purified TIMP-2 protein concentration had been doubled from 70ng to 140ng.

All the control gels therefore appear to verify that the bands of inhibition seen on the reverse zymograms in Figure 65 and Figure 68 were most likely to be TIMPs.

Chapter 7 Potential role of Hypoxia-inducible factor -1alpha (HIF-1 α) in the pathogenesis of AAA

7. Potential role of Hypoxia-inducible factor -1alpha (HIF-1 α) in the pathogenesis of AAA

7.1 Introduction

The global oxygen sensor HIF-1 α responds to low tissue oxygen by up-regulating hypoxia responsive genes, controlling the expression of over seventy genes involved in intracellular adaptive responses to hypoxia (Semenza., 2004). HIF-1 α targets genes by directly binding to hypoxia responsive elements within the promoters of these genes which also include VEGF (Forsythe *et al.*, 1996) and Ets-1 (Oikawa *et al.*, 2001) which are both of interest to this AAA study. HIF-1 α is also known to modulate MMPs in other research areas such as joint disease and cancer studies (Pufe *et al.*, 2004, Yamaguchi *et al.*, 2005, Krishnamachary *et al.*, 2003).

In AAA, expression of HIF-1 α has been found to be significantly higher in aneurysm tissue than in normal aorta (Hu *et al.*, 2004) which was later corroborated by our Group (Erdozain *et al.*, 2011) who found expression of Hif-1 α , MMP-2 and Ets-1 strongly up-regulated within HASMC and inflammatory infiltrate of the tunica media of the aortic wall. Arterial wall hypoxia has been investigated in other conditions such as atherosclerosis, intimal hyperplasia and myointimal hyperplasia and animal models of AAA have also demonstrated low oxygen tension (pO₂) within the aortic media (Bjornheden *et al.*, 1999). Preliminary human data have shown that hypoxia exists *in vivo* in patients with aneurysms (Vorp *et al.*, 1998, 2001). Additionally, the earlier results (Chapter 4, section 4.4.2.2) exposing HASMCs to prolonged hypoxia resulted in increased secretion of MMP-2 leading to the hypothesis that HIF-1 α could be a potential initiating factor in the pathway leading to MMP up-regulation. Sun *et al.*,(2007) designed a numerical model of oxygen transport in AAA which demonstrated O₂ levels of 1% and lower within the thickest regions of the ILT and subsequently led this study to evaluate the effect of more severe hypoxia (1%) on MMP secretion, following on from our earlier study (Erdozain *et al.*, 2011) where oxygen tension was sequentially reduced from 5% down to 2% over 72 hrs.

7.2 Chapter Aims

1. To determine if HIF-1 α is expressed in cultured SMC and if so analyse its expression after exposure to severe hypoxia (1%) by immunoblotting techniques
2. To examine the differences in HIF-1 α expression in whole cell HASMC lysates between normoxia and hypoxic HASMC samples in preparation for further experimentation later in the study
3. To confirm the localisation of HIF-1 α in control and aneurysmal tissue using immunohistochemical techniques.

7.3 Immunoblotting

7.3.1 Materials List

- ECLTM Western Blot Detection Reagents, HybondTM-C extra nitrocellulose (Amersham Biosciences UK Ltd. Buckinghamshire, UK).
- Kodak BioMax XAR Film (VWR International Ltd. Dorset, UK).
- Polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma Aldrich Company Ltd. Dorset, UK).
- Anti-human HIF-1 α primary antibody (Novus Biologicals., Littleton, CO, USA).

7.3.2 Western Blot Protocol

All materials and recipes for sample buffer, running buffer, blotting buffer, washing buffer, blocking buffer, the stacking gel, and the resolving gel are shown in Appendix 1.

HASMC total cell lysate samples were mixed with reducing loading buffer and heated to 100°C for 5 mins and separated by 7.5% SDS-PAGE gel electrophoresis at 120V for approximately one hour or until the gel-front

neared the end of the resolving gel. Subsequently the proteins were transferred onto a nitrocellulose membrane under reducing conditions at 150mA for approximately 2 hours.

Immunodetection of the nitrocellulose-bound proteins began with a one hour incubation in 5% blocking buffer at room temperature with continuous agitation. After this step the blocking buffer was removed and replaced with 1:500 mouse monoclonal anti-human HIF-1 α primary antibody (Novus Biologicals., Littleton, CO, USA) diluted in fresh blocking buffer.

The nitrocellulose membrane was incubated with the 1 $^{\circ}$ antibody overnight at 4 $^{\circ}$ C after which the blocking buffer was discarded and any unbound 1 $^{\circ}$ antibody was removed by 6 x 5mins wash steps in excess washing buffer. The membrane was next incubated in 1:200 polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody (DAKO UK Ltd., Cambridgeshire, UK) prepared in blocking buffer for approximately one hour. Unbound 2 $^{\circ}$ antibody was removed by further 6 x 5mins wash steps in excess washing buffer. Following the wash steps the nitrocellulose membrane was covered in ECL detection reagents (prepared according to the manufacturers instructions) and luminescence was detected on Kodak film.

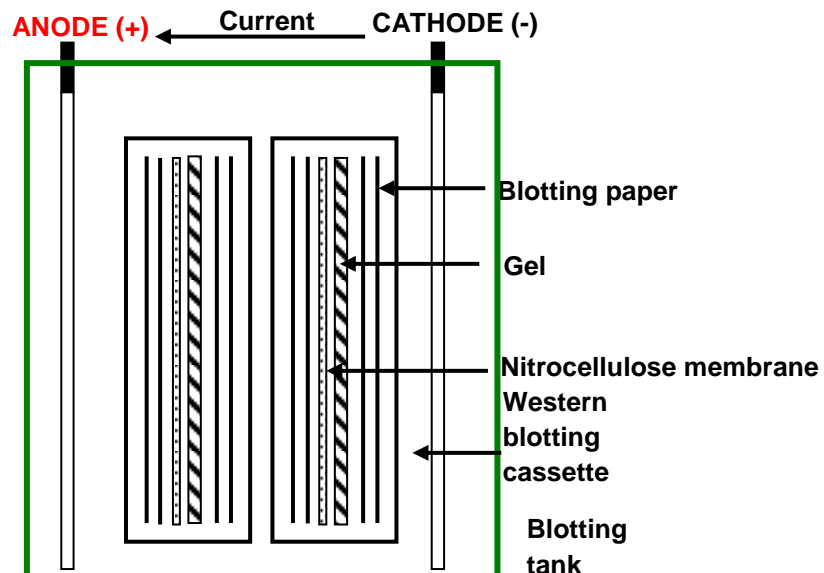


Figure 74 Schematic Diagram of the Blotting Apparatus

The cassettes containing the acrylamide gel and nitrocellulose membrane are placed inside the blotting tank which is then filled with transfer buffer. It is vital that the nitrocellulose is placed nearest the positive electrode to enable the transfer of proteins from the gel onto the membrane and to ensure the proteins are not lost. The blot is run at 150 mA for 2 hours.

7.3.3 Results of HIF-1 α Immunoblotting

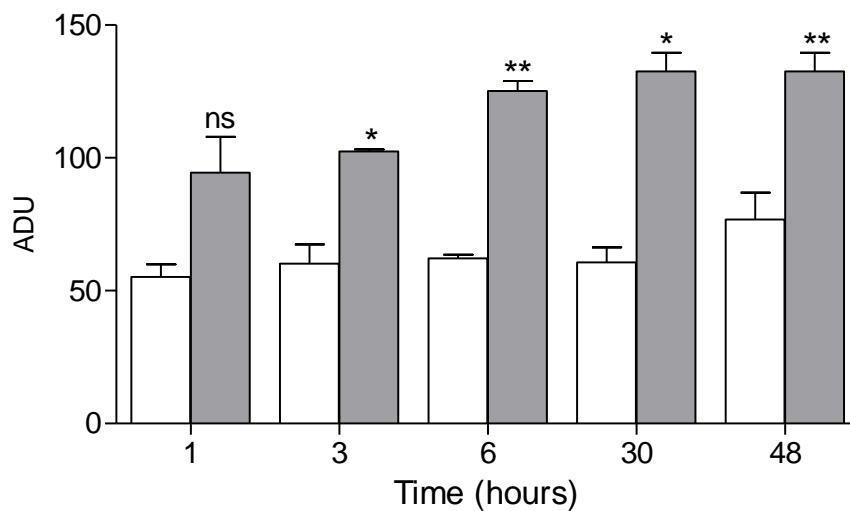


Figure 75 HIF-alpha Protein levels in HASMC Total Cell Lysate

HIF-1 α protein levels in total HASMC lysate after exposure to 1% hypoxia (filled bars) or normoxia (open bars) for 48 h assessed by immunoblotting. Density analysis was carried out using Scion Imaging Software (version 4.0.2; www.scioncorp.com). Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. * p < 0.05; ** p < 0.01. Results are from three independent experiments repeated in triplicate. n = 3. Statistical analysis was performed using Student's t-tests.

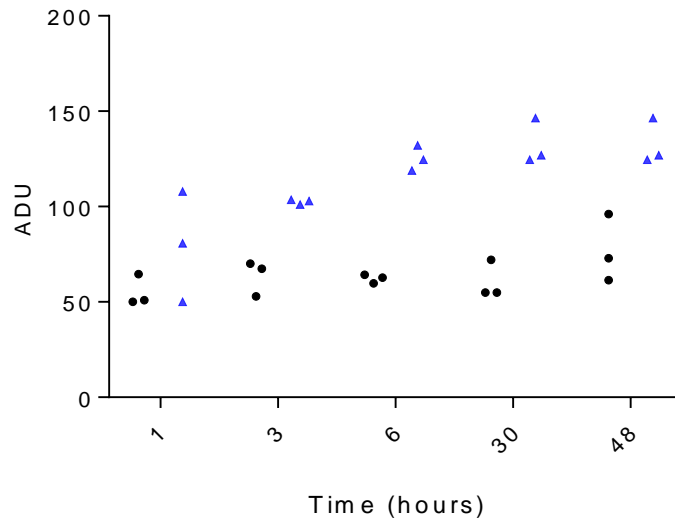


Figure 76 Scatter Plot of HIF-1alpha Protein Levels in HASMC Total Cell Lysate

HIF-1 α protein levels in total HASMC lysate after exposure to 1% for 48 h assessed by immunoblotting. Density analysis was carried out using Scion Imaging Software (version 4.0.2; www.scioncorp.com). Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. Results are from three independent experiments repeated in triplicate. n = 3. Statistical analysis was performed using Student's t-test. Normoxia (black circles), 1% hypoxia (blue triangles).

In Figure 75 HIF-1 α immunoblot analysis established the presence of increased HIF-1 α in total cell lysate from HASMCs exposed to over 48hrs of severe (1%) hypoxia. After 1 hour of hypoxia, there was an increase in levels of HIF-1 α protein between the hypoxic and normoxic samples, although not statistically significant, but after 3 hours, 6 hours, 30 hours and 48 hours of hypoxia, a significant rise was demonstrated in the hypoxic samples ($p < 0.05$, $p < 0.01$, $p < 0.05$ and $p < 0.01$ respectively) in comparison to normoxic control samples.

7.4 Distribution of HIF-1 α in AAA

Immunohistochemistry was performed using 5 x human AAA donors and 5 x normal aortic control tissue donors (n =10) to demonstrate HIF-1 α was present within these tissues and also to illustrate its distribution.

7.4.1 Materials List

- Mouse monoclonal anti-human HIF-1 α (Abcam, Cambridge, UK).
- Alkaline phosphatase-conjugated avidin/biotin complex anti-mouse secondary antibody (ABC) kit (Vector Laboratories, Peterborough, UK).
- Levamisole (Vector Laboratories, Peterborough,UK).
- Horse serum (Sigma-Aldrich Ltd (Dorset, UK).
- Fast Red TR/Naphthol AS-MX Tablets (Sigma-Aldrich Ltd (Dorset, UK).
- Mayers haematoxylin (Sigma-Aldrich Ltd (Dorset, UK).
- Monoclonal IgG1 (Dako UK Ltd, Cambridgeshire, UK).

7.4.2 Immunohistochemistry Protocol

With local ethics approval, AAA tissue was obtained from consented patients undergoing elective surgical repair. Sections were taken from the proximal distal and body of the aneurysm. Normal tissue free from signs of aortic disease was obtained from the aortic cuff of renal transplant donors. At the time of retrieval tissue samples were immediately fixed in 0.9% NaCl (10%v/v) histological formaldehyde and subsequently processed using a Shandon Hypercentre II firowax-embedding station (Shandon Inc., Pennsylvania, USA).

After processing, tissue sections (5µm) cut from the paraffin embedded wax blocks were de-paraffinised, then rehydrated in preparation for staining. Sections were evaluated using mouse monoclonal anti-human HIF-1α (Abcam) which was incubated overnight at 4°C. Subsequently the sections were incubated in a biotinylated anti-mouse secondary antibody solution (1:100) for 2 hours at room temperature followed by a minimum 30 minute incubation in Vectastain's ABC alkaline phosphatase solution. Endogenous alkaline phosphatase was inhibited using Levamisole. Visualisation of HIF-1α was achieved by incubating the sections in Fast Red substrate solution until a red precipitate appeared and counterstaining with Mayers haematoxylin. Monoclonal IgG was used as a negative control or sections prepared minus the primary antibody.

7.4.3 Results: Distribution of HIF-1α

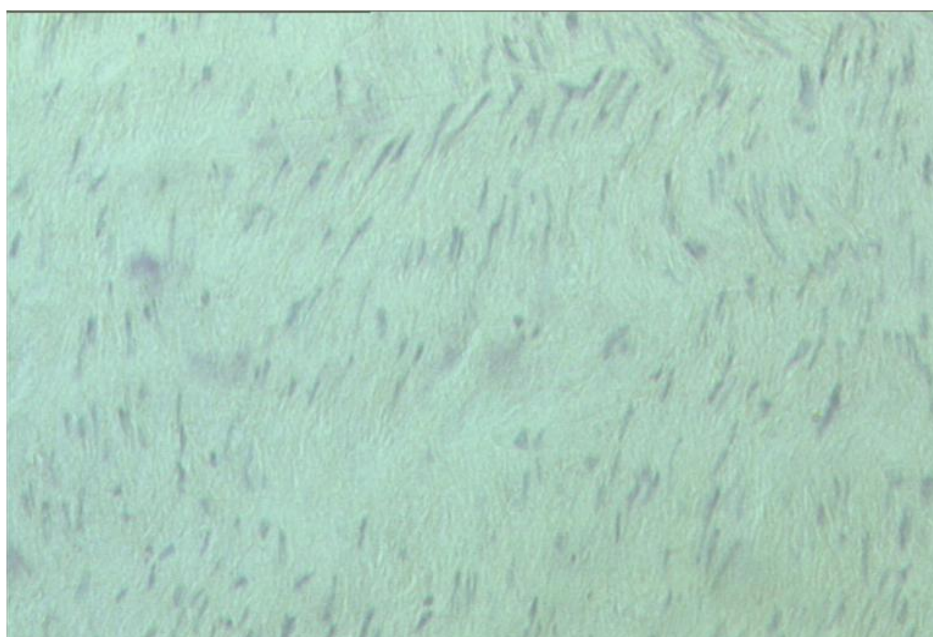


Figure 77 Photomicrograph Showing Localisation of HIF-1 Alpha in the Media of Normal Aorta (x63)

Control specimens of normal human aorta were probed with mouse anti-human HIF-1 alpha using a biotinylated horse anti-mouse secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayers Haematoxylin for comparison with identically treated sections of human aneurysm tissue. The section appears negative for nuclear HIF-1α although some cytoplasmic may be expected under normal circumstances. (n = 5)

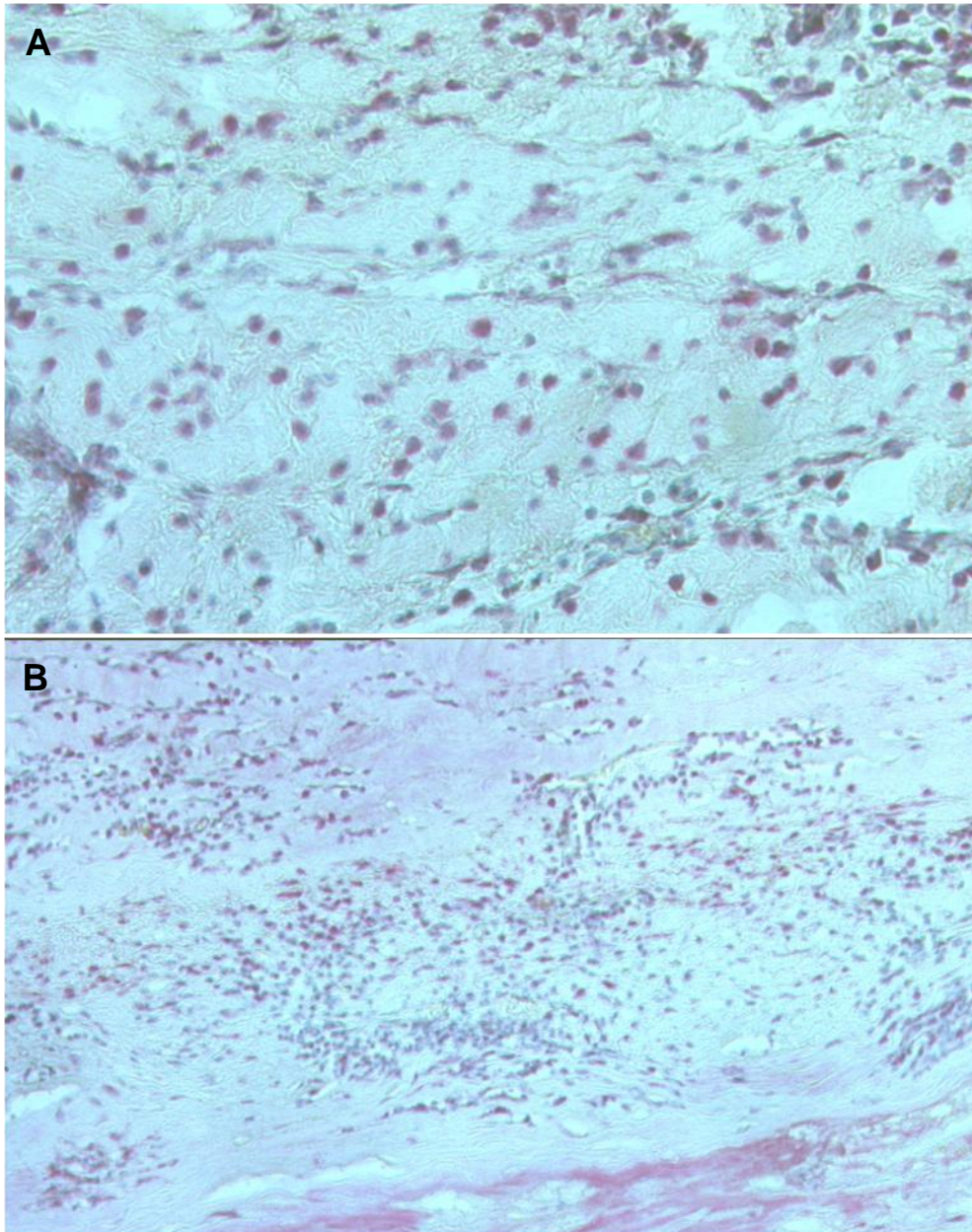


Figure 78 Photomicrograph Showing the Immunolocalisation of HIF-1 Alpha in the Body Region of AAA Tissues

Sections of human aneurysmal tissue were probed with mouse anti-human HIF-1 alpha and a biotinylated horse anti-mouse secondary antibody (1:200) (Vector Laboratories) followed by counterstaining with Mayer's haematoxylin. HIF-1 alpha Immunopositive nuclei are present on both slides. Slide A: Demonstrates the nuclear immunolocalisation of HIF-1 alpha (red) in a human AAA tissue section (x126) Slide B: Lower power magnification showing the nuclear immunolocalisation of HIF-1 alpha in a human AAA tissue section (x63). (n = 5).

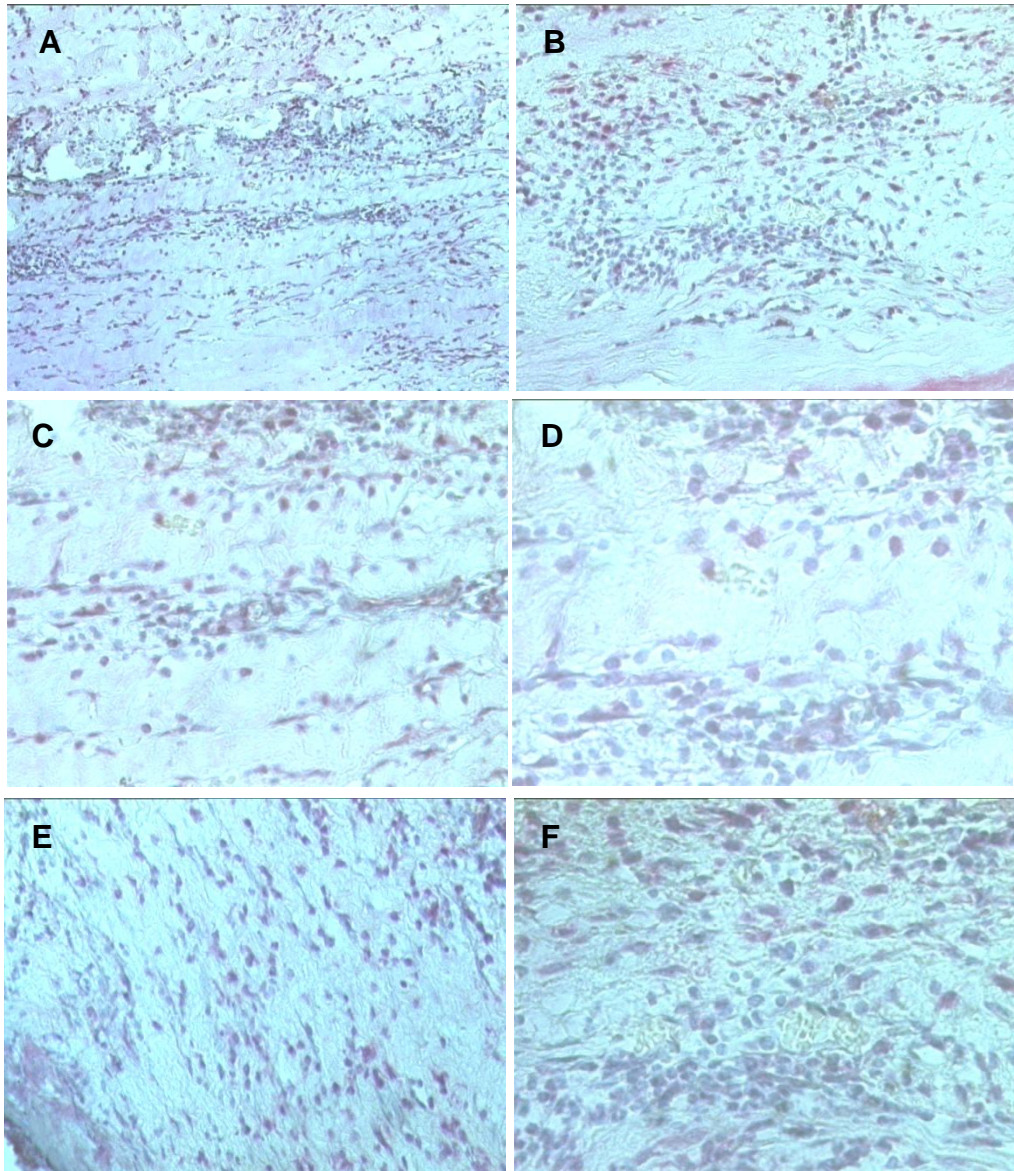


Figure 79 Photomicrographs Showing the Immunolocalisation of HIF-1 Alpha (Red) in AAA Tissue Sections (x126)

Sections of human aneurysmal tissue were probed with mouse anti-human HIF-1 alpha followed by a biotinylated horse anti-mouse secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayers haematoxylin to demonstrate HIF-1 α positivity within the inflammatory infiltrate and VSMCs of the aortic media. Proximal AAA regions: Slide A (x 63) and Slide B (x126), AAA Body regions: Slide C (x126) and Slide D (x252), AAA Distal regions: Slide E (x 126) and Slide F (x252). (n = 5).



Figure 80 Photomicrograph Showing the Immunolocalisation of HIF-1 Alpha Surrounding the *Vasa Vasorum*

Sections of human aneurysmal tissue were probed with mouse anti-human HIF-1 alpha and a biotinylated horse anti-mouse secondary antibody (1:200) (Vector Laboratories). *Vasa vasorum* supply blood carrying oxygen to the vascular tissues but are scarce within the infrarenal region of the aorta. In this section of the media HIF positivity appears less pronounced within the area of the *vasa vasorum* which correlates to the improved oxygenation within this area. (n = 5).

Chapter 8: Expression of Vascular endothelial growth factor (VEGF) and Ets-1 in AAA and analysis of VEGF and Ets-1 levels in HASMCs exposed to severe hypoxia

8. Expression of Vascular endothelial growth factor (VEGF) and Ets-1 in AAA and analysis of VEGF and Ets-1 levels in HASMCs exposed to severe hypoxia

8.1 Introduction

8.1.2 Vascular endothelial growth factor (VEGF) and AAA

Vascular endothelial growth factor (VEGF) is a family of homodimeric glycoproteins vital for vascular development and is induced in cells in response to hypoxia or ischemia stimulating angiogenesis in order to restore the blood supply to inadequately oxygenated tissues. The VEGF family covers a number of proteins from two families that result from alternate splicing of mRNA from the single 8-exon VEGF gene. All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation eliciting a variety of functions between all the family members (Holmes *et al.*, 2007).

Overexpression of VEGF can contribute to pathological conditions and is crucial for tumour angiogenesis in cancer (Shibuya, 2013). Nishibe *et al.*, (2010) demonstrated the expression of VEGF in abdominal aortic aneurysm tissue samples and its absence in the normal abdominal aorta leading them to suggest that VEGF may play an important role in aneurysm formation via its direct and/or indirect actions.

Interestingly they also found two thirds of their AAA samples displayed VEGF positivity localized within the SMCs. We know VEGF expression is induced by low oxygen in a number of cell types including SMCs and that Forsythe *et al.*, (1996) had demonstrated the involvement of the master hypoxic transcription factor HIF-1 α in the activation of VEGF transcription so therefore increased expression of VEGF in AAA tissue through an unknown mechanism could possibly be via a signalling cascade involving HIF-1 α (Hu *et al.*, 2004).

8.1.3 Ets-1 and AAA

Although many of the events that occur within the signalling cascade that govern aneurysm development have been elucidated, a large amount is still unknown. In order to further explicate the tissue markers of hypoxia, AAA tissue samples were analyzed for the transcription factor Ets-1. Additionally, levels of this transcription factor in SMC total cell lysate from cells exposed to severe hypoxia were also investigated.

Ets-1 is the founding member of the Ets oncogene family originally identified in the E26 avian erythroblastosis virus and is expressed in a variety of cells including vascular smooth muscle cells. Ets-1 is considered important for the transcriptional regulation of several MMPs including MMP-2 and MMP-9 which contain Ets-1 binding sites in their promoters (Dittmer, 2003., Ito *et al*, 2004., Nakamura *et al.*, 2004) and which are both proven to play significant roles in the pathogenesis of AAA.

Hypoxia has been shown to induce Ets-1 via the activity of HIF-1 α (Oikawa *et al.*, 2001) and Peters *et al.*, (2004) also suggested that hypoxia and HIF-1 α may be involved in the up-regulation of Ets-1 when they discovered that Ets-1 and HIF-1 α co-localize in the hypoxic synovium of inflamed rat joints in adjuvant induced arthritis.

TIMP-1, in addition to MMPs, is also a gene target of Ets-1 (Logan *et al.*, 1996) and levels of TIMPs in HASMCs exposed to hypoxia were found to be elevated in hypoxic samples compared to controls (Chapter 6, section 6.2.3). It seems reasonable to hypothesize that Ets-1 could be involved in the signalling pathway terminating in MMP up-regulation as observed in these hypoxia studies.

8.2 Exposure of HASMCs to Severe Hypoxia (1%) for 30 hours

Human aortic smooth muscle cells purchased from TCS Cellworks were passaged into 6-well plates, grown to sub-confluence under normal conditions in the presence of 10% FCS in DMEM with additions of 2mM L-glutamine, penicillin (10,000 IU/ml) and streptomycin (10,000 IU/ml). The cells were then maintained in serum-free medium under severe hypoxia (5%CO₂, 94%N₂ and 1%O₂) inside a sealed Minimac hypoxia chamber at 37°C for up to 48 hours. Controls were maintained throughout the 48 hour period in an incubator at 37°C in 95% air, 5%CO₂. At termination of allotted time-points the media was removed from wells, cells washed with PBS and subsequently lysed with ice-cold lysis buffer (0.5% Nonidet, 100mM NaCl, 10mM Tris HCL pH 7.5). Total cell lysate was stored at -80°C for future analysis.

8.3 Ets-1 and VEGF Western blotting

Samples standardised for protein were loaded onto 7.5% SDS-PAGE gels, then transferred onto nitrocellulose membranes and probed with mouse monoclonal anti-human VEGF (1:200) (Santa Cruz Inc.) or rabbit polyclonal anti-human Ets-1 (1:200) (Santa Cruz Inc.) and finally polyclonal horseradish peroxidase-conjugated goat anti-mouse or swine anti-rabbit secondary antibodies (1:10000)(Dako UK Ltd., Ely, UK). Proteins were visualised using an Amersham ECL Advance TM Western blotting detection kit (GE Healthcare Life Sciences, Buckinghamshire, UK).

8.4 Results of Ets-1 and VEGF Western Blotting

8.4.1 Results: Levels of Ets-1 in HASMC total cell lysate after exposure to 1% hypoxia for 30 hours



Figure 81 Representative Immunoblot Showing Levels of Ets-1 in HASMC Lysate

HASMC whole cell lysate (7 μ g) was loaded onto 7.5% gels and analyzed by immunoblotting using rabbit polyclonal anti-human Ets-1 primary antibody (1:200) followed by a polyclonal horseradish peroxidase-conjugated swine anti-rabbit secondary antibody (1:10000) and visualised using chemiluminescence. 1: Negative (lysis buffer) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

The RSMs, also known as a molecular weight ladders do not appear on the blots in Figures 81, 83 and 85. Prior to transfer they were visible on the SDS-PAGE gels but failed to transfer across to the nitrocellulose membrane during the Western blot protocol. The protein samples were transferred over a 2 hour period to ensure complete transfer, but it is possible the ladder transferred too far and was lost or the SDS present in the transfer buffer stripped the dye from the ladder.

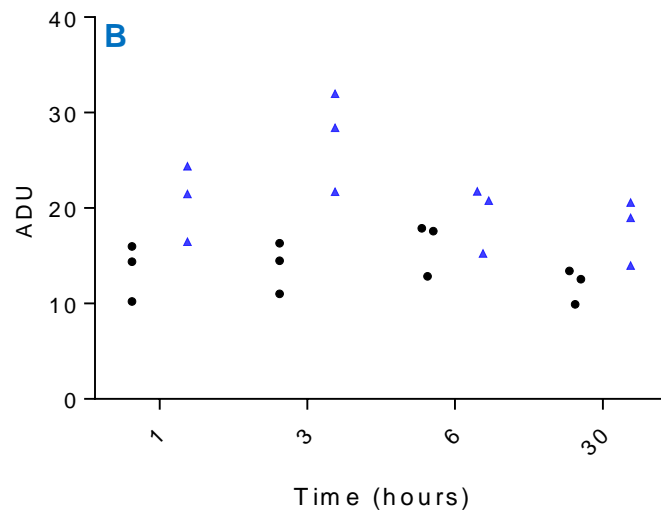
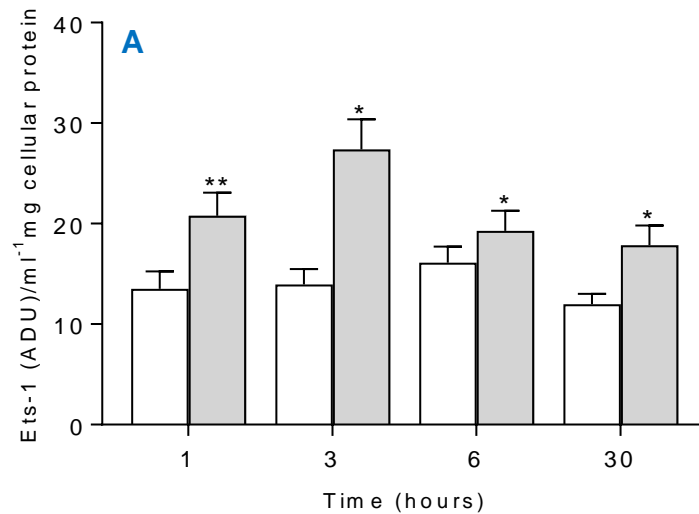


Figure 82 Bar and Scatter Graphs Showing Levels of Ets-1 in HASMC Total Cell Lysate After Exposure to Hypoxia

Levels of Ets-1 protein in total HASMC lysate after exposure to 1% hypoxia for 30 hours and assessed by immunoblotting. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values (* $p < 0.05$; ** $p < 0.01$). Results are from three independent experiments with 3 replicates. ($n = 3$). Statistical analysis was performed using Student's t-tests.

A: Normoxia (open bars), 1% hypoxia (grey filled bars).

B: Scatter plot : Normoxia (black circles), 1% hypoxia (blue triangles)

Results of Ets-1 Western blot showed significant induction in Ets-1 protein levels in the hypoxic samples at the 1 hour time-point ($p < 0.01$) which was sustained at 3 hours ($p < 0.05$), 6 hours ($p < 0.05$) and at 30 hours ($p > 0.05$).

8.4.2 Results: Levels of VEGF in HASMC total cell lysate after exposure to 1% hypoxia for 30 hours

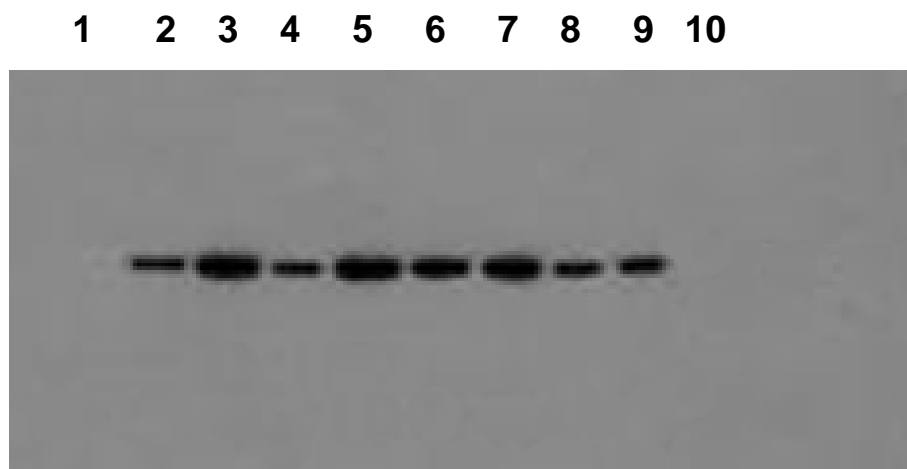


Figure 83 Representative Immunoblot Showing Levels of VEGF in HASMC Lysate

Whole cell lysate (7 μ g) was loaded onto 7.5% gels and analyzed by immunoblotting using mouse monoclonal anti-human VEGF primary antibody (1:200) followed by a polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10000) and visualised using chemiluminescence.

1: Negative (lysis buffer) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

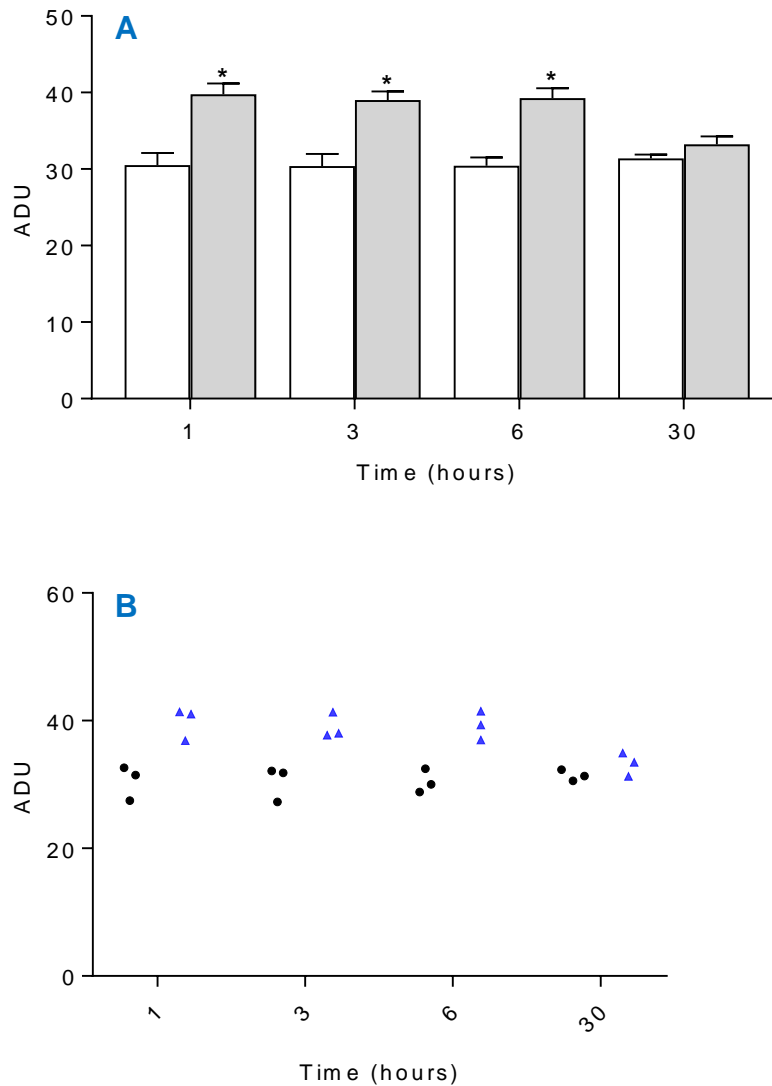


Figure 84 Bar and Scatter Graphs Showing Levels of VEGF in HASMC Total Cell Lysate After Exposure to Hypoxia

Levels of VEGF protein in total HASMC lysate after exposure to hypoxia for 30 hours and assessed by immunoblotting. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values (* $p < 0.05$). Results are from three independent experiments with 3 replicates. ($n = 3$). Statistical analysis was performed using Student's t-tests.

A: Normoxia (open bars), 1% hypoxia (grey filled bars)

B: Scatter plot : Normoxia (black circles), 1% hypoxia (blue triangles)

Results of the VEGF Western blot found VEGF protein levels significantly up-regulated in the hypoxic samples at the 1 hour time-point ($p < 0.05$), at 3 hours ($p < 0.05$) and 6 hours ($p < 0.05$). At the 30 hour time-point there was a small increase in VEGF levels in the hypoxic sample in comparison to the control but this was not statistically significant.

8.4.3 Beta Actin Loading Controls

To ensure equal sample loading and to check that there has been even transfer from gel to nitrocellulose membrane across the whole gel beta actin (Abcam, Cambridge, UK) loading controls were carried out.

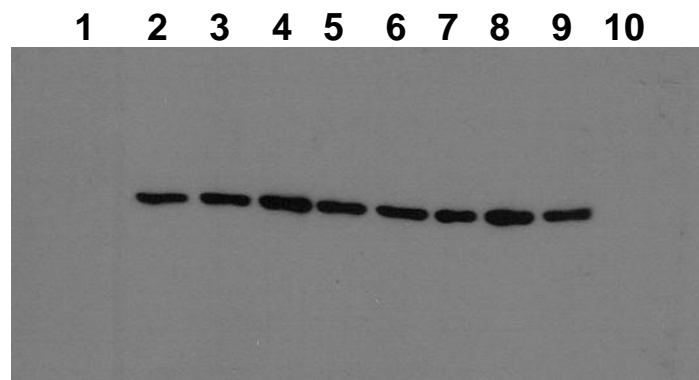


Figure 85 Representative Beta Actin Loading Control Immunoblot

Whole cell HASMC lysate (7 μ g) was loaded onto 7.5% gels and analyzed by immunoblotting using a mouse monoclonal anti-human beta actin (1:5000) (Abcam) followed by a polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10000) and visualised using chemiluminescence.

1: Negative (lysis buffer) 2: 1hr N 3: 1hr H 4: 3hrs N 5: 3hrs H 6:6hrs N 7: 6hrs H 8:30hrs N 9: 30hrs H 10: RSM (N: Normoxia, H: Hypoxia, RSM: Rainbow size marker).

8.5 Distribution of VEGF and Ets-1 in AAA

Immunohistochemistry was performed on human AAA and normal aortic control tissue to demonstrate the presence of VEGF and Ets-1 within these tissues and also to illustrate distribution of these two proteins within aneurysmal tissue. With local ethics approval, AAA tissue was obtained from 5 consented patients undergoing elective surgical repair and normal aortic tissue was obtained from the aortic cuff of 5 renal transplant donors (n = 10).

8.5.1 Materials List

All materials were purchased from Sigma-Aldrich Ltd (Dorset, UK) unless stated otherwise.

- Rabbit polyclonal anti-human Ets-1 (Santa Cruz Inc, Heidelberg, Germany)
- Mouse monoclonal anti-human VEGF (Santa Cruz Inc, Heidelberg, Germany)
- Alkaline phosphatase-conjugated avidin/biotin complex anti-mouse or anti-rabbit secondary antibodies (ABC) kit (Vector Laboratories)
- Monoclonal IgG1 (negative control) (Dako UK Ltd., Cambridgeshire, UK).
- Levamisole (Vector Laboratories).
- Naphthol AS-MX phosphate/fast red TR Fast TM substrate solution.
- Horse serum
- Goat serum
- Mayers haematoxylin

8.5.2 Immunohistochemistry Protocol

With local ethics approval, AAA tissue was obtained from consented patients undergoing elective surgical repair. Sections were taken from the proximal distal and body of the aneurysm from 5 x donors. Normal tissue free from signs of aortic disease was obtained from the aortic cuff of 5 x renal transplant donors (n = 10). At the time of retrieval tissue samples were immediately fixed in 0.9% NaCl (10%v/v) histological formaldehyde and subsequently processed using a Shandon Hypercentre II firowax-embedding station (Shandon Inc., Pennsylvania, USA).

After processing, tissue sections (5µm) cut from the paraffin embedded wax blocks were de-paraffinised, then rehydrated in preparation for staining. Sections were evaluated using mouse monoclonal anti-human VEGF (1:200) or rabbit polyclonal anti-human Ets-1 (1:200) which were incubated overnight at 4°C.

Subsequently the sections were incubated in a biotinylated anti-mouse or anti-rabbit secondary antibody solution (1:100) for 2 hours at room temperature followed by a minimum 30 minute incubation in Vectastain's ABC alkaline phosphatase solution. Endogenous alkaline phosphatase was inhibited using Levamisole.

Visualisation of the proteins was achieved by incubating the sections in Fast Red substrate solution until a red precipitate appeared and counterstaining with Mayers haematoxylin. Monoclonal mouse IgG1 directed against *Aspergillus niger* glucose oxidase (1:200) was used as a negative control or sections prepared minus the primary antibody.

8.5.3 Results of Ets-1 Immunostaining of AAA and Normal Tissue

8.5.3.1 Localisation of Ets-1 in Normal Aortic Tissue

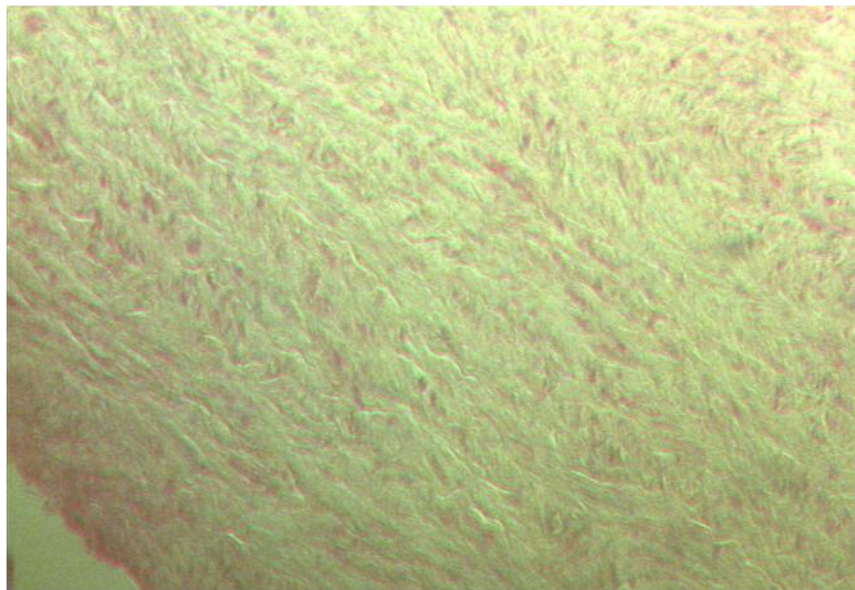


Figure 86 Photomicrograph Showing the Distribution of Ets-1 Within the Normal Aortic Media (x126)

Control specimens of normal human aorta without evidence of aneurysmal disease were probed with rabbit anti-human Ets-1 (1:200) followed by a biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Laboratories) and counterstaining with Mayer's haematoxylin for comparison with identically treated sections of human aneurysm tissue. Some cytoplasmic staining (red) is evident but there does not appear to be any Ets-1 immunopositivity within the nuclei where as a transcription factor Ets-1 would generally be localized in order to bind to the Ets-1 binding sites within the promoter of target genes and enable transcription of those genes. (n = 5).

8.5.3.2 Localisation of Ets-1 in AAA

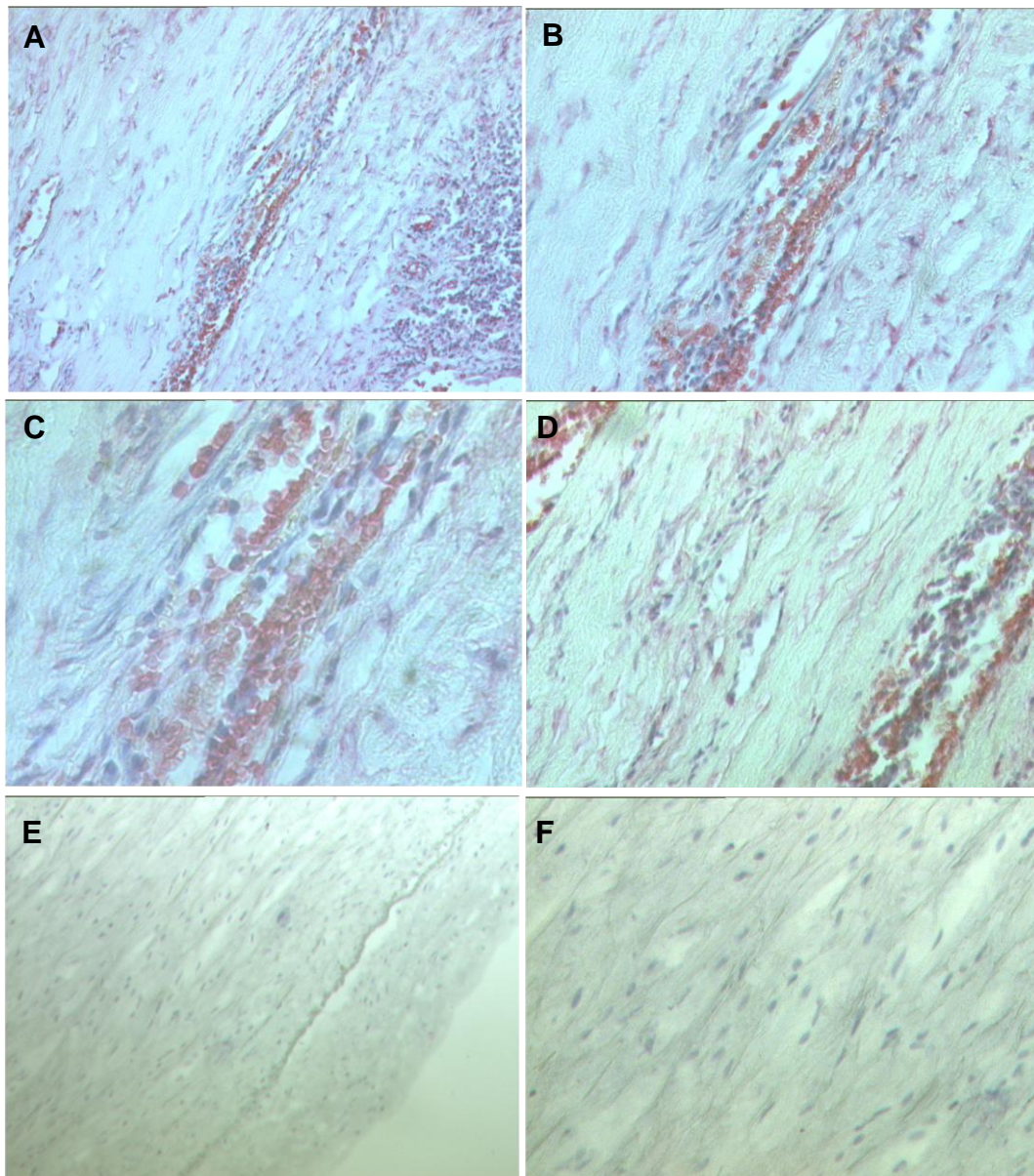


Figure 87 Photomicrographs Showing the Localisation of Ets-1 Within the Body and Distal Regions of AAA and Control Sections of Normal Aorta

Aneurysmal tissues were probed with rabbit anti-human Ets-1 (1:200) followed by a biotinylated goat anti-rabbit secondary antibody (1:200) and counterstained with Mayers haematoxylin. Slides A, B and C show the same areas of the body region of AAA from the same slide at different magnifications (x63, x126 and x252 respectively). Slide D shows the distal region of a AAA section (x126) and all demonstrate Ets-1 positivity (red) within the inflammatory infiltrate. Slides E and F show sections of normal aortic tissue probed with monoclonal mouse IgG1 directed against *Aspergillus niger* glucose oxidase (1:200) as a negative control at x63 and x126 magnifications respectively. (n = 10).

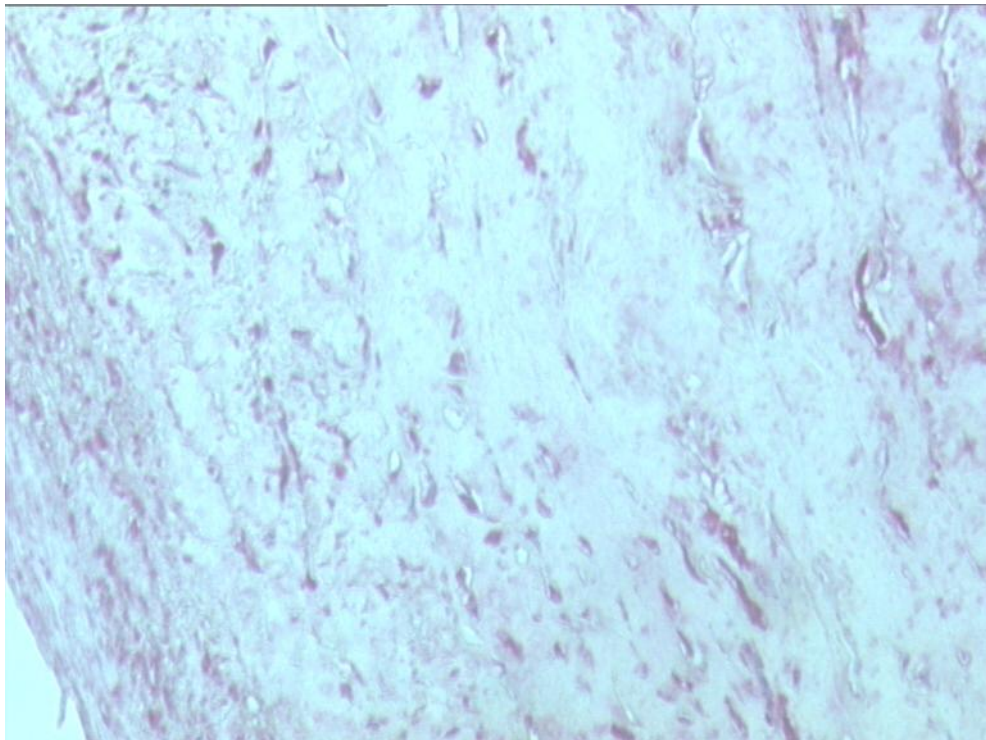


Figure 88 Photomicrograph Showing the Distribution of Ets-1 Within the Distal Region of AAA Tissue (x126)

Sections of human aneurysmal tissue were probed with rabbit anti-human Ets-1 (1:200) followed by a biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayer's haematoxylin. Diffuse Ets-1 immunopositivity can be seen throughout the tunica media of this distal region of aneurysmal tissue. (n = 5).

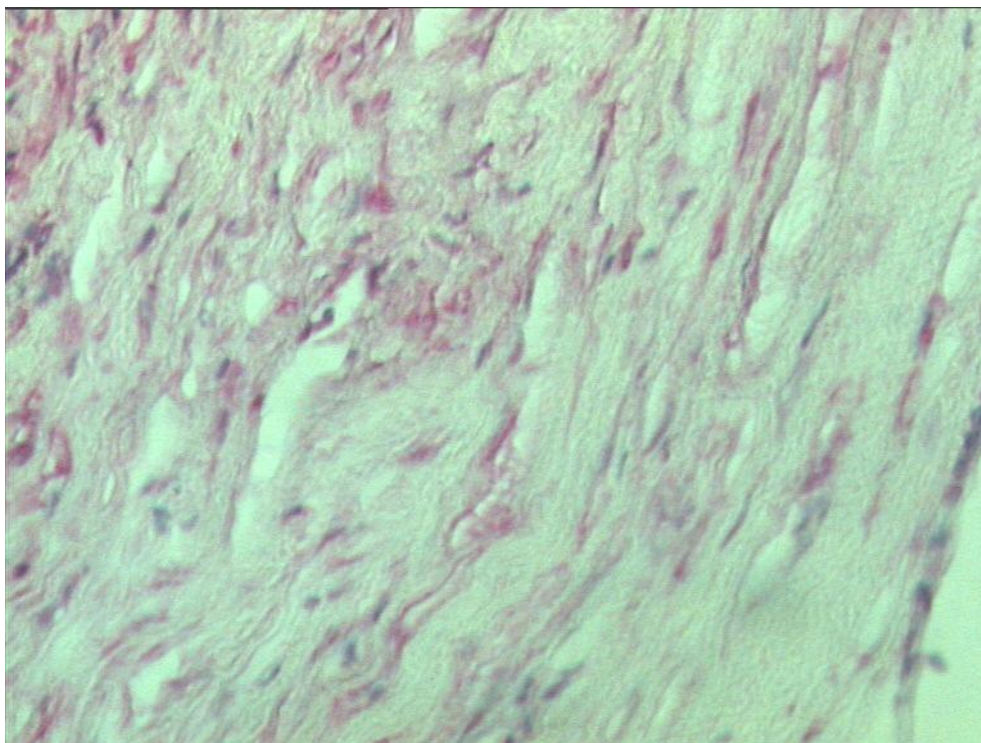


Figure 89 Photomicrograph Showing the Localisation of Ets-1 Within the Distal Region of AAA Tissue (x 126)

Sections of human aneurysmal tissue were probed with rabbit anti-human Ets-1 (1:200) followed by a biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayers haematoxylin. This slide demonstrates Ets-1 nuclear and cytoplasmic immunopositivity within the VSMCs of the medial region of AAA tissue. Typical tears are also present in this tissue section which is a common problem occurring when trying to cut sections from aneurysm tissue which is often brittle and friable in comparison to cutting normal aortic sections. (n = 5).

8.5.3.3 Distribution of of VEGF in AAA

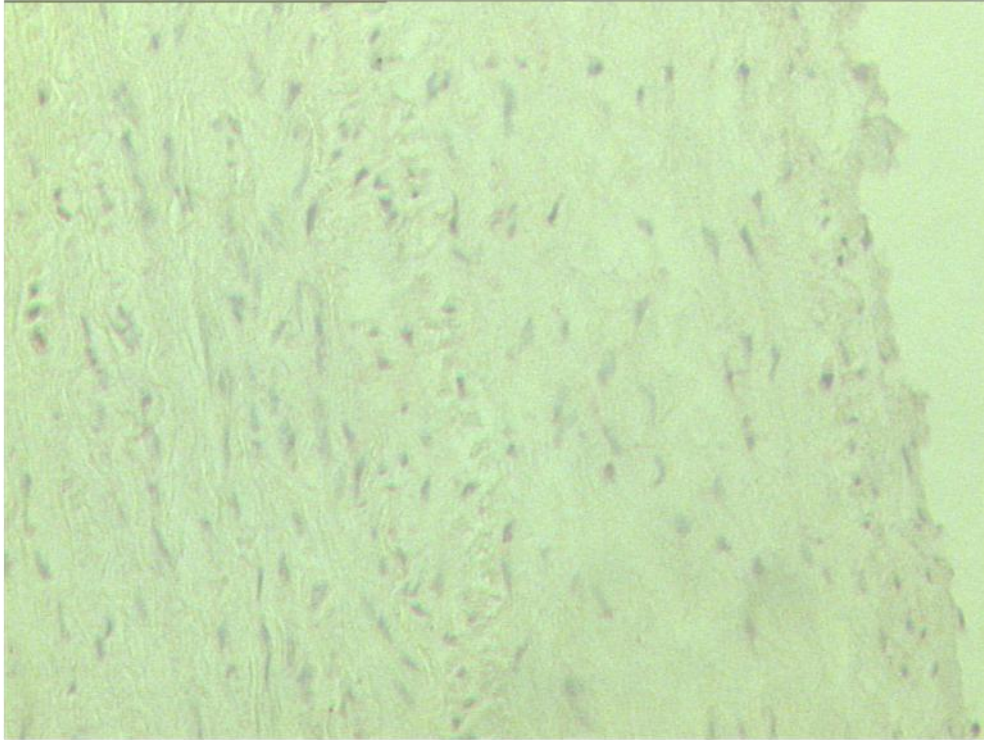


Figure 90 Photomicrograph of Normal Aortic Tissue Probed for VEGF (x 126)

Sections of normal human aortic tissue were probed with mouse monoclonal anti-human VEGF(1:200) followed by a biotinylated goat anti-mouse secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayer's haematoxylin. This slide does not appear to show any clear VEGF immunopositivity (n = 5).

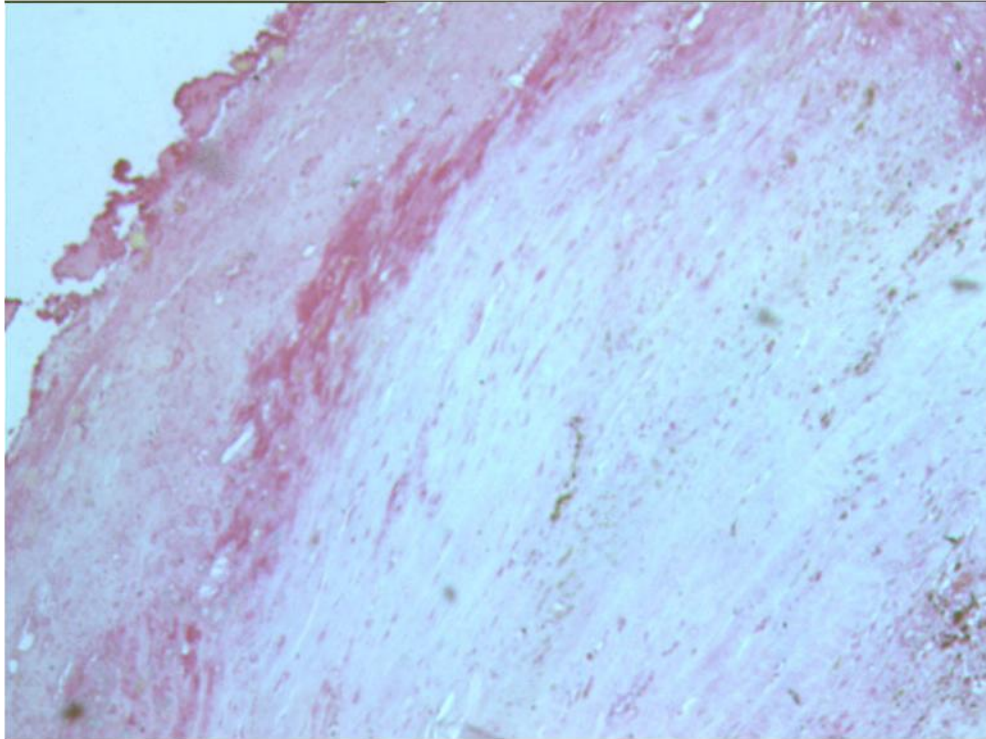


Figure 91 Low Power Photomicrograph (x 31.5) Showing the Distribution of VEGF Within the Distal Region of AAA Tissue

Sections of AAA tissue were probed with mouse monoclonal anti-human VEGF(1:200) followed by a biotinylated goat anti-mouse secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayers haematoxylin. Widespread and diffuse VEGF immunopositivity is seen within the tunica adventia and tunica media regions of the aneurysmal tissue (n = 5).

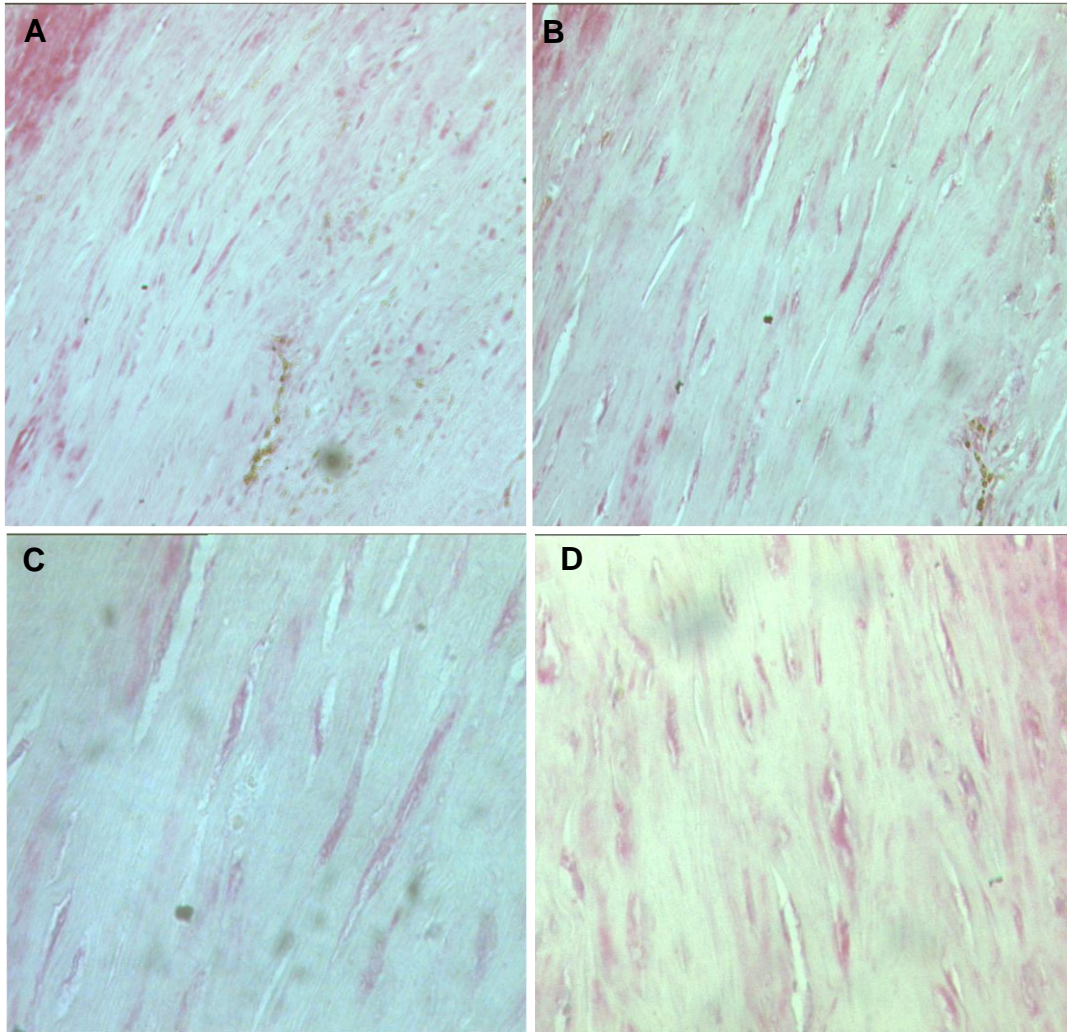


Figure 92 Photomicrograph Showing the Localisation of VEGF Within the Body and Distal Region of AAA

Sections of AAA tissue were probed with mouse monoclonal anti-human VEGF(1:200) followed by a biotinylated goat anti-mouse secondary antibody (1:200) (Vector Laboratories) and counterstained with Mayer's haematoxylin. Slides A, B and C show the same areas of the body region of AAA on the same slide at different magnifications (x63, x126 and x252) respectively). Slide D shows the distal region of a AAA section (x126) and all clearly demonstrate cytoplasmic VEGF staining within the SMCs of the aortic media. (n = 5).

**Chapter 9: Analysis of MMP-2
levels in HASMCs Exposed to
Severe Hypoxia (1%) after
Inhibition of HIF-1 alpha.**

9. Analysis of MMP-2 levels in HASMCs Exposed to Severe Hypoxia (1%) after Inhibition of HIF-1 alpha

9.1 Introduction

As described in Chapter 2, section 2.3.1, HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes involved in increasing oxygen delivery or facilitating the metabolic adaptation to hypoxia. Our earlier study (Erdozain *et al.*, 2011) described strong immunopositivity for HIF-1 α , MMP-2 and Ets-1 within the tunica media of all AAA tissue sections evaluated in comparison to normal aortic tissue controls.

In Chapter 4, section 4.4.2.2 results demonstrated significantly increased expression of MMP-2 in HASMCs exposed to severe hypoxia for 48 hours. In order to further elucidate if HIF-1 α has any involvement in the signalling pathway ending with the up-regulation of MMP-2 as seen in this hypoxia study, it will be necessary to inhibit HIF using bisphenol A (BpA) and subsequently evaluate HASMC samples for any changes in MMP-2 expression. The presence of an active Hsp90 is necessary for HIF-1 activation in hypoxia and BpA is an environmental endocrine disruptor which Kubo *et al.*, (2004) found promoted HIF-1 α degradation by dissociation of the chaperone protein Hsp90 from HIF-1 α .

Under normal oxygen levels HIF-1 α is hydroxylated on its proline residue by prolyl hydroxylases which result in its ubiquitination by the von Hippel-Lindau tumor suppressor protein (pVHL) and degradation by the proteasome (Ivan *et al.*, 2001., Jaakola *et al.*, 2001).

During hypoxia, proline hydroxylation is inhibited and HIF-1 α is not ubiquitinated and HIF-1 α translocates and accumulates in the nucleus of hypoxic cells resulting in activation of its target genes. Disruption of Hsp90 function by BpA (which has a similar effect to the antibiotic geldanamycin) under hypoxic conditions is followed by HIF-1 degradation and therefore no transcription of target genes.

9.1.2 Aims of the chapter

- 1: To determine the optimal concentration of BpA necessary to inhibit HIF-1 α in HASMCs during 48 hours of 1% hypoxia.
- 2: To treat HASMCs with 250 μ M BpA during a 48 hour period of 1% hypoxia.
- 3: To assess the effect of HIF-1 α inhibition if any, on the expression of MMP-2 secretion into HASMC conditioned medium.

9.2 HIF-1 α Inhibition using BpA

9.2.1 Cell Culture

The primary HASMC culture was seeded into T25 flasks and routinely grown in DMEM supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin or Smooth Muscle Cell Complete Medium which was replaced every two to three days. The cells were kept in 5% CO₂, 95% air at 37°C in a humidified Jencons Millenium incubator (Jencons Ltd. Bedfordshire, UK). Upon reaching confluence (100% monolayer covering flask base) the cells were split 1:5 into T75 flasks. HASMCS were washed in PBS then dissociated from the flasks using 2mls Accutase /T25 and incubated at 37°C for approx 2-5 minutes. DMEM was added to inactivate the Accutase and the cells were brought out of suspension by centrifugation at 1600rpm for 5 minutes. The supernatant was removed and the pellet resuspended in fresh DMEM. For hypoxia studies cells were passaged into 6-well plates or for cell staining experiments grown on sterilised 13mm diameter glass coverslips in 6/12-well plates or chamber slides.

9.2.2 Preparation of Cells for BpA Treatment

For this hypoxia study cells were passaged into 6-well plates or into 6/12-well plates containing sterilised 13mm diameter glass coverslips ready for staining procedures. They were maintained as before in a standard incubator at 37°C until the start of the experiment. Cells were maintained in serum-free media for 24 - 48 hours prior to the start of the hypoxia study.

9.2.3 Experimental Trial of BpA

Prior to the main hypoxia study, a small trial was set up to determine the optimum concentration of BpA necessary to completely inhibit the activity of HIF-1 α . Whilst the cells were subconfluent and 24 hours prior to the start of the trial the media was removed and replaced with serum-free medium. At the start of the trial, under sterile conditions BpA (Enzo Life Sciences Ltd, Exeter, UK) was initially dissolved in ethanol which was then added dropwise to serum-free media whilst stirring to minimise the possibility of precipitation to make a 5mM BpA stock solution. The stock solution was further diluted with serum-free media to make 50 μ M, 100 μ M, 200 μ M and 250 μ M concentrations of BpA.

HASMC were subsequently cultured for 48 hours inside a sealed Minimac chamber at 37°C and 1% hypoxia (Don Whitley Scientific DG250 Workstation) in the presence of BpA (0, 50, 100, 200 and 250 μ M). Controls were maintained in a standard incubator at 37°C under normoxic conditions (5% CO₂, 95%). At termination of the trial, media was removed and HASMCs lysed with ice-cold lysis buffer (0.5% Nonidet, 100mM NaCl, 10mM Tris HCL pH 7.5) and stored at -80C.

Immunoblotting analysis of trial HSMC lysate samples was carried out as described earlier in Chapter 7, section 7.3.2, using mouse monoclonal anti-human HIF-1 α (1:500)(Novus Biologicals) followed by a polyclonal horseradish peroxidase-conjugated goat anti-mouse secondary antibody

(1:200) (Dako UK Ltd). Proteins were visualised using an Amersham ECL Advance™ Western blotting detection kit (GE Healthcare Life Sciences).

9.2.3.1 Results of BpA Trial

Subsequently the 250µM BpA concentration was chosen for the main study. Vehicle controls were also carried out to ensure ethanol used to initially to dissolve BpA was not interfering with results.

9.3 Results: Expression of MMP-2 in BpA Inhibited HASMCs After 48 Hours of 1% Hypoxia

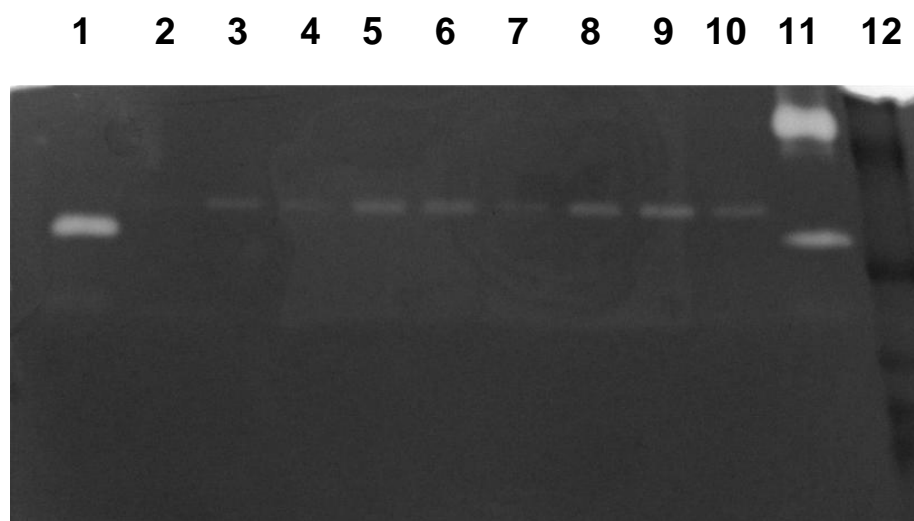


Figure 93 Levels of MMP-2 in HASMC Supernatant (Gel 1)

Representative zymographic analysis of MMP-2 activity in culture supernatants from HASMCs treated with 250µM of the HIF-1α inhibitor BpA and exposed to 1% hypoxia or normoxia for 48 hours.

1: Positive control (Purified human MMP-2) 2: 1hr N 3: 1hr H 4: 1hr + BpA 5: 3hrs N 6: 3 hrs H 7: 3hrs + BpA 8: 48 hrs N 9: 48hrs H 10: 48hrs + BpA 11: Purified MMP-9 12: RSM. N (normoxia) H (hypoxia) BpA (bisphenol A).

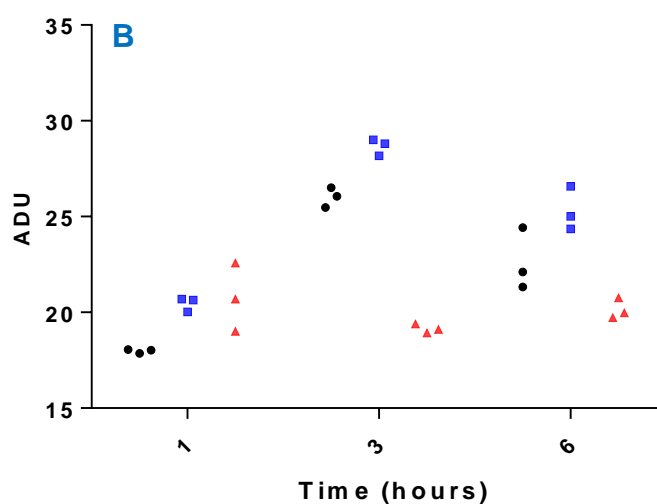
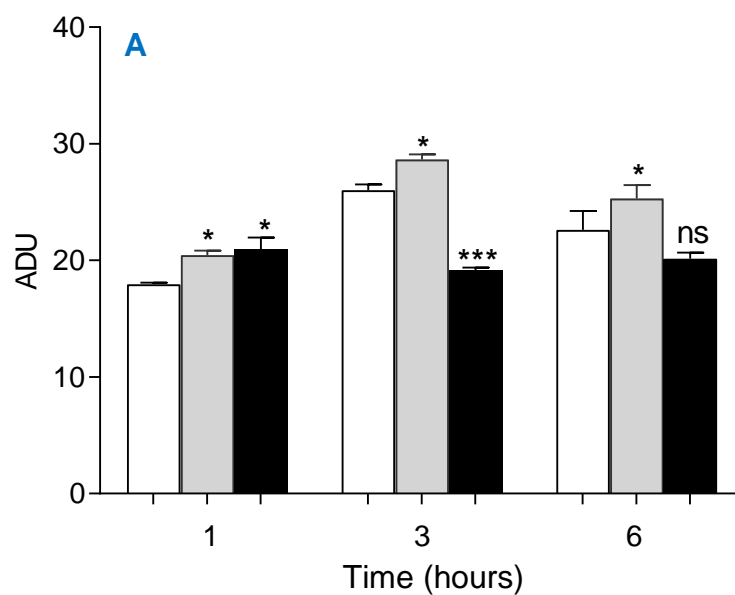


Figure 94 Bar and Scatter Graphs Showing Levels of MMP-2 in HASMC Conditioned Media (Gel 1)

Analysis of HASMCs treated with 250 μ M of the HIF-1 α inhibitor BpA and exposed to 1% hypoxia or normoxia for 48 hours. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. Results are from three independent experiments repeated in triplicate. (n = 3). Statistical analysis was performed using Student's t-test and correction for multiple comparisons using the Holm-Sidak method. Statistical significance was indicated by p values < 0.05.

A: Bar graph: Normoxia (open bars), 1% hypoxia (grey filled bars), bisphenol A (black filled bars).

B: Scatter plot: Normoxia (black circles), 1% hypoxia (blue squares), bisphenol A (red triangles).

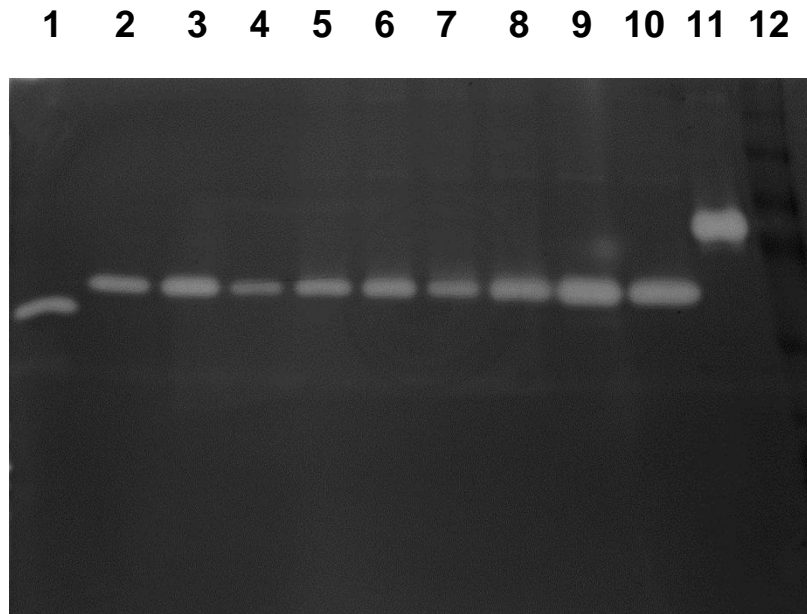


Figure 95 Representative Gel Showing Levels of MMP-2 in HASMC Supernatant After Inhibition of HIF-1 α (Gel 2)

Representative zymographic analysis of MMP-2 activity in culture supernatants from HASMCs treated with 250 μ M of the HIF-1 α inhibitor BpA and exposed to 1% hypoxia or normoxia for 48 hours.

1: Positive control (Purified human MMP-2) 2: 6hrs N 3: 6hrs H 4: 6hrs + BpA 5: 30hrs N 6: 30 hrs H 7: 30 hrs + BpA 8: 48hrs N 9: 48hrs H 10: 48hrs + BpA 11: Purified MMP-9 12: RSM. N (normoxia) H (hypoxia) BpA (bisphenol A).

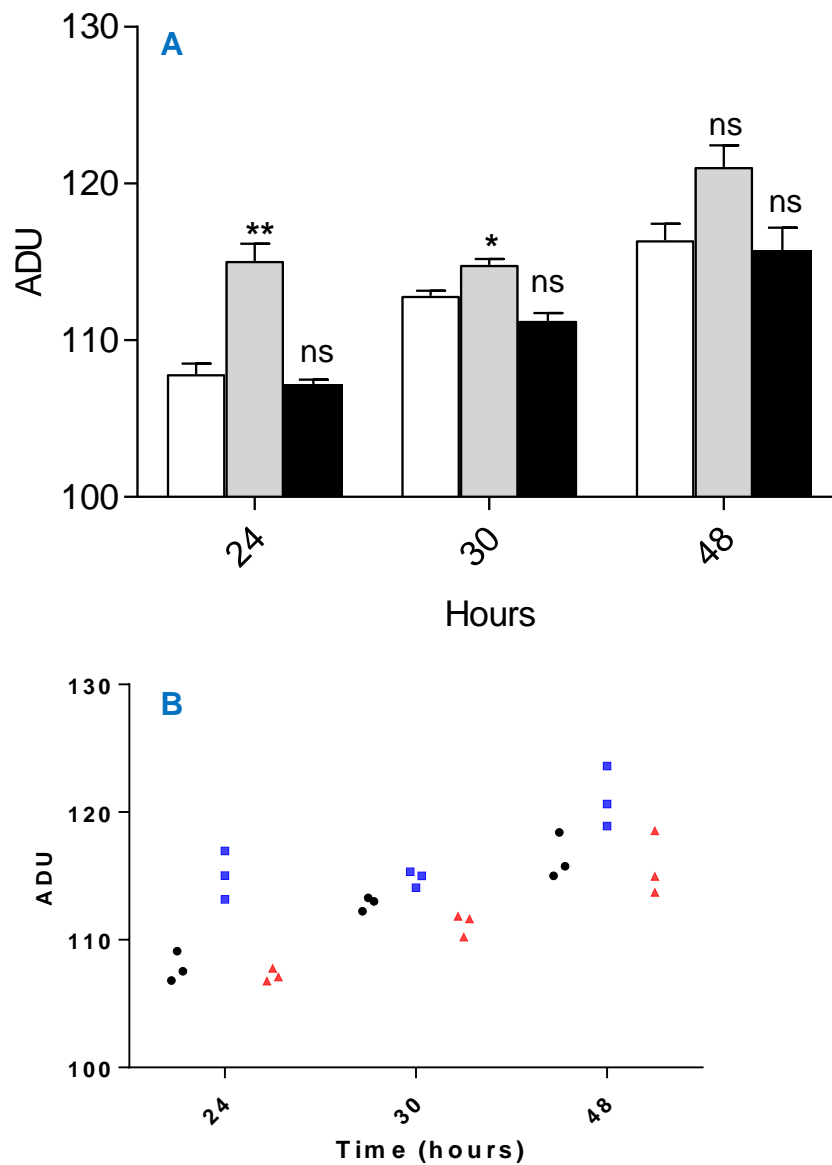


Figure 96 Bar and Scatter Graphs Showing Levels of MMP-2 in HASMC Supernatant (Gel 2)

Analysis of HASMCs treated with 250 μ M of the HIF-1 α inhibitor BpA and exposed to 1% hypoxia or normoxia for 48 hours. Results expressed as arbitrary density units (ADU) per unit standardised total protein. Data expressed as means \pm SEM of triplicate values. Results are from three independent experiments repeated in triplicate. n = 3. Statistical analysis was performed using Student's t-test and correction for multiple comparisons using the Holm-Sidak method. Statistical significance was indicated by p values < 0.05.

A: Bar graph: Normoxia (open bars), 1% hypoxia (grey filled bars), bisphenol A (black filled bars).

B: Scatter plot: Normoxia (black circles), 1% hypoxia (blue squares), bisphenol A (red triangles).

Results of the MMP-2 zymography carried out after HASMCs were treated with 250 μ M of the HIF-1 α inhibitor BpA and exposed to 1% hypoxia or normoxia for 48 hours yielded promising results. Hypoxic samples minus BpA had some significant increases in MMP-2 activity at the 1 hour time-point ($p < 0.05$), 3 hours ($p < 0.05$), 6 hours ($p < 0.05$) (Figure 94), 24 hours ($p < 0.01$) and 30 hours ($p < 0.03$) (Figure 96) versus normoxic controls. Elevations were sustained at 48 hours (Figure 96) but not statistically significant. These results were as anticipated, demonstrating higher MMP-2 levels in hypoxic samples as seen in my earlier results (Chapter 4, section 4.4.2.2). Hypoxic samples treated with the HIF inhibitor showed decreased MMP-2 activity comparable to the normoxic control samples and were statistically not significant versus normoxic controls. (Figures 94 and 96) The only exceptions occurred at the 1 hour time-point (Figure 94) which showed higher MMP-2 activity than the 1 hour control normoxic control (explanation given later during the discussion) and at the 3 hour time-point (Figure 94) which showed significantly lower MMP-2 activity ($p < 0.001$) than the normoxic control (Figure 94).

10. Discussion

10.1 Discussion

The aim of this study was to investigate whether hypoxia is an initiating factor in the MMP up-regulation seen AAA, to determine the effects of hypoxia on TIMPs the natural inhibitors of MMPs and to examine whether HIF-1 α , VEGF and Ets-1 are mediating factors in the progression of aneurysmal disease.

The existing clinical approach to patients diagnosed with AAAs is surveillance of aortic dimensions with a view to perform open or endovascular surgical repair when the aortic diameter has enlarged sufficiently to be at high risk of rupture. There are limited options for medical treatment beyond risk factor modification as currently no pharmacological therapy to limit human AAA progression or rupture has been validated through randomised clinical trials highlighting the importance of ongoing AAA research (Robertson *et al.*, 2014).

The first introductory Chapter focuses on the vascular biology of the human aorta by giving an overview of the main aortic structural components and important aspects of its physiology which are relevant to understanding the pathology of AAA. The aorta is the largest artery in the body and is composed of three layers known as the tunica intima, tunica media and tunica adventitia containing the major structural components collagen and elastin which are vital for the tensile strength and elasticity of the aorta. Other specialised proteins of the aortic ECM such as fibrillin, fibronectin, laminin and proteoglycans have also been described here as well as cells of the arterial intima including intimal endothelial cells, medial SMCs and adventitial fibroblasts. These vascular cells have the capacity to express components of the ECM, growth factors and proteases including MMPs which are calcium-dependent zinc-containing endopeptidases which are responsible for tissue remodelling and degradation of the aortic ECM under normal physiological conditions and are tightly controlled by their natural

inhibitors. The central pathological role MMPs play in the progression of AAA is discussed in Chapter 2. Section 2.2.1.

To enable the reader to gain insight into the pathophysiology of AAA Chapter 2 outlines factors known to be associated with AAA formation. It should be emphasised that this research concentrates on AAAs and does not include thoracic aortic aneurysms (TAAs) because, although they both occur within the aorta, they are located in different anatomical locations and have differing aetiologies. For example, AAAs are associated with older age, male gender and risk factors such as smoking and high blood pressure (Lerderle *et al.*, 1997) whereas TAAs occur frequently at younger ages without obvious gender predominance (Thompson *et al.*, 2006). In AAA, whilst there is no single genetic defect or polymorphism responsible, there is familial clustering, a common HLA subtype, and several altered gene expressions suggesting some genetic role in pathogenesis (Tilson and Seashore, 1984). Hereditary studies estimate that 20% of TAAs have a positive family history and are also linked to known genetic syndromes such as Marfan Syndrome or Ehlers - Danlos Syndrome involving mutations affecting the fibrillin-1 and procollagen genes (COL3A1) respectively (Dietz *et al.*, 1991, Pope *et al.*, 1975). Furthermore, as TAAs are predominantly located in the ascending aorta and AAAs in the infrarenal region they are therefore subjected to differing haemodynamic forces in addition to the progressive decrease in collagen to elastin ratio resulting in the infrarenal area being thinner and stiffer (Wolinsky and Glagov, 1969). Importantly, for this study where hypoxia is being investigated as a factor in AAA, it should be noted that the thoracic aorta is well oxygenated by *vasa vasorum* as opposed to the infrarenal aorta which is normally devoid of medial *vasa vasorum* and derives its oxygen and nutrient supply by simple diffusion from the lumen (Zarins *et al.*, 2001).

Although the pathogenesis of AAA remains unexplained, we know its initiation and progression is multifactorial. A large body of existing evidence has demonstrated increased MMP levels are the major factor destroying the integrity of the ECM and leading to the degradation of major structural elements such as elastin and collagen within the aortic wall. Currently,

research has not fully elucidated the chain of events such as the exact signalling pathway that terminates in MMP-2 up-regulation. Factors already known to increase MMP levels such as inflammation and reactive oxygen species were discussed in Chapter 2. A prominent pathological feature of AAA is the inflammatory response during which oxidative stress is invariably increased, further contributing to the pathophysiology of inflammation and progression of AAA. Associated with this inflammatory response, Martinez-Pinna *et al.*, (2014) noted an increased presence of haemoglobin (Hb) - derived iron in the adventitia of AAA patients. This observation correlated with haematoxylin and eosin stained AAA tissue sections of which a representative example is shown in Figure 56, slide B showing extensive inflammatory infiltrate and adventitial haemosiderin deposition. Haemosiderin is an iron-storage complex seen as a yellowish-brown granular intracellular pigment produced from the breakdown of Hb and is often found in macrophages which are also known to be present within AAA inflammatory infiltrate. Recently Rubio-Navarro *et al.* (2015) reported that the presence of Hb in the adventitial AAA wall promotes the migration and differentiation of activated circulating monocytes in AAA patients, explaining the existence of a protective CD163-macrophage phenotype that could take up the Hb present in the AAA-wall.

Our group (Erdozain *et al.*, 2011) proposed that hypoxia may be an initiating factor in the up-regulation of MMP-2 in AAA. Immunohistological studies by Erdozain *et al.*, (2011) found that the global hypoxic transcription factor HIF-1 α co-localised with high expression of MMPs in the tunica media and pro-inflammatory infiltrate of AAA tissue and that MMP-2 immunopositivity was observed in the VSMCs within the region of tunica media distant from the *vasa vasorum*. These observations showing localisation of MMP-2 to AAA medial SMCs, is supported by earlier research showing that MMP-2 production is elevated in SMCs derived from aortic aneurysms (Crowther *et al.*, 2000). MMP up-regulation driven by hypoxia and HIF-1 α has also been explored in other research areas such as cancer studies. Krishnamachary *et al.*, (2003) showed that HIF-1 regulates the expression of genes including cathepsin D and MMP- 2 in HCT116 human colon carcinoma cells whilst

Choi *et al.*, (2011) found HIF-1 α was stabilized under hypoxic conditions and stimulated MMP-9 expression which affected the tumour invasiveness of breast cancer cells. A further study by Jing *et al.*, (2012) also determined that HIF-1 α contributed to hypoxia-induced invasion and metastasis of oesophageal carcinoma by promoting MMP-2 expression.

Arterial wall hypoxia has been investigated in other conditions such as atherosclerosis and intimal hyperplasia (Heughan *et al.*, 1973, Crawford *et al.*, 1991., Martin *et al.*, 1991, Bjornheden *et al.*,1999, Lee *et al.*, 2000, Santilli *et al.*, 2000). Research has shown that hypoxia exists *in vivo* in patients with aneurysms (Choke *et al.*, 2006) and that intraluminal thrombus (present in approximately 75% of all cases of AAA) contributes to aortic wall hypoxia and weakening (Vorp *et al.*, 1996, 1998, 2001). In view of all this evidence of hypoxia-driven MMP up-regulation it was decided to explore if this also occurred in AAAs.

In our first study (Erdozain *et al.* 2011) HSMCs were maintained in FCS-containing medium at decreasing oxygen levels (5% down to 2%) over a 72 hour period and the results showed significant increases in MMP-2 and MMP-9 activity. In the studies presented here, I sought to further investigate more severe hypoxia as a driving force behind elevated HASMC MMP secretion and assess the induction times of the response to severe hypoxia (1% O₂). In this instance the cells were maintained in serum-free media to rule out foetal calf serum as a potential confounding factor. It was important to investigate MMP levels subjected to more severe hypoxia as Sun *et al.*, (2007) had designed a numerical model of oxygen transport in AAA with intraluminal thrombus (ILT) which demonstrated O₂ levels of 1% and lower within the thickest regions of the ILT which are known to be present in more than 75% of all cases of AAA (Harter,*et al.*, 1982). In Chapter 4, section 4.4.2.2, results of HASMCs exposure to severe hypoxia (1%) revealed that hypoxic samples in comparison to normal controls showed significant increases in secreted pro and active MMP-2 at all time-points except at 48 hours which showed a sustained elevation but was not statistically significant. These findings suggest that smooth muscle cells, the major cell

type found within the aortic tunica media respond to severe hypoxic stress (1% O₂) by secreting MMP-2 strengthening the hypothesis that localised hypoxia could be an initiating factor in the pathogenesis of AAA.

The identification of MMPs was achieved by comparison with known molecular weight standards but MMP-2 immunoblotting analysis of samples (Chapter 4, section 4.7.2.2) was carried out to further corroborate that MMP-2 protein was present in the HASMC culture supernatant and verify the increases in enzymatic activity seen in the hypoxic HASMC samples in comparison to normoxic samples were due to MMP-2 activity (Chapter 4, section 4.4.2.2).

MMP-9 was not detected in normoxic or hypoxic HASMC conditioned samples after a 48 hour exposure to 1% oxygen. This data appears to correlate with our earlier findings (Erdozain *et al.*, 2011) where a significant increase in MMP-9 was only seen at 2% hypoxia after 72 hours of decreasing O₂ levels, suggesting the driving force behind MMP-9 exportation is a sustained sequential decrease in O₂ over time and which also seems more comparable to the chronic hypoxic conditions found within the aneurysm. Additionally MMP-2 is constitutively expressed while MMP-9 tends to be inducible by growth factors and inflammatory stimuli so this may account for lack of this enzyme in the HASMC-conditioned media. In our original study (Erdozain *et al.*, 2011), HASMC conditioned medium from hypoxic samples demonstrated elevated levels of MMP-9 but those HASMCs were maintained in medium containing foetal calf serum (FCS) during the period of hypoxia; FCS contains growth factors capable of potentially inducing release of MMP-9 as opposed to my present study where cells were maintained in serum-free media.

The decision to investigate MMP-7 levels in HASMCs subjected to severe hypoxia was made following our previous findings (Erdozain *et al.*, 2011) of significant transcriptional up-regulation of MMP-7 ($p < 0.001$) in HASMCs potentiated by hypoxia, suggesting a role for this factor in the development of AAA. MMP-7, also known as Matrilysin, has a broad proteolytic activity

against a variety of extracellular matrix substrates and has been implicated in tumour metastasis, inflammation and is expressed in SMCs under hypoxic conditions.

It was important to assess whether the marked MMP-7 mRNA up-regulation was also seen at the MMP-7 protein level in hypoxic HASMC samples. MMP-7 and its involvement in AAA pathogenesis is largely unknown, although immunohistochemical analysis of aneurysmal aortae has indicated differences in the distribution of this MMP from normal aorta (Borges *et al.*, 2009). More recently, Lyon *et al.*, (2015) also detected increased MMP-7 activity in human AAA sections compared to normal human aortae, in addition to demonstrating that MMP-7 can modify the behaviour of VSMCs by cleaving N-cadherin, thereby modulating AAA formation and progression. Burke *et al.*, (2003) also identified a putative HRE containing HIF-1 binding sequences within the promoter of the MMP-7 gene although they subsequently found it to be incapable of conferring hypoxic inducibility but considered other possibilities that the hypoxic inducibility of MMP-7 transcription is mediated by a different HIF binding site within the promoter.

Yu and Frederick Woessner (2000) suggested heparin might enhance MMP-7 activity during zymography by either inducing a conformational change that increases activity, facilitating refolding, reducing autolysis, or by helping to anchor the enzyme in the gel overnight. Therefore, levels of MMP-7 protein were investigated using heparin-enhanced zymography, ensuring that even low levels of MMP-7 could be detected.

Results of zymographic analysis proved inconclusive for MMP-7 protein in both conditioned media and whole cell lysate, even after sample volume was considerably increased and time incubated in development buffer extended (Chapter 5, section 5.2.2). One explanation for these results may be because the HASMCs were exposed to severe hypoxia for only 48 hours the hypoxic exposure period was not extensive enough for it to translate at the MMP-7 protein level.

Interestingly, research by Yamasaki *et al.*,(2013) determined that expression of MMPs-7, -9, -14, and -15 were significantly up-regulated in LNCaP human

prostate cancer cells but only after exposure to chronic hypoxia (1%) of up to 6 months but not under acute hypoxia. Furthermore research has also demonstrated increased levels of MMP-7 in the aneurysm wall in AAA, seemingly expressed by cells within the aortic wall such as SMCs rather than the luminal thrombus (Fontaine *et al.*, 2002, Kazi *et al.*, 2005) suggesting that MMP-7 may play a late role in aneurysm progression through promotion of continued cellular attrition within the aortic wall (Ikonomidis *et al.*, 2007).

Additionally, the absence of MMP-7 could be attributed to being maintained in serum-medium throughout the period of hypoxia. Certain genes contain serum response elements within their promoters which respond to serum response factors, if MMP-7 does contain a serum response element, it could modify MMP-7 expression negatively. In fact, during our earlier study (Erdozain *et al.*, 2011) the HASMCs were maintained in medium containing FCS, and interestingly, MMP-7 mRNA was detected and found to be elevated in those hypoxic samples. Research investigating the role of MMP-13 in AAA by Mao *et al.*, (1999) may also support this theory, as they noted that human aortic SMCs did not express MMP-13 when maintained in a basal medium supplemented with only 2.5% fetal calf serum and thought it likely that additional factors were necessary for the induction of SMC MMP-13 expression *in vitro*.

After establishing that HASMC MMP-2 levels were significantly upregulated by severe hypoxia (Chapter 4, section 4.4.2.2), it was important to investigate if the global hypoxia transcription factor HIF-1 α also played a role in the signalling pathway as it is a key regulator responsible for the induction of genes that facilitate adaptation and survival of cells under hypoxic conditions (Wang *et al.*, 1995, Semenza, 1998). HIF-1 α immunoblotting analysis established the presence of increased HIF-1 α in HASMC total cell lysate following exposure to severe hypoxia (1%) over 48 hours (Chapter 7, section 7.3.3) and immunohistological staining showed the presence of HIF-1 α in the inflammatory infiltrate and medial SMCs of AAA tissue (Chapter 7, Figures

78, 79) demonstrating HIF-1 α expression was significantly higher in AAA in comparison to non-aneurysmal control tissue (Chapter 7, Figure 77) which supports the findings of Hu *et al.*, (2004) and our earlier results (Erdozain *et al.*, 2011). Importantly, immunohistological staining of AAA tissue also established that HIF-1 α immunopositivity was principally localised in the nucleus signifying that nuclear translocation had taken place (Chapter 7, Figures 78,79). When identifying transcription factors It is important to differentiate between true nuclear staining and that of cytoplasmic staining because it is only when HIF stabilisation within the nucleus has occurred that it can then associate with the HRE regions of promoters of hypoxia-responsive target genes. Weak cytoplasmic HIF-1 α staining seen in normal control aortic sections is likely to be transient prior to being targeted for proteosomal degradation during normal oxygen levels (Semenza., 2000).

The idea that HIF-1 α is an initiating factor in the pathway leading to MMP up-regulation is also consistent with research which demonstrated elevated levels of TNF α to naïve or small AAAs (<50mm) far higher than levels seen in larger AAAs (Harmano *et al.*, 2003) along with the knowledge that HIF-1 α is also proven to be up-regulated in response to inflammatory TNF α (Hellwig-Bürgel *et al.*, 1999) appears to suggest these are early events in the pathogenesis of AAA.

During hypoxia HIF-1 α activates the expression of certain genes by directly binding to a 50-base pair *cis*-acting hypoxia responsive element located in their enhancer and promoter regions (Semenza *et al.*, 1991) In order to examine additional tissue markers of hypoxia AAA tissue samples were probed for the transcription factor Ets-1 and the angiogenic factor VEGF. Research by Forsythe *et al.*, (1996) had previously demonstrated the activation of VEGF gene transcription by HIF-1 α and a later study established Ets-1 can also be induced by VEGF (Hashiya *et al.*, 2004). It has also been suggested that a positive feedback loop may exist for the Ets-1/VEGF interaction and that by inducing Ets-1 expression, VEGF may trigger its own production leading to further Ets-1 synthesis (Dittmer, 2003). Hypoxia, in addition to VEGF has been shown to induce Ets-1 via the

activity of HIF-1 α (Oikawa *et.al.* 2001), therefore it could be surmised that Ets-1 and VEGF may participate in the AAA signalling pathway terminating in MMP up-regulation as seen in these present hypoxia studies.

VEGF immunohistological staining of AAA tissues demonstrated widespread and diffuse VEGF immunopositivity within the tunica adventia and tunica media (Chapter 8, Figure 91) but no obvious VEGF immunopositivity was noted in normal aortic tissue (Chapter 8, Figure 90). This corresponds with research carried out by Nishibe *et al.*, (2010) who found VEGF was expressed in AAA tissue samples and absent in the normal abdominal aorta leading them to suggest that VEGF may play an important role in aneurysm formation via its direct and/or indirect actions. They also found two thirds of their AAA samples displayed VEGF positivity localised within the aortic SMCs which was also consistent with my findings in this present study where marked cytoplasmic VEGF staining was seen within VSMCs of aneurysmal tissue (Chapter 8, Figure 92).

Ets-1 immunohistochemical analysis (Chapter 8) also demonstrated an obvious difference in Ets-1 immunopositivity in AAA tissues in comparison to normal aorta tissue samples (Figures 86, 87, 88, and 89) and was localised mainly to the nuclei of matrix cells within the tunica media and inflammatory cells of the diffuse infiltrate, although some cytoplasmic staining was visible. AAA tissues were found to display a marked increase in immunopositivity of both Ets-1 and HIF-1 α transcription factors, when compared with the results obtained for normal aortic tissue (Chapter 8, Figure 86). Both factors appear localised to the VSMC and inflammatory infiltrate and predominately expressed within the medial layer of the aneurysm wall. This too is consistent with research carried out by Peters *et al.*, (2004) in our group, who also found co-localisation of HIF-1 α and Ets-1 in areas of hypoxia in the synovium of inflamed rat joints in adjuvant-induced arthritis and suggested HIF-1 α may be involved in the up-regulation of Ets-1. Furthermore immunohistological staining of AAA tissues had shown MMP-2 is also localised to the same regions which may be of relevance as research by Okuducu *et.al.* (2006) demonstrated MMP-2 and MMP-9 are target genes

of Ets-1. Burke *et al.* (2003) suggested hypoxia could act on the MMP-7 promoter via HIF-1 α mediated up-regulation of Ets-1 as its promoter contains two binding sites for Ets-1 for up-regulation of this MMP.

It had been established earlier in the study that severe hypoxia induced HASMCs to secrete significantly higher levels of MMP-2 into conditioned media in comparison to normoxic controls (Chapter 4, section 4.4.2.2). In order to further investigate the possible role of VEG and Ets-1 in the hypoxic induction of MMP-2, levels of VEGF and Ets-1 in total HASMC cell lysate after exposure to severe hypoxia were analysed by the Western blotting technique (Chapter 8, Figures 81 - 84). VEGF is a known target of HIF- α during hypoxia (Forsythe *et al.*, 1996) and significant increases of VEGF were noted in hypoxic samples. Additionally, HASMCs exposed to hypoxia also demonstrated significantly increased levels of Ets -1 (Chapter 8, Figure 82) which is consistent with Watanabe *et al.*, (2004) who established that VEGF and hypoxia increased Ets-1 expression in cultured bovine retinal endothelial cells. More recently, Qiao *et al.*, (2015) determined that severe hypoxia induced a rapid increase in Ets-1 in pancreatic β cells within one hour which also corresponds with the findings of this study. Furthermore they suggested that this pattern of activity defined Ets -1 as an early response gene which is interesting as immunoblotting results of this study indicate levels of HIF-1 α , Ets-1 and VEGF rise earlier than MMP-2. Initiation of the hypoxic response would begin with cellular hypoxia being sensed by the prolyl-4-hydroxylases of HIF-1 α leading to stabilisation of the HIF-1 α subunit and translocation from the cytoplasm to the nucleus where it dimerises with HIF-1 β (Huang *et al.*, 1996, Kallio *et al.*, 1997) to bind with the HREs in the regulatory regions of VEGF and Ets-1 resulting in up-regulation of these target genes. Subsequently this could be followed by up-regulation of MMP-2 by Ets-1 accounting for the delayed rise seen in the MMP-2 immunoblot. Similarly, Ghosh *et al.*, (2012) hypothesized that in the human ovarian carcinoma cell line SKOV-3, VEGF induces Ets-1 through the activation of the PI3K/AKT and p38 MAPK pathways after which Ets-1 is enriched in the nucleus followed by the activation of specific MMP genes.

Chapter 6 focused on the potential role of the endogenous inhibitors of MMPs known as TIMPs in the formation of AAAs. Although the activity of MMPs has been shown to be essential in cell biological processes it is vital that they are tightly controlled by TIMPs under normal physiological conditions to provide a balancing mechanism to prevent excessive degradation of the ECM. Under pathological conditions associated with unbalanced MMP activities such as in AAA, changes in TIMP levels are considered to be important because they directly affect the level of MMP activity. Another important feature of TIMP-2 is its role in the activation of proMMP-2 where levels of TIMP-2 are crucial for MMP-2 activation. Often the production of TIMPs is elevated by the same stimuli that promote MMP expression (Thompson *et al.*, 2002). Therefore, as this study has established that hypoxia is a factor in increasing the expression of MMPs it would seem prudent to analyse TIMP levels during experimental hypoxia. Furthermore, our earlier study (Erdozain *et al.*, 2011) found significant transcriptional up-regulation of TIMP-1 mRNA using PCR analysis of HASMC lysate after exposing HASMCs to hypoxia for up to 48 hours. TIMP-1 and TIMP-2 levels in HASMC conditioned media from cells exposed to severe hypoxia over a 48 time period were analysed using the reverse zymography technique. Results did in fact show that there were significant increases in TIMP-1 and TIMP-2 protein levels in the hypoxic HASMC supernatant samples in comparison to controls (Chapter 6, Figures 65-69) rather than decreases in TIMP expression which might be expected due to the increased MMP-2 levels found in hypoxic samples earlier in the study. However, these results are consistent with substantial evidence that the production of TIMPs is actually increased within the aneurysm wall (Tamarina *et al.*, 1997, Nishimura *et al.*, 2003). Misra *et al.*, (2010) also found hypoxia significantly increased levels of TIMP-1 and TIMP-2 in murine fibroblasts and more recently Tefã-Silva *et al.* (2013) using an experimental AAA rat model which combined inflammation and turbulent blood flow found dramatic increases in levels of HIF-1 α , MMP-2 and -9, TIMP-1 and TIMP-2. Although little is known about TIMP behaviour in aneurysmal formation, increases in TIMP-1 expression noted in this study may be explained by research carried out to examine if hypoxia promotes fibrogenesis in human renal fibroblasts by

Norman *et al* (2000). They identified a HRE in the TIMP-1 promoter and established that the hypoxic induction of TIMP-1 expression was dependent on the binding of HIF-1 α to a HRE in the 5' promoter. My findings in this study had already established HIF- α levels are increased in hypoxic HASMCs therefore if TIMP-1 is hypoxia-inducible via a HIF-1 α dependent pathway this may account for the increased TIMP-1 expression in hypoxic HASMCs samples. TIMP-1 levels in normoxic HASMCs could be due to constitutive expression as Fabunmi *et. al.* (1998) had found TIMP-1 and TIMP-2 were constitutively expressed in cultured human SMCs during their vascular research. TIMP-2 protein levels were also found to be significantly increased in HASMCs subjected to hypoxia even though Norman *et al.* (2000) noted that HIF-1 α binding motifs were absent from the TIMP-2 promoter. However another possibility is that TIMP-2 could be hypoxia-inducible via HIF-1 α mediated up-regulation of Ets-1, as research carried out into the renal expression of proto-oncogene Ets-1 on matrix remodelling in experimental diabetic nephropathy demonstrated that Ets-1 may be involved in the transcriptional regulation of MMP-2 and TIMP-2 (Lui *et al.*, 2011). Constitutive TIMP-2 expression would account for the lower levels found in normoxic HASMC control cells conditioned media.

In this study, when carrying out image analysis of band intensity and size using Scion Imaging Software, it may have been advantageous to have quantified MMPs, TIMPs or other proteins. They were analysed by defining the arbitrary densitometry units but could have been calibrated against a standard curve prepared from a purified enzyme which would have made quantification more accurate when comparing MMP/TIMP ratios. However the limited number of wells per zymogram gel did not allow a full standard curve, as normoxic and hypoxic samples were run side-by-side for each time-point for easier visual comparison. Nonetheless, this did not prevent the study from establishing that exposure to severe hypoxia significantly increased the levels of the aforementioned proteins in comparison to normoxic controls. Another point to note is that these results represent three independent experiments in triplicate at different times. Three different primary sources of HASMCs (different donors) were used during these studies, however experimental results from one batch was discounted and

not used even though up-regulation of MMP-2 was also noted on analysis, as during the hypoxic experimental period at some time-points the cells lifted off from the base of the 6-well plates possibly due to the cells being too confluent prior to the start of the study. However I believe sufficient biological variance has been satisfied and reproducible experimental results achieved using primary cells from different donors. Additionally, our earlier study (Erdozain *et al.*, 2011), looking at the effect of progressive hypoxia (5% down to 2%) on the activity of HASMC MMPs and again using different HASMCs isolated and cultured from normal human aorta, also demonstrated similar results. Human aneurysmal and normal control aortic tissue rather than primary cells were used during histological analysis, with aneurysm tissue from 5 consented patients undergoing elective surgical repair and normal aortic tissue from 5 renal transplant donors (n=10).

The final chapter explains the effect of inhibiting HIF-1 α on MMP-2 up-regulation in HASMCs exposed to severe hypoxia. Using a HIF-1 α inhibitor seemed the logical next step to determine whether hypoxia and HIF-1 α are an initiating factor in the MMP-2 up-regulation observed in this study and consequently in AAA formation. It was important to select the correct HIF inhibitor and Bisphenol A (BpA) which promotes HIF-1 α degradation by dissociation of the chaperone protein Hsp90 was chosen. BpA works in a similar fashion to the antibiotic geldanamycin, inhibiting Hsp90 followed by degradation although their structures are quite different and BpA is more economical to use (Minet *et al.*, 1999, Kubo *et al.*, 2004). Preliminary experimentation was carried out to ascertain the optimum concentration of BpA necessary to inhibit HIF-1 α in HASMCs during hypoxia. BpA required solubilisation in ethanol prior to being further diluted in serum-free media to the final concentration therefore care needed to be exercised to ensure the ethanol was sufficiently diluted in media before being applied to the HASMCs to prevent any detrimental effects to them. Cell viability assays were also carried to ensure the BpA was not harmful to the cells in addition to vehicle controls to make certain experimental results were unaffected by ethanol. Controls during this final hypoxia study included HASMCs kept

under normoxic conditions, HASMCs kept under 1% hypoxia and HASMCs plus BpA kept under 1% hypoxia.

Following the HIF-1 α inhibition experiment, levels of MMP-2 activity in HASMC conditioned media were analysed by zymography and results yielded very promising results (Chapter 9, section 9.3). Although at the 1 hour time-point, both the hypoxic and hypoxic plus BpA samples were significantly higher than normoxic controls. It is understandable that the BpA containing sample matched the hypoxic sample as the inhibitor would require time to be taken up by the cells and to take effect. All subsequent time-points up to 48 hours demonstrated significant increases in MMP-2 levels in hypoxic samples compared to normoxic controls as seen in the earlier study (Chapter 4). Hypoxic samples containing the inhibitor BpA which promoted HIF-1 α degradation under hypoxia on the contrary showed much lower MMP-2 levels and were not significantly different from control normoxic samples. The only exception was the 3 hour time-point in which the BpA inhibited sample was significantly lower than the normoxic control.

I believe that it is reasonable to state, from these results, that hypoxia signalling via HIF-1 α up-regulates MMP-2 as HIF-1 α inhibition prevents its stabilisation and thus down-regulates MMP-2 during hypoxia. Furthermore it suggests that the hypoxia-induced MMP-2 activation in early aneurysm formation is mediated by a signalling cascade initiated by HIF-1 α and potentially may play a primary role in AAA.

Importantly, recent work by Tsai *et al.*, (2016) has further corroborated my research. Firstly, using AngII induced AAA in a hyperlipidemic mouse model they stabilised HIF-1 α using deferoxamine (DFO) a prolyl hydroxylase inhibitor and found increased HIF-1 α expression was associated with increased MMP-2 and MMP-9 levels in the DFO treated mice which is consistent with the hypoxic induced stabilisation of HIF-1 α and subsequent up-regulation of MMP-2 seen in the hypoxic cultured HASMCs. Secondly, administration of HIF-1 α inhibitors decreased AngII-mediated HIF-1 α and VEGF in addition to down-regulating MMP-2 and MMP-9 expression in the

mouse aortic homogenates which also correlates to the decreased MMP-2 levels seen in hypoxic HASMCs after treatment with the BpA inhibitor during this research (Chapter 9, Figures 93-96).

10. 2 Future Work

During the present study, analysis of TIMP levels in hypoxic and normoxic HASMC conditioned media after inhibition of HIF-1 α with BpA was carried out. However, bands were not detected on the reverse zymograms even though the concentrations of MMP and gelatin were the same as those used in earlier reverse zymograms and in addition to further concentration modifications. Therefore it would be important to repeat the experiment to see if inhibiting HIF-1 α affects TIMP levels. I believe the lack of bands occurred as the original purified MMP-2 protein incorporated into the gel was not available to purchase and therefore a new protein had to be trialled which obviously was not successful. Further types of MMP-2 protein would need to be acquired and optimised in order to obtain good bands as reverse zymography is critically dependent on the source of gelatinase activity used to define the zones of TIMP inhibition.

Additionally VEGF and Ets-1 levels could be evaluated in HASMC total cell lysate after HIF-1 α inhibition under hypoxic and normoxic conditions. This could also be followed by sequential inhibition of VEGF and Ets-1 in HASMCs under hypoxia and subsequent analysis of HASMC MMP-2 levels which could also give further insight into the sequence of events in the hypoxic signalling pathway if MMP-2 levels are changed by either of the inhibited proteins. Clearly HIF should not be inhibited prior to VEGF or Ets-1 inhibition as we now already know that HIF inhibition affects MMP-2 levels but need to establish if this is in conjunction with either VEGF or Ets-1.

Like VEGF and Ets-1, NF- κ B is also known to be markedly activated in AAA. NF- κ B is a transcription factor that has a major role in regulating pro-

inflammatory genes which are implicated AAA and also regulates the transcription of MMP-1, MMP-2, MMP-3 and MMP-9 (Bond *et al.*, 1999, Takeshita *et al.*, 1999, Kim *et al.*, 2000). Culver *et al.*, (2010) demonstrated the activation of NF- κ B in response to hypoxia. Hypoxia and inflammation are intimately connected at the cellular and molecular level (Biddlestone *et al.*, 2015) and Bandarra and Rocha, (2015) also demonstrated crosstalk between HIF-1 α and NF- κ B. Therefore it could also be useful to block NF- κ B in HASMCs under hypoxic and normoxic conditions and then re-evaluate HASMC MMP levels to determine the effects of NF- κ B inhibition.

During the present study gelatin zymograms only demonstrated bands of MMP activity which was verified using EDTA inhibition assays which abolished all MMP activity thus resulting in the absence of bands from treated gels. However, Wilder *et al.* (2011) reported a zymography protocol to detect and distinguish proteolytic activity for different cathepsin family members, modifying individual cathepsin substrate preferences, varying pH and electrophoretic mobility. Therefore adaptations to the present zymography protocol could be used to assess any possible cathepsin activity in HASMCs exposed to experimental hypoxia. If analysis did show elevated hypoxia-driven expression of these proteases then it would be prudent to see if the up-regulation was mediated by HIF-1 α by inhibiting HIF as in the present study.

Another useful procedure is sequential double immunofluorescence using a selection of different fluorophores after which separate fluorescent images can be digitally captured and overlaid using software such as Photoshop which would definitively allow visual clarification of localisation and co-localisation of proteins of interest.

10.3 Conclusion

Although there is further research required to elucidate the precise mechanism for AAA induction, and the knowledge regarding the role of hypoxia and HIF-1 α in the pathogenesis of aneurysm formation is still limited, results from this study will add to the pool of knowledge within the field of AAA research. Targeting HIF-1 α or disruption of the HIF-1 pathway could represent a novel pharmacological approach for small AAAs and the many aspects of HIF-1 regulation provides a number of possibilities for therapeutic intervention. Clearly the mechanisms involved in AAA formation are multiple and complex. Nevertheless I believe that this work provides insight into a possible mechanism for the progression, if not the initiation, of aneurysmal disease. The work undertaken here shows that hypoxia is a prime candidate for driving matrix metalloproteinase regulation, expression and activation and may have serious consequences in the inflamed abdominal aorta.

Appendix-1

Haematoxylin and Eosin Stain Protocol

Tissues sections mounted on slides were treated by submersion in the following solvents for the times indicated:

- Ice cold acetone : 1-5mins (Tissue Fixation)
- Tap water : 5mins (Rehydrate tissue)
- Harris Haematoxylin (prepared 1 part Haematoxylin : 3 parts water) : 5mins
- Tap water : 5mins
- Acid alcohol (1% HCl in IMS) : 1min with gentle agitation
- Tap water : 5mins
- Eosin : 0.5min
- Tap water : 5mins
- IMS : 1min
- IMS : 1min
- Xylene : 1min
- Xylene : 1min
- A coverslip was mounted onto the treated tissue sample with excess DePeX mounting medium.

SDS-PAGE, Western and Dot Blot Recipes

SDS-PAGE Resolving Gel (8%)

- 10.66mls dH₂O
- 4.0mls 40% Acrylamide (Anachem. Bedfordshire, UK)
- 5.02mls 1.5M Tris/Base pH8.8 (Promega UK Ltd. Southampton, UK)
- 100µl 20% SDS (Sigma Aldrich Company Ltd. Dorset, UK)
- 200µl 10% Ammonium Persulphate (Sigma Aldrich Company Ltd. Dorset, UK)
- 15µl N, N, N', N'-Tetra-methyl-ethylenediamine (TEMED) (Sigma Aldrich Company Ltd. Dorest, UK)

SDS-PAGE Stacking Gel (4%)

- 4.98mls dH₂O
- 660µl 40% Acrylamide
- 840µl 1.0M Tris/Base pH6.8
- 33.4µl 20% SDS
- 66µl 10% Ammonium Persulphate
- 8µl TEMED

SDS-PAGE Running Buffer

- 14.4g Glycine (VWR International Ltd. Dorset, UK)
- 3.06g Tris/Base
- 1.0g SDS
- 1000mls dH₂O

SDS-PAGE Sample Buffer

- 6.75mls dH₂O
- 0.75mls 1M Tris/Base pH6.8
- 2.0g Sucrose (VWR International Ltd. Dorset, UK)
- 1mg Bromophenol Blue (Promega UK Ltd. Southampton, UK)
- 0.5mls 2-mercaptoethanol (Sigma Aldrich Company Ltd. Dorset, UK)

Western and Dot Blot Transfer Buffer

- see SDS-PAGE Running Buffer but made with 20% Methanol (VWR International Ltd. Dorset, UK)

Western and Dot Blot Washing Buffer

- 0.5% Tween 20 (Sigma Aldrich Company Ltd. Dorset, UK) in PBS

Western and Dot Blot Blocking Buffer

- 5% Marvel non-fat dried milk powder in Washing Buffer

Gelatin Zymography

2x Sample Buffer Solution (reducing buffer)

- 5ml stacking gel buffer solution
- 8ml 10% SDS solution
- 1ml dH₂O
- 4ml glycerol
- 2ml 2 – mercaptoethanol and mix well (use fume hood)
- Small amount (1-2mg of bromophenol-blue indicator dye)
- Divide sample buffer into 1ml portions (eppendorfs) and store at -70°C or -20°C for short periods
- NB – warm to dissolve crystallized SDS

2x Sample Buffer (non-reducing)

- 5ml stacking gel buffer solution
- 8ml 10% SDS solution
- 3ml dH₂O
- 4ml glycerol
- Small amount (1-2mg of bromophenol-blue indicator dye)
- Divide sample buffer into 1ml portions (eppendorfs) and store at -70°C or -20°C for short periods

Stacking Gel Buffer Solution

- Dissolve 15.2g Tris base in 200ml water
- Adjust to pH 6.8 by slow addition of concentrated HCL
- Add dH₂O to give 250ml final volume

0.5M Tris-HCL Stock solution

- Dissolve 60.5 g Tris Base in 900ml dH₂O
- Adjust to pH 7.6 by slow addition of concentrated HCL
- Make up to a volume of 1L (1000mls)

10% SDS

- 10g SDS (Lauryl Sulfate) into 90ml of dH₂O (main shelf) Make up to 100ml
- SDS is difficult to dissolve so warm made-up solution in a tub of hot water

Resolving Gel Buffer Solution

- 90.6g Tris Base in 350ml dH₂O
- Adjust to pH 8.8 by slow addition of concentrated HCL
- Add dH₂O to a final volume of 500ml

or

- pH 9.2 as this enhances resolution and shortens run time

Water Saturated Butanol Solution

- 100ml n-Butanol and 5ml dH₂O
- Shake and use top layer

Zymogram Development Solution

- 100ml 0.5M Tris-HCL solution
- 100ml 50mM Calcium Chloride Solution (7.35g CaCl₂ in 1 litre = 50mM) (main shelf)
- 800ml dH₂O

Gelatin Solution

- 300mg gelatin (substate for gel) in 20ml H₂O (main shelf)(Sigma G-2500)

Coomassie Blue Staining Solution

- Dissolve 2g Coomassie Brilliant Blue R-250 in
- 500ml Methanol
- 100ml glacial acetic acid (17.4M)
- 400ml acetic
- Mix well and filter through a Watman filter and store at below 25°C

Destaining Solution

- 1.6L or (400ml) Methanol
- 400ml or (100ml) glacial acetic acid
- 2L or (500ml) dH₂O
- Mix and store at below 25°C

Renaturing Buffer (allows MMPs to renature and digest gel)

- 12ml Triton-X-100 in 488mls dH₂O (gives a 2.5% solution)

Ammonium Persulphate

- 100mg in 1ml dH₂O – must be prepared fresh (acts with TEMED to set gel)

4 x Tank Buffer (4 x Electrophoresis Tank Buffer Solution)

Dissolve

- 48g Tris Base (shelf by IMS)
- 230.4g glycine (shelf by IMS)
- 16g SDS into 4 litres of dH₂O

or

- 12g Tris Base
 - 57.6 glycine
 - 4g SDS
- into 1 litre dH₂O

1 x Tank Buffer Solution

- Mix 1 part (4 x Tank Solution) to 3 parts dH₂O to get 1 x Tank Buffer
- Eg. 200ml 4 x Tank Buffer in 600ml dH₂O (total 800ml)

Stacking Gel

- 1ml acrylamide/bis 37.5:1 (neat)
- 2.5ml stacking gel buffer
- 6.3ml dH₂O
- 0.2ml or 200µl 10% SDS
- 0.2ml or 200µl ammonium persulphate
- 20µl TEMED (only put in just before filling gel plates or will set in tube)

Resolving Gel

- 10mls acrylamide/bis 37.5:1 soln (neat) made by Amresco)(Fridge 1)
- 10mls resolving gel buffer
- 15.5mls dH₂O
- 4mls made-up gelatine solution (main shelf)
- (0.8ml)800µl 10%SDS (Lauryl sulphate) (main shelf)
- (0.6ml)600µl ammonium persulphate (main shelf)
- 40µl TEMED (put in just before filling gel plates) (main shelf)

Protocol

- Make up resolving and stacking gels as per recipes
- Make up gelatin in 20mls (heat to dissolve gelatin)
- Prepare 2 x sets glass gel plates(protein cupboard), place on blue tissue and spray with acetone Place gasket on gel plate – raised edge

of gasket goes against raised edge of glass plate (do when wet from acetone –easier)

- with water or acetone.
- Use a glass pipette to fill gel plate with resolving gel (add TEMED just before)
- Using a glass pipette drizzle water saturated butanol on top of the resolving gel to reduce bubbles and to give the gel a level top
- Leave to polymerise

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