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STRUCTURAL REMODELLING OF THE VASCULAR WALL IN RELATION TO AGEING AND ARTERIAL STIFFENING

Marie McNulty

A thesis submitted for the degree of

Doctor of Philosophy

to

University of Dublin, Trinity College

2006

Declaration

This thesis is being submitted for the degree of doctor of philosophy to the University of Dublin, Trinity College, and has not been previously submitted as an exercise for a degree at this or any other university.

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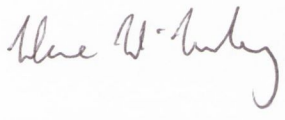

Marie McNulty

Summary

Structural remodelling of the vascular wall has been investigated in this study in terms of ageing and the process of vascular stiffening. While common structural adaptations are often presumed for ageing and vascular stiffening, recent evidence suggests that independent alterations may occur.

Age-associated vascular remodelling was investigated in both the central elastic artery- the aorta and the smaller muscular artery- the internal mammary artery (IMA) by the quantification of matrix metalloproteinase-2 and -9 activities in the arterial homogenates, using gelatin zymography. MMP-2 and -9 activities were localised within the vessel wall using *in situ* zymography. These studies demonstrated an age-associated increase in aortic MMP-2 activity and activation, confirming the results of previous animal studies. Age-related changes in matrix degrading activity were not present in the IMA.

Structural remodelling of the vessel wall in relation to both ageing and arterial stiffening was investigated using human vascular tissue samples from subjects following the measurement of arterial stiffness. Aortic stiffness was measured by pulse wave velocity (PWV), and augmentation index (AIx) was obtained to provide indices of systemic arterial stiffness. MMP-2 and -9 activities were quantified in the arterial homogenates to assess the activity of these enzymes in relation to measures of vascular stiffness. Collagen content was also measured in vascular tissue following Sirius red staining, as was intimal thickness and elastin tissue fragmentation following elastin staining. The results demonstrated increased aortic MMP-9 expression in subjects with stiffer arteries and increased

disruption of the elastic tissue architecture. There was also a trend towards increased collagen accumulation in the aortic media in subjects with stiffer arteries. As for vascular ageing, vascular stiffness related alterations of the structural proteins and enzyme expression was demonstrated only in the aorta, and was not evident in the IMA.

Circulating markers of vascular remodelling were also investigated in relation to both age and stiffness-associated vascular remodelling. Plasma MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2 and metabolites of collagen type I turnover (procollagen type C-terminal propeptide and C-terminal telopeptide) were quantified in plasma using commercial immunoassays and zymography, following the measurement of PWV and AIx in hypertensive and normotensive subjects. The results demonstrate the importance of collagen type I degradation in determining the arterial stiffness of both the aorta and smaller muscular arteries, and provide further evidence of MMP-9 involvement in aortic stiffening.

The involvement of cross-link formation on vascular structural proteins was also investigated in relation to vascular ageing and vascular stiffening. Plasma levels of advanced glycation end-products (AGEs) were quantified in hypertensive and normotensive subjects following the measurement of PWV and AIx. The results demonstrate increased circulating AGEs in the hypertensive subjects where the levels are related to aortic but not systemic arterial stiffness.

In summary, the work of this thesis has demonstrated that specific alterations occur in the ageing human aorta, which may in part explain the increased incidence of both cardiovascular disease and vascular stiffness in older subjects. Other aspects of vascular remodelling are specifically related to vascular stiffening irrespective of age.

Acknowledgements

My sincere thanks to Professor John Feely whose enthusiasm has provided access to a wide range of clinical samples and measurements, and whose constant advice has been vital in the development of all aspects of this project.

Thanks also to Dr Paul Spiers, for his invaluable guidance in the lab and also his assistance and encouragement during the establishment of various lab techniques that were used in this study.

I would also like to thank Dr Azra Mahmud, not only for carrying out the measurements of arterial stiffness, but also for her advice and expertise in this area.

My thanks to all the nurses at the Cardiothoracic Surgery Unit, St James's Hospital and also to cardiothoracic surgeons Mr Vincent Young, Mr Michael Tolan and especially Ms. Eilis McGovern, for their involvement in the collection of human vascular tissue, and also for allowing the measurement of arterial stiffness in their patients.

I would also like to thank Mary and Linda for their patience and generosity with their time while obtaining measurements of arterial stiffness from patients in the Cardiac Surgery Unit.

Thanks to Ken and Pierce –their enthusiasm (and humour) is greatly appreciated.

Also thanks to Ms Teresa Mulroy, whose help has often gone far beyond the call of duty.

Thanks to my family, for their never-ending encouragement. And to Garrath who always gave me support and perspective.

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Abbreviations

±	With or without
AAA	Abdominal aortic aneurysms
ACE	Angiotensin converting enzyme
AGE	Advanced glycation end-product
AGE-BSA	AGE-bovine serum albumin
AIx	Augmentation index
APS	Ammonium persulphate
AT_1	Angiotensin II receptor type 1
Au	Arbitrary units
BSA	Bovine serum albumin
CA	Coronary aneurysms
CABG	Coronary artery bypass grafting
CEL	N(epsilon)-carboxyethyl lysine
cGMP	Cyclic guanosinemonophosphate
CML	N(epsilon)-carboxymethyl lysine
DBP	Diastolic blood pressure
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ESS	Enzyme stock solution
EWS	Enzyme working solution
GAG	Glycosaminoglycan
HRP	Horseradish peroxidase
ICAM	Interstitial cell adhesion molecule
ICTP	C-terminal telopeptide
IEL	Internal elastic lamina
IL-1	Interleukin-1
IMA	Internal mammary artery

kDa	Kilodalton
LH	Lithium heparin
m/sec	Metres per second
MAP	Mean arterial pressure
MBP	Mean blood pressure
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane type-MMP
NF-κB	Nuclear factor- κB
PDGF	Platelet-derived growth factor
PIIIP	N-terminal procollagen III peptide
PIP	Procollagen type C-terminal propeptide
PMN	Polymorphonuclearneutrophils
PP	Pulse pressure
PPE1	Porcine pancreatic elastase 1
PVDF	Polyvinyl difluoride
PWV	Pulse wave velocity
RNA	Ribonucleic acid
ROI	Region of Interest
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SBP	Systolic blood pressure
SDS	Sodium doecyl sulphate
SHR	Spontaneously hypertensive rat
SMC	Smooth muscle cell
SVAS	Supravalvular aortic stenosis
TEMED	N, N, N, N-tetramethylethylene diamine
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-alpha
VSMC	Vascular smooth muscle cell
WKY	Wistar-Kyoto

Chapter 1

Introduction

1.1 ARTERIAL STIFFNESS

In clinical practice, blood pressure is defined in terms of systolic (SBP) and diastolic blood pressure (DBP), which refers to a pulsatile phenomenon, with SBP and DBP representing the extremes of the blood pressure oscillation around a mean value, the mean arterial pressure (MAP). However the blood pressure curve may be considered as the summation of a steady component and a pulsatile component, the pulse pressure (PP). The determinants of PP and SBP are the cushioning capacity, or compliance of the arteries along with the timing and intensity of arterial wave reflections. The term 'stiffness' is used to as an alternative to indicate qualitatively the elastic vessel wall properties and hence the cushioning ability. Arterial stiffness is ultimately a measure of the vessel compliance.

1.1.2 Arterial Stiffness and Cardiovascular Mortality

Large artery stiffness is a principal determinant of PP and both are related to cardiovascular mortality independently of other classic risk factors.(1-4) It has also been shown that a single measurement of aortic stiffness is strongly associated with the presence and extent of atherosclerosis(5) and that this measurement is highly related to cardiovascular risk as assessed by the standard Framingham equations. PWV is significantly increased in patients with type-2 diabetes compared to age-matched control subjects(6). PWV has also been shown to be independent predictor of later mortality in both control subjects and in patients with type-2 diabetes (7). In addition, aortic stiffness, measured as

PWV has been demonstrated to be significantly associated with the risk of stroke death in patients with essential hypertension, even after classic cardiovascular risk factors have been considered (8).

1.1.3 Factors Contributing to Arterial Stiffening

Arteries become 'stiffer' with age(9;10) and also during pathological processes such as hypertension.(11) During the progression of diabetes mellitus(12) and atherosclerosis(13) arterial stiffness is also enhanced. This is also true of end-stage renal disease.(14) Chronic alcohol intake stiffens arteries(15) as does cigarette smoking.(16) Females experience a greater increase in large artery stiffness with age than males of a similar age, and the time-course of changes occurring in the large arteries of females is suggestive of sex steroid modulation.(17)

The processes underlying these factors are varied. They include mechanical stress, inflammation, genetic influences, growth factors and neural and hormonal influences. Sex steroids are known to regulate enzymes which can cause degradation in the vessel wall.(18) Genetic variations in extracellular matrix proteins have been associated with stiffer arteries.(19;20)

Although modulation of vascular tone may impact upon measures of arterial stiffness, any of these factors, which ultimately modulate the intrinsic compliance of the vessel, is impacting on the relationship of the structural components. The structure and composition of these components will determine the passive stiffness of the vessel in question. A clearer understanding of the structural

processes that contribute to large artery properties may provide novel approaches to therapy.

1.1.4 Pulse Wave Analysis and Arterial Stiffness

The elastic properties of large arteries can be assessed non-invasively by determination of the velocity along the respective arterial segment as pulse wave velocity (PWV). The aortic pressure curve is considered to be the sum of an incident pressure wave that is propagated at a given velocity (PWV) along the arterial tree and a reflected wave which travels backward from the peripheral reflecting sites, toward the ascending aorta and the heart. This can be recorded non-invasively by applanation tanometry, a technique which various studies have deemed to show good repeatability and low inter-observer variation (21). This technique of aortic pulse wave analysis, depends on accurate recordings of the radial or carotid artery, it's calibration against brachial pressure, then generation of the ascending aortic pressure wave-form through use of a generalised transfer function in a computerized process. For the aorta, measurement of carotid-femoral PWV provides an index of aortic stiffness. PWV has been shown to be an independent risk factor for cardiovascular mortality in hypertensive subjects with end-stage renal disease.(4) Calculation of cardiovascular risk using Framingham equations (5) indicates that in subjects with essential hypertension and preserved renal function, the 10-year cardiovascular risk consistently increases with an increase in aortic PWV.

1.1.5 Augmentation Index

Increased PWV leads to an early return of reflected waves from the periphery to the ascending aorta, and thus generating a late aortic systolic peak, which is a measure of systolic augmentation in the aorta and expressed as the augmentation index (AIx). The AIx is a global estimate of arterial stiffness and depends upon PWV, the magnitude of reflected waves from the periphery and pattern of left ventricular ejection. The early or systolic reflected waves have detrimental consequences for ventricular vascular interaction; the heart faces increased left ventricular after-load and also looses the diastolic boost to coronary perfusion pressure. Thus AIx has been shown to be an independent prognosticator for all-cause and cardiovascular mortality in patients with end-stage renal disease(22) and more recent data shows it be a significant predictor of coronary artery disease in men under the age of 60 years.(23)

1.1.6 Physiological Implications of Increased PWV

As arteries stiffen, PWV increases, causing the reflected wave to return to the ascending aorta earlier, during systole. Such early wave reflection leads to increases in SBP, PP and decreased pressure throughout diastole. An increase in SBP leads to increased myocardial oxygen demands, whereas a fall in DBP leads to decreased coronary perfusion, both predisposing to myocardial ischaemia. Furthermore, increased SBP leads to increased ventricular afterload, generating left ventricular hypertrophy. Early aortic wave reflection also causes a loss of PP amplification between central and peripheral arteries. PP is much higher in the

peripheral than in the central arteries. The loss of this gradient with arterial stiffening is most likely due to the early return of the backward pressure wave towards the heart, because of the increase in the velocity of the aortic pulse wave. This amplifies the aortic systolic pressure, increases end systolic stress and further promotes increased ventricular hypertrophy.(24) The turbulent flow caused by vessel stiffening may(25) also lead to endothelium damage which contributes substantially to cardiovascular disorders such as atherosclerosis, hypertension and heart failure leading to vascular occlusion, reduced blood supply to organs and end-organ damage.(26)

1.2 ARTERIAL MORPHOLOGY

1.2.1 Biology of the Normal Arterial Wall

The basic morphological structure of larger arteries consists of cells and matrix arranged in three transmural zones, the tunica intima, the tunica media and the tunica adventitia (Figure 1.1). The intima is the region bound on the luminal side by a one cell thick layer of endothelial cells on top of basement membrane. This endothelium, the inner lining of blood vessels, is located between the circulating blood and vascular smooth muscle. The endothelium is more than a barrier to the passage of substances from the blood to the vessel wall; it also has anti-coagulant properties and is involved in the regulation of vascular tone.(27;28) The endothelium releases humoral factors that control relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition and is therefore important in blood pressure control, blood flow and vessel patency.(26)

The intima is bound peripherally by the internal elastic lamina (IEL), a fenestrated sheet of elastic fibres, which provides support for the endothelial cells. In between, vascular smooth muscle cells (VSMCs) and various components of the extracellular connective tissue matrix are the predominant features. The intima does not contribute much mechanically to the vessel wall, but is a rich source of substances and signal transduction mechanisms that influence the properties of the whole vessel wall. Not all species form an intima unless their arteries are injured. In humans, the intima develops spontaneously after birth.(29) Intimal formation also occurs in response to ageing and to injury, including atherosclerosis.(30) An intima formed in response to injury is referred to as a 'neointima'.

The medial layer represents the main basis of mechanical properties of arteries. It is built up by a network of fenestrated elastic lamellae, thinner than the IEL and an inter-lamellar zone, which consists of circular layers of smooth muscle cells, with a slight helical orientation. VSMCs are the exclusive cellular component of the medial layer, but do not represent a homogenous cell population. They have different mixtures of phenotypes, including contractile, proliferative, synthetic, or apoptotic behaviour.(31) The relative occurrence of each of these phenotypes depends on age, location in the vascular tree and prevailing pathological conditions. Despite these variations, it has been shown that the medial smooth muscle cells (SMCs) do not contribute significantly to the passive properties of the vessel wall.(32) In between the SMCs are obliquely arranged collagen and interconnecting bands or fibrils of elastic tissue between the elastic laminae. This

forms small compartments in which SMCs lie in a proteoglycan matrix. A connecting molecular grid consists mainly of mucopolysaccharides. The arrangement of lamellar units distributes stress and maintains the viscoelastic properties of the wall.(33) The external elastic lamina (EEL) separates the media from the outermost layer, the adventitia.

The adventitia is particularly abundant in the more centrally located large arteries and contains blood vessels (vasa vasorum) and nerves (nervi vascularis) embedded in collagen rich connective tissue with fibroblasts and fat cells. The high proportion of collagen in the adventitia confers strength and stability to the entire vessel. Collagen fibres surrounding the fibroblasts of the adventitial are the primary source of the tensile strength of the vessel wall.

1.2.2 The Extracellular Matrix and it's Role in Vessel Wall Remodelling

The three layers of the vascular wall- the intima, the media and the adventitia are embedded in the extracellular matrix (ECM). The ECM can therefore be viewed as the scaffolding that supports the cellular elements of the vessel wall. However, in addition to their structural functions, the components of the ECM are involved in cell proliferation, migration and cell-cell interactions.(34-36) As these processes occur they influence the matrix composition and lead to the formation of specialised ECM structures. Changes in matrix composition also require the removal of extracellular components. In non-pathological tissue, the matrix removal and synthesis occur simultaneously in an ordered fashion. However, during pathological conditions such as heart failure and atherosclerosis there is an

imbalance in matrix turnover and subsequent alterations of the components of the ECM.(34)

Figure 1.1

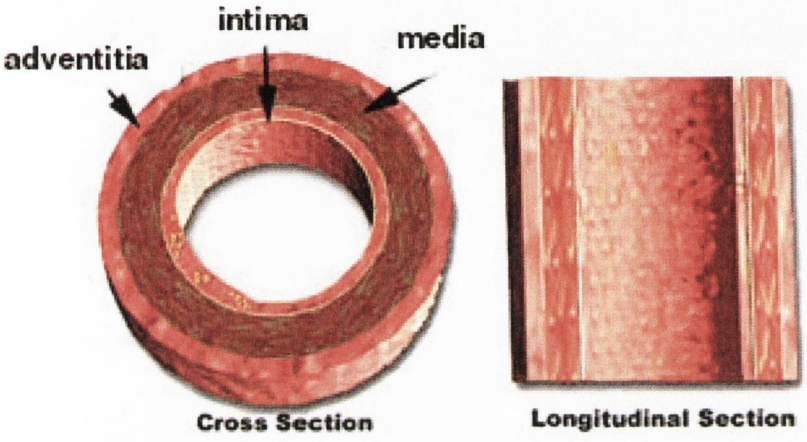


Figure 1.1: The three main layers of the artery, shown as a cross and longitudinal section. The innermost layer the intima is bound on the lumen side by the endothelium and peripherally by the internal elastic lamina. The middle layer, the media is separated from the outer layer, the adventitia by the external elastic lamina.

The basement membrane is a specialised ECM structure, important in cell growth and differentiation that surrounds the VSMCs and remains in close proximity to the cell it was formed by. The main constituents of the basement membrane are laminan, collagen type IV, entactin and the heparin sulphate proteoglycan, perlecan. The basement membrane gives mechanical support to the cell and also regulates the transmigration of cells and exchange of macromolecules. It also acts a substrate for cell adhesion and migration.(37;38)

The role of fibronectins is to attach cells to a variety of extracellular matrices (Figure 1.2) (except collagen type IV which uses laminin as the adhesive molecule). Fibronectins are glycoproteins, existing as dimers of two similar peptides. Fibroblasts, endothelial cells and VSMCs secrete, bind and assemble fibronectin into fibrils in the ECM.(39) Integrins are important transmembrane receptors in fibronectin polymerisation (Figure 1.2). Fibronectins in the vascular ECM have binding sites for VSMCs, collagen, fibrin and heparin, and the polymerisation of fibronectin into the ECM must be tightly regulated to ensure that the adhesive information in the ECM is correct.(18) Unlike laminin, there is little passive accumulation of fibronectin, which is processed actively at specialised areas on the cell surface.(40)

Proteoglycans are protein-polysaccharide complexes that consist of a protein core to which one or more glycosaminoglycan (GAG) chains are covalently attached (Figure 1.2). These are chondroiton sulfate, dermatan sulfate, heparan sulfate and

Figure 1.2

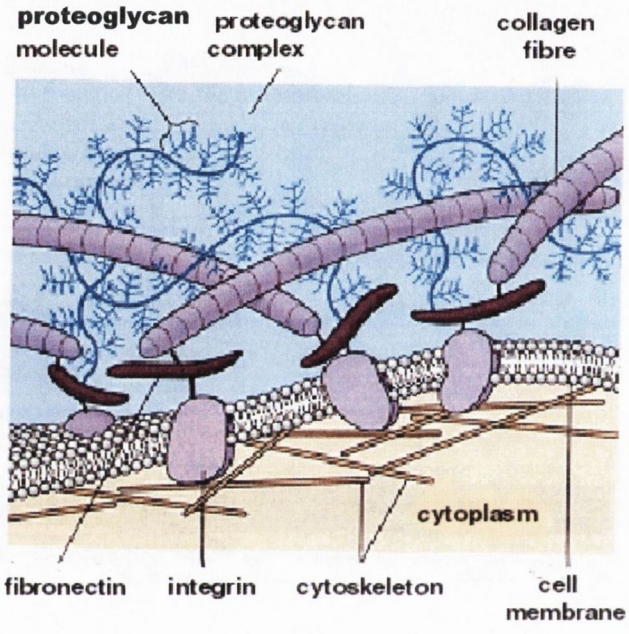


Figure 1.2: Diagram showing cell membrane/extracellular matrix interface and the relationship between glycoproteins (laminan and fibronectin), collagen and proteoglycans and the resulting structure of the extracellular matrix.

Modified from www.uwinnipeg.ca/~simmons/Cell%2Otour/sld025.htm

keratan sulfate. Hyaluronan is a GAG existing as a free molecule in the ECM and can promote processes such as cell migration.(41)

The major proteoglycans in the vascular interstitial matrix are versican, decorin and biglycan. Versican has multiple roles. It interacts with hyaluronan forming aggregates that bind water providing tissue hydration and the facilitation of gliding and sliding movements. They also form a large part of proteoglycan aggregates in-pressure resisting tissue such the arterial wall.(42) The smaller glycoproteins, biglycan and decorin help to stabilize and link collagens and proteoglycans to the cell surface. It has also recently been shown that biglycan and decorin induce morphological and cytoskeletal changes in fibroblasts, resulting in an increase in migration.(43)

1.3.1 THE STRUCTURAL PROTEINS OF THE EXTRACELLULAR MATRIX

Collagen and elastin, the connective tissue proteins are the two major extracellular constituents of the vascular wall (Figure 1.3). The absolute and relative quantities of these two constituents largely determine the biomechanical properties of major arteries and veins.(44) The collagen and elastin form the fibrous scaffolding of the vessel while the proteoglycans and glycoproteins (along with solutes and water) form the inter-fibrillary matrix. The structural proteins, collagen and elastin play pivotal roles in maintaining the structural integrity of the arterial wall. In the aorta and other large vessels, the collagen and elastin not

Figure 1.3

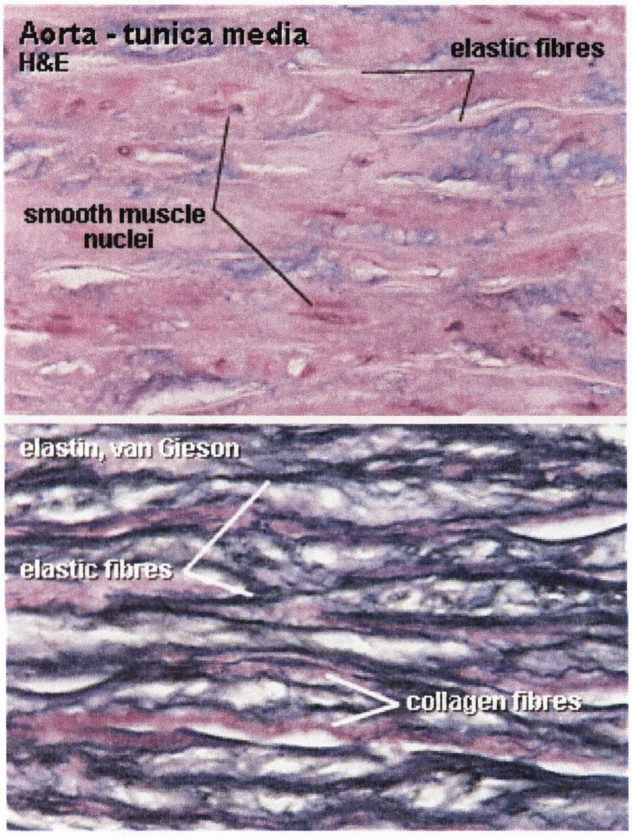


Figure 1.3: Section of human aorta (elastic artery) stained with hematoxylin and eosin (top) demonstrates smooth muscle cell nuclei and elastic fibres. The elastin van Gieson stain (bottom) demonstrates the large proportion of collagen and elastin fibres in the vessel wall.

From: http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm

only contribute to wall mass, but determine the passive physical properties of the vessel wall. Collagen fibres bear load in the circumferential direction, whereas elastic fibres provide longitudinal as well as circumferential support. The relative proportions of collagen and elastin, along with their physical arrangement determine the mechanical properties of the arterial wall, largely in the tunica media. The ratio between collagen and elastin varies at different locations in the arterial tree. In the pulmonary artery, the medial component is composed of about 27% collagen and 9% elastin. In the adventitia of the same vessel the collagen increases to around 63%, elastin only accounting for around 1.5%. In the thoracic aorta the proportion of collagen in the media is greater than that of the pulmonary artery. Collagen makes ups around 37% of the media and 77% of the adventitia. The proportions of elastin are also greater in the aorta, around 24% in the media and 2.4% in the adventitia. The large proportions of collagen in the aortic

media lend strength, allowing the vessel to withstand the pulsatile load imposed upon it from the heart. The large proportion of elastin allows for elastic recoil and returns mechanical energy to the vessel.(45)

The more distal and smaller arteries- the muscular arteries receive an already-smoothed blood flow from the larger elastic arteries. They have a more limited dampening effect on blood flow but are involved in regulation through active constriction or dilation.(46;47) For these reasons, the wall is rich in VSMCs and poor in structural proteins. In muscular arteries the large number of elastic lamellae have become reduced to two in number, one on the inside (IEL) and one

on the outside (EEL) of the tunica media with some elastic fibres between the large number of SMCs.

1.3.2 Collagen

The term 'collagen' is used as a generic term for proteins forming a characteristic triple helix of three polypeptide chains, and all members of the collagen family form supra-molecular structures in the ECM- although their size, function and tissue distribution vary considerably. Collagen is the most abundant protein in the ECM(48) and so far 26 genetically distinct collagen types have been described.(49-56) Although the different collagen types are designated by Roman numerals in the approximate order in which they were discovered, collagens are classified based on their structure, supramolecular organization and function.

Thirteen collagen types are found in the vessel wall or are expressed by cells of the vessel wall in vitro, although none of these collagens are restricted to the vessel wall. In contrast to the large, parallel running, densely packed collagen fibrils of tendon or bone, vascular collagens fibrils are small, more widely spaced apart and form three dimensional webs or networks. These networks are soft, highly compliant matrix and therefore able to withstand multidirectional stress without permanent effects.(57-59)

Vascular collagens are divided into distinct groups based on their macromolecular structure – fibril forming collagens, non fibril forming collagens, (subdivided into

basement membrane collagens, fibril associated collagens, micro-fibrillar collagens and anchoring collagens) and network forming collagens.(60)

Fibril forming collagens, which are the most abundant collagen family, account for about 90% of all collagens. Collagen types I, II, III, V and XI make-up the fibril forming family, of this family types I, III and V have been identified in the aorta and are mainly responsible for tensile strength and elastic resilience(61-63) and lend stability and strength to the tissues in which they are present.(49;64;65) Type I and type III account for 80-90% of total aortic collagen and occur in the intimal, medial and adventitial layers. Both type I and type III collagens are characterised by the presence of a long, continuous triple helical structure with each chain comprising about 330 repeats of the amino acid sequence –glycine, proline and hydroxyproline. Type III collagen is co-localized with type I collagen in tissues such as the blood vessels and skin(66-68) and is essential for collagen I fibrillogenesis, demonstrated during experiments on mice lacking the Col3a1 gene, which codes for type III procollagen.(69) Electron microscopy of aortic tissue from the Col3a1$^{-/-}$ mice showed aortic medial collagen fibrils were missing and collagen fibrils in the adventitia were irregular in size. The modulation of collagen type I fibrils by collagen type III is supported by the fact that collagen fibrils formed by self-assembly *in vitro* are of uniform diameter,(70;71) but variable in different tissue or the same tissue at different stages of development, where the ratio of type III to type I collagen is different.(72-74)

The precise role and location of type V is uncertain(75) although it has been located in the sub-endothelial space and diffusely distributed throughout the media in atherogenesis.(76) Collagen type V has also been localised in various human arteries to the endothelial basement membrane and basement membranes of SMCs of the intima and media, and diffusely distributed through the intercellular space of the intima.(77) The elastic lamellae of human thoracic aortae were associated with collagen V fibres, along with collagen types I and III.(78)

Collagen type IV is a basement membrane collagen and is also present in the vessel wall, along with collagen XV, XVIII and XIX. Type IV collagen is involved the process of cell adhesion(79;80) and assembling into meshworks due to the more flexible triple helix.

Collagen type VI, a member of the beaded filament forming family is found in all layers of the aorta. This collagen is highly disulphide cross-linked and contributes to a network of beaded filaments interwoven with other collagen fibrils(81) and acts as a bridge between elastin and smooth muscle.(78)

Collagen type VIII, a network forming collagen, is present in the sub-endothelial space of arteries and is associated with the endothelial basement membrane and interacts with micro-fibrils associated with elastic fibres.(60) The 3-D network formed by type VIII collagen may stabilize basement membranes by creating a structure that is resistant to compression but has an open porous configuration.(82;83)

The function of fibril associated collagens (FACIT)- collagens XIV and type XVI in the vessel wall is poorly understood although these collagens appear to function primarily in modifying the properties of other collagens by associating with their surface. The collagens were designated FACIT collagen as they have shorter triple-helical segments interrupted by non-helical polypeptides (Fibril Associated Collagens with Interrupted Triple Helix).

Endothelial cells have been shown to express collagens type VII and XIII.(84;85) Other collagen types present in minor amounts are listed in table 1.1.

These different collagen types are characterised by considerable complexity and diversity in their structure and their function. The relative proportions of various collagen types within the tissue therefore has profound effects on subsequent tissue properties.

1.3.3 Collagen Turnover

The bulk of vascular collagen is produced by VSMCs, but may also be produced by endothelial cells, adventitial fibroblasts and macrophages.(86-88)

Fibrillar collagen is synthesized as pro-collagen containing an N-terminal and a C-terminal propeptide. Pro-collagen is then secreted into the extracellular space and the propeptides are removed by specific proteinases, allowing integration of the rigid collagen triple helix into the growing fibril.(89) The 100-kDa procollagen type C-terminal propeptide (PIP) is cleaved from procollagen type I during the synthesis of fibril forming collagen type I and is released into the bloodstream (Figure 1.4).

Figure 1.4

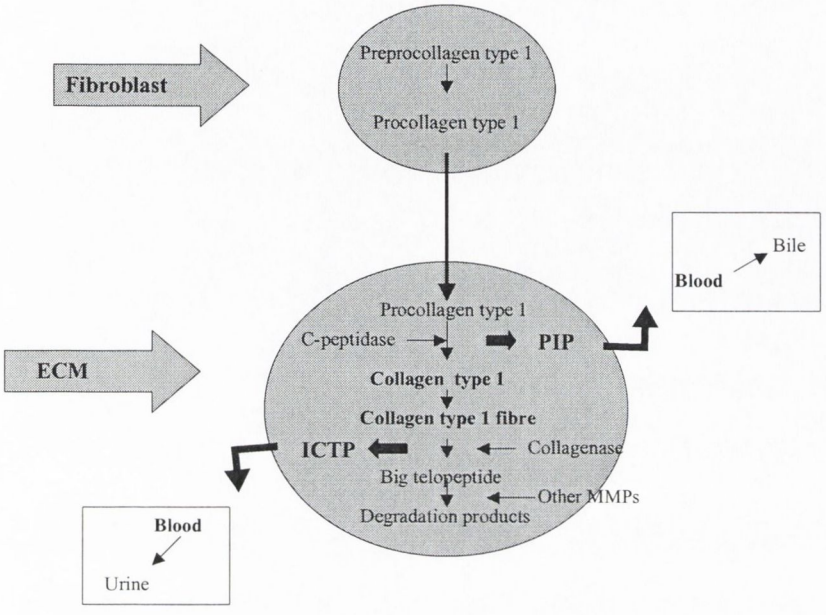


Figure 1.4: Diagrammatic depiction of the different compartments of fibrillar collagen turnover. The origin and destination of the serum markers of collagen type I synthesis (procollagen type I C-terminal propeptide or PIP) and collagen type I degradation (collagen type I pyridinoline cross-linked C-terminal telopeptide or ICTP) are indicated.

From; Biochemical assessment of myocardial fibrosis in hypertensive heart disease, Lopez B *et al* 2001.

Table 1.1 Classification and tissue distribution of collagen

Type	Subgroup	Molecular forms	Distribution
I	Fibrillar	$[\alpha1(I)]_2\alpha2(I)$ $[\alpha1(I)]_3$	Most tissues
II	Fibrillar	$[\alpha1(II)]_3$	Cartilage, cornea, vitreous humor, intervertebral disc.
III	Fibrillar	$[\alpha1(III)]_3$	Soft tissues, with type I collagen
IV	Network-forming	$[\alpha1(IV)]_2\alpha2(IV)$ $[\alpha1(IV)]_2\alpha4(IV)$ other types	Basement membranes
V	Fibrillar	$[\alpha(V)]_3$ $\alpha1(V)\alpha2(V)\alpha3(V)$ other forms	Minor amounts in most tissues with collagen type 1
VI	Beaded filament-forming	$\alpha1(VI)\alpha2(VI)\alpha3(VI)$	Minor amounts in most tissues
VII	Anchoring fibril-forming	$[\alpha1(VII)]_3$	Skin, cervix, oral mucosa
VIII	Network forming	$[\alpha1(VIII)]_2\alpha2(VIII)$	Many tissues
IX	FACIT	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	With collagen type II.
X	Network forming	$[\alpha1(X)]_3$	Hypertrophic cartilage
XI	Fibrillar	$\alpha(XI)\alpha2(XI)\alpha1(II)$	With collagen type II
XII	FACIT	$[\alpha1(XII)]_3$	Many tissues with type I collagen

XIII	Transmembrane domain	Unknown	Minor amounts in many tissue
XIV	FACIT	$[\alpha1(XIV)]_3$	Many tissues with collagen type I
XV	MULTIPLEXINS	unknown	Many tissues
XVI	FACIT	$[\alpha1(XVI)]_3$	Many tissues
XVII	Transmembrane	$[\alpha1(XVII)]_3$	Hemidesmosomes of stratified squamous epithelia
XVIII	MULTIPLEXINS	unknown	Liver, kidney, placenta etc,
XIX	FACIT	unknown	Several tissues.
XX	FACIT	α1(XX)	Corneal epithelium, tendon
XXI	FACIT	α1(XXI)	Heart, stomach, kidney, skeletal muscle.
XXII	FACIT	unknown	Cartilage
XXIII	Transmembrane	unknown	Metastic tumor cells
XXIV	Fibrillar	unknown	Bone, cornea
XXV	Transmembrane	unknown	Brain, neurons
XXVI	FACIT	unknown	Testes and ovaries

MULTIPLEXINS= proteins with multiple triple helix domains and interruptions

FACIT= fibril associated collagen with interrupted helices

Table constructed from references appearing in the text.

A stoichiometric ratio of 1:1 exists between the number of collagen type I molecules produced and that of PIP released. Circulating PIP is cleared from the blood by the liver.(90) Serum peptides derived from the tissue synthesis and degradation of fibrillar collagen can be measured to monitor collagen turnover. These markers of collagen synthesis and degradation in the serum have been used as markers of collagen turnover in hypertensive heart disease.(91;92) Observations have also shown that high serum levels of PIP, measured by radioimmunoassay reflect fibrosis in organs such as the liver and the lung.(93) The serum or plasma

concentration of PIP can therefore be used as a marker of collagen synthesis in conditions of preserved liver function.

The degradation of collagen type I fibrils occurs through catalytic cleavage by interstitial collagenase (Figure 1.4). Interstitial collagenase, also known as matrix metalloproteinase-1 (MMP-1) cleaves all 3 α-chains of collagen at a single specific locus. The resulting 36-kDa and 12 k-Da telopeptides maintain their helical structure and are resistant to further proteolytic degradation. The big telopeptide spontaneously denatures into non-helical gelatin derivatives, which in turn are completely degraded by the interstitial gelatinases or MMP-2 and MMP-9. The small 12-kDa pyridinoline cross-linked C-terminal telopeptide (ICTP) resulting from the cleavage of collagen type I, is found in an immunochemically intact form in the blood and a stoichiometric ratio of 1:1 exists between the number of collagen type I molecules degraded and that of ICTP released (Figure 1.4).(94) In clinical studies, the serum concentration of ICTP was found to be

related to the intensity of the degradation of collagen type I fibres.(93) Where renal function is preserved, the serum or plasma concentration of ICTP is a marker of collagen type-I degradation.

By the measurement of these circulating collagen metabolites, it has been demonstrated that the tissue synthesis of collagen type I and type III (PIP and PIIIP) are increased in hypertensive compared to normotensive subjects.(95) In the hypertensive subjects, serum PIP (a marker of collagen type I synthesis) was directly related to anatomical alterations of the left ventricle, and serum PIIIP (marker of collagen type III synthesis) levels were related to functional alterations of the left ventricle. As the increased PIP and PIIIP were normalized by treatment with an ACE inhibitor, it may be that the renin-angiotensin-aldosterone system is related to myocardial fibrosis in hypertension. In a subsequent study, the increased levels of serum PIP were also related to histological markers of myocardial fibrosis in hypertensive heart disease.(96)

A change in the proportions of collagen subtypes could potentially affect not only the mechanical properties of the myocardium, but also of the vessel wall, as collagen subtypes show considerable diversity in their structure and function. The predominant collagen subtypes found in the normal human arterial wall are type I (70% to 75%) and type III (20% to 25%), with type V comprising 1% to 2%.(97) Although there is disagreement regarding absolute collagen accumulation in vessel stiffening, studies in animal tissue have demonstrated alterations in the composition of the collagen subtypes in hypertensive rats with stiffer arteries. In genetically hypertensive rats, large artery wall stiffness was

affected not only by hypertension itself, but also by a higher density of collagen type III, but not type I fibres.(98) In addition, a study which examined changes in collagen subtypes in SHRs, found decreased amounts of collagen type I, accompanied by an increase in collagen type V.(99) The alteration in the composition of collagen subtypes was accompanied by increased aortic stiffness. Despite this, a recent study has shown that markers of collagen type I synthesis (PIP) in hypertensive subjects are related to increased vascular stiffness, and both vessel stiffness and PIP levels were reduced by ACE inhibition.(100)

1.3.4 Collagen Changes and Ageing

Arterial changes, associated solely with the ageing process, distinct from pathological causes such as hypertension is the subject of increasing interest.(101) Ageing is associated with adverse haemodynamic and metabolic alterations, which can accelerate vascular degenerative changes in both men and women.(102) Ageing is associated with an increase in LDL cholesterol and a reduction in HDL cholesterol,(103) an increase in SBP and PP.(104) This increase in SBP is known to be primarily caused by stiffening of the large arteries,(105;106) which is associated with the ageing process.(107) The age–related changes in stiffness and elasticity have been ascribed to changes in collagen and elastin concentrations in the aorta.(32;108;109) During maturation and ageing, collagen fibres become increasingly insoluble and more refractory to the actions of enzymes. They also show progressive increases in tensile strength.

These intrinsic alterations are brought about by the formation of intermolecular cross-links and changes in collagen concentration and amount.

Studies regarding age-related collagen changes in the vasculature have been carried out and the results are often directly conflicting. There are disagreements within the results of animal studies, human studies and comparing the two to each other.

Several studies examining changes in the rat aorta have found that the collagen concentration increases until a certain age and then reaches a plateau.(109-111) However a recent study carried out examining rat aorta found that the age-associated increase in vessel stiffness was accompanied by an overall increase in the vessel diameter resulting in a decreased collagen concentration (collagen per mm^2 aortic wall).(112) There was no change in the absolute amount of collagen associated with increasing age but there was a decrease in the amount of type I collagen relative to type III collagen with increasing age. The authors suggest this decreased collagen concentration may be due to an increase in components such as glycoproteins and proteoglycans. In cultured rabbit arterial SMCs, the synthesis of both collagen types I and III decreased with age, although type I declined at a greater rate than type III, resulting in increased synthesis of collagen type III.(113)

Studies carried out on collagen changes in normotensive human thoracic aorta have published opposing results. *Cattell et al* (114)found a highly significant negative correlation between the amount of collagen (mg per 1cm diameter full wall thickness) per aorta sample and age (years) ($p<0.0001$). The amount of

collagen per sample fell from 53 mg at 14 years to 10.5 mg for a 90-year-old, a decrease of 80%. However, the collagen concentration (mg/mg dry weight) in the thoracic aortic wall samples significantly increased with age ($p<0.0002$), the concentration at 92 years being 72% above that of the aorta of a 14 year-old. It is suggested that because the amount of collagen is decreasing with age, the increased concentration may be due to the loss of other components in the arterial wall. Another study also carried out on human thoracic aorta (115) has found that ageing was associated with an increase in collagen amount (expressing results as mg/cm^2 -comparable to mg/1cm diameter of full wall thickness or $mg/0.785cm^2$ as in *Cattell et al*), but results did not display the same levels of significance as the more recent study.

The correlation between decreased collagen amount and increasing age was most significant in the ascending aorta, relative to the descending aorta. In addition, the correlation between collagen concentration in the aorta and ageing became more significant in regions of the aorta further from the heart.(114)

1.3.5 Collagen Changes and Vascular Disease

The structure and mechanical properties of arteries are altered in hypertension.(116-119) The compliance and distensibility of the elastic arteries are reduced in established(120) as well as borderline hypertension(121) and increased aortic stiffness, measured by carotid-femoral PWV is increased in essential hypertension.(122) It has been reported that the main structural change in the arterial wall during hypertension relates to increased collagen content.

Collagen synthesis in spontaneously hypertensive rats (SHRs) has been shown to exceed control levels in a prehypertensive period, decrease during the development of hypertension and increased again in the established hypertensive state.(123) In another study involving SHRs, total aortic collagen was found to be significantly higher than in placebo Wistar-Kyoto (WKY) rats. The aortic accumulation in the SHRs was prevented by angiotensin II inhibition through AT_1 receptors.(124) Another study found no difference in aortic collagen content between SHRs and WKYs.(125) Changes in the total and proportions of various collagen types have been investigated in hypertensive rats.(99) Collagen concentration was decreased by 16% in both 6-week and 20-week old SHR compared to WKY rats, although collagen synthesis was around two-fold higher in the hypertensive rats, suggesting increased collagen degradation in the hypertensive rats. Although collagen type V represents a small proportion of the collagen in the WKY rats (5%), it was two-fold higher in the SHRs. This increase was accompanied by a reduction in the proportion of type I collagen, and no change in the proportion of type III. These collagen changes in the hypertensive rat were accompanied by an increased aortic stiffness. Induced hypertension and hyperlipidemia in rabbits displayed up-regulation of collagen types I and III with a differential distribution across the arterial wall. Collagen type I was mainly distributed in the intima, the outer media and the adventitia. Collagen type III was spread more uniformly across the wall, including the adventitia.(126)

Collagen changes in the hypertensive heart also occur. Myocardial fibrosis is known to occur in humans with left ventricular hypertrophy associated with hypertension.(127) The myocardial fibrosis results in an increase in the interstitial and perivascular content of fibrillar collagen types I and III.(128)

Collagen changes occur in atherosclerosis. Increased staining intensity for collagens type IV, V and VI was found in vessels that had more severe atherosclerotic lesions (Table 1.2).(129) Increased accumulation of collagen types I and III has also been reported in atherosclerotic lesions (Table 1.2).(130) Experimentally induced atherosclerosis in sheep displayed reduced staining for collagen type IV as the lesion progressed, and was localised in the basement membrane in control tissue.(131) Collagen type VI exhibited a diffuse immunostaining throughout the vessel wall in the atherosclerotic sheep, with more marked concentrations around the SMCs.(131) Type VIII collagen is present in the atherosclerotic media and intima(60) and in hypercholesterolemic rabbits, the *in situ* distribution patterns of collagen type VIII in the carotid artery, implies a role for this collagen type in neointimal growth in early phases of atherogenesis.(132)

The collagen content of atherosclerotic arteries of smokers is reported to be higher than the atherosclerotic arteries of non-smokers.(133)

Table 1.2 Collagens of the normal vessel wall and expression in atherosclerotic lesions

Morphological Structure	Collagen Type	Atherosclerotic Plaque
Fibril	I	↑
	III	↑
	V	↑
Fibril associated	XIV	
	XVI	
Microfibril	VI	↑
Basement membrane	IV	↑
Associated	XV	
	XVIII	
	XIX	
Membrane bound	XIII	
Anchoring	VII	
Short chain, network forming	VIII	↑

↑, accumulated in atherosclerotic lesions

From: Vascular collagens: spotlight on the role of type VIII collagen in atherogenesis, Plenz GAM et al 2003. (60)

1.3.6 Elastin

The elastic properties of the aorta depend largely on the presence of elastin in the wall which enable it to act as a 'windkessel'- after aortic distension caused by blood flowing from the heart. Elastin allows recoil during diastole without the use of energy(25) and keeps the blood flow in the vascular system relatively constant.(134)

Elastic fibres mainly consist of a core of elastin and a surrounding lattice of microfibrils.(135) Relatively little is known about the intracellular steps involved in the biosynthesis of elastin. It is synthesised as a soluble precursor molecule called tropoelastin which has a relative molecular mass of 72 kDa. Tropoelastin consists mainly of beta-turns which become helically ordered by hydrophobic folding into beta-spirals. These sequences play an important role in the hydrophobic elasticity of elastin. Alpha-helical sequences which are interspersed among beta-turns are enriched in alanine and lysine residues and are involved in the cross-linking process.(136-138) In its mature form, insoluble elastin is a polymer of tropoelastin chains randomly cross-linked into a highly extensible, three dimensional network. Elastin may be deposited as elastic fibres or may alternatively be deposited as laminae or sheets.

Microfibrils, which along with elastin make up elastic fibres are found in tissues that contain elastin and those that do not. The major microfibrillar components- fibrillin 1 and fibrillin 2,(139;140) consist of repeated globular domains connected by thin fibrillar domains.

Elastic fibres and elastic sheets are present in the blood vessel wall. Elastic fibres are present in the adventitia while elastic lamellae are present in the media. The lamellae are fenestrated and interconnected by branches of elastin so that a three-dimensional meshwork is present, which is able to transfer stress uniformly throughout the vessel wall (Figure 1.3).(141)

Elastic lamellae also occur as solitary structures in the form of the IEL and the EEL. In elastic arteries, such as the aorta, medial elastin is arranged in multiple concentric lamellae, interspersed with smooth muscle and collagen, along with a complex meshwork of microfibrils throughout the wall. The elastic fibres are responsible for dilation and recoil, while the microfibrils are flexible links allowing elastic arteries to function effectively. The number of elastic lamellae is greatest in the proximal part of the aorta. In the thoracic aorta, elastin accounts for about 40-50% of the dry weight of the tissue, and is a major component in the abdominal aorta and carotid arteries.

1.3.7 Elastin Turnover

Organization of a functional elastin fibre involves interactions between tropoelastin, secreted by SMCs and glycoprotein microfibrils. The process is not well understood but has been shown to be mediated at the cell surface by the elastin receptor(142) and seems to be dependent on the presence the C-terminal domain of the molecule.(143) The elastin receptor is a 67 kDa multifunctional protein that contains a binding site that recognizes a hydrophobic binding sequence in elastin and a carbohydrate binding site that associates with

galactoside sugars. Tropoelastin binds with high affinity to the receptor at the protein binding site.(144) The receptor which is often referred to as the elastin-laminin receptor, serves as a molecular chaperone facilitating intracellular transport and extracellular assembly.(142) It is expressed on fibroblasts, SMCs, chondroblasts, leukocytes and certain cancer cell types. Following deposition on a pre-existing microfibrillar scaffold, cross-links are synthesised by the lysyl oxidase enzyme(135) – namely lysinonorleucine, desmosine and isodesmosine.(145) After cross-linking, elastin becomes insoluble and elastic. Experiments in animals have shown that the synthesis of elastin in the medial layer of developing arteries is influenced by local haemodynamic conditions. At birth in rabbits, when pulmonary and systemic pressures are equal, the ratio of elastin to collagen in the pulmonary artery and the aorta are the same. At the age of two months, the pulmonary pressure has decreased from 40 mmHg to 15 mmHg and the systemic pressure has increased to 80 mmHg, the ratio of elastin to collagen in the aorta is nearly twice that of the pulmonary artery.(146)

Mature cross-linked elastin is degraded very slowly by elastases. It has been reported that the turnover of arterial elastin is very slow, or does not happen at all under normal physiological situations.(147)

The degradation of elastin occurs through the action of elastases, which mainly belong to the MMP family, together with cysteinyl and serine proteinases.(148) It has been shown that around 80%-90% of serum elastase activity is due to matrix metalloproteinases.(149) Of the MMP family, MMP-9/ 92-kD gelatinase

has the greatest affinity for aortic elastic fibres(150) although other MMPs can also degrade elastin, with varying affinity.

1.3.8 Elastin Changes and Ageing

By middle age, the human aorta has undergone around two billion cycles of expansion and contraction. This fatiguing effect of cyclic stress leads to the fracture of elastin fibres and transfer of stress to collagen fibres.(151) This process can be visualised microscopically as fragmentation and loss of regularity in elastic layers of the medial layer. The gradual loss of elastin is accompanied by a reduction in vascular compliance.

It has been shown that various defects in the IEL, including fragmentations and interruptions occur with age in different human arteries. These defects are more pronounced in males than in females and may be related to the subsequent development of intimal thickening, arteriosclerosis and atherosclerosis.(152-154) Study of the elastic fibre morphology of the human aortic arch and lower abdominal aorta demonstrated a loss of elastic tissue with ageing, most notably in the lower abdominal aorta.(155)

Increased vessel stiffness due to ageing in rats was shown to be related to increased fenestration of the elastic lamina. Although the total amount of elastin was unchanged, an increase in diameter in the aortic wall, which led to an overall decreased concentration of elastin, accompanied increased vessel stiffness.(112)

A study carried out using normotensive human thoracic aorta found that the amount of elastin in the aorta fell from 26.8mg/sample to 10.2mg/sample in a 90-

year-old, a decrease of 62%. Concentration of elastin as opposed to amount in the thoracic aorta showed an inverse pattern, with a marked increase in the concentration of elastin with age. The mean values in the study demonstrated the elastin concentration for a 14-year old is 0.091mg/mg dry weight, and this rises to 0.22mg/mg by the age of 90, an increase of 140%.(114)

1.3.9 Elastin Changes and Vascular Disease

Rupture and fragmentation of the IEL in arteries are common features of patients with heritable connective tissue diseases.(156;157) Supravalvular aortic stenosis (SVAS) is a congenital vascular disease causing the narrowing of large arteries.(158) The condition can lead to hypertension, cerebrovascular accident and heart failure. Vascular lesions in affected arteries show disorganised, thickened elastic fibres, excessive hypertrophied SMCs and extensive deposition of collagen. A deficiency in the elastin gene and therefore a quantitative deficiency in elastin can lead to SVAS.(158;159) It may be that the elastin deficiency makes the elastic fibres more susceptible to haemodynamic stress and damage or that the elastic fibre assembly may be influenced by the ratio of elastin to other elastic fibre components.

Mutations in the fibrillin-1 gene, and sometimes the gene coding for elastin itself give rise to Marfan syndrome (MFS),(160) a connective tissue disorder of which the main manifestation is dilation of the aortic root. Vascular tissues of MFS patients display disorganised and fragmented elastic fibres.(161) Studies carried out using mice with mutations in the fibrillin-1 gene(162) have implicated

fibrillin-1 in maintaining homeostasis of elastic fibres and it seems that a decrease in quality and quantity of the microfibrils below a critical threshold leads to dissecting aneurysm.

Elastin gene studies involving mice lacking elastin have also highlighted the role for elastin in arterial development.(163) Late arterial development was affected as the arterial diameter declined and the lumen eventually obliterated. These changes were a result of sub-endothelial accumulation of SMCs, a process involving cell proliferation, migration and reorganisation, a process therefore determined by elastin.

In normotensive Brown Norway (rats, which are particularly susceptible to rupture of the IEL), angiotensin-converting enzyme (ACE) inhibitors protect against rupture of the IEL, suggesting a role for the renin-angiotensin system in the IEL rupture.(164) Angiotensin II, which induces hypertension, has been shown to be related to elastin gene expression in the affected arteries.(165) Sustained hypertension, which is associated with increased vessel stiffness, is associated with the destruction and the reconstruction of elastin fibres.(166) A deficiency in the elastin gene has been suggested as a cause for essential hypertension.(167) Mice with an elastin insufficiency ($Eln^{+/-}$) had a decreased elastin to collagen ratio compared to their wild-type counterparts. These mice were hypertensive from birth with stiffer arteries, displaying only moderate cardiac hypertrophy and lived a normal lifespan.

In a carotid artery balloon injury rat model, expression of potent elastolytic cysteine proteases is increased.(168) Expression of two of these elastolytic

enzymes- cathepsin S and K, was increased and preferentially located to the developing neointima, suggesting an association with pathological intimal formation.

A drug that contains porcine pancreatic elastase 1 (PPE1) and therefore causes elastin degradation has been used since 1981 for the prevention of arterial ageing and atherosclerosis in elderly patients in Japan. The age-related increases in PWV were found to be lower, as was the atherosclerotic index of the carotid artery, in those who take PPE1 than in controls.(169) These results are somewhat paradoxical to the opinion of the pathogenic role of elastin-degrading enzymes.

1.4.1 THE MATRIX METALLOPROTEINASES

The MMPs are a family of calcium and zinc dependent enzymes that can collectively degrade the entire ECM. Although many proteases can cleave the ECM molecules, the MMPs are believed to be the normal, physiologically relevant mediators of matrix degradation. MMPs are therefore involved in many normal processes such as embryogenesis, ovulation, wound healing, pregnancy and morphological growth changes that require breakdown and remodelling of the ECM.

1.4.2 Classification of MMPs

At present 26 human MMPs have been identified all of which have a high degree of similarity in their domain structures. The nomenclature for the MMPs is confusing as many of the enzymes have been given different names by different groups. Furthermore, MMPs may be named based on their substrate specificity, their molecular weight or by Arabic numeral. MMPs have been divided into four groups based on substrate specificity- the collagenases, the gelatinases, the stromelysins and membrane-type metalloproteinases (MT-MMPs). It is however important to remember that many of MMPs degrade components of the ECM with varying affinity, and can often degrade components other than those suggested by the substrate grouping. In fact the distinctions are becoming less clear as more becomes known about them (Table 1.3).

The collagenases comprise interstitial collagenase-1 (MMP-1), neutrophil collagenase (MMP-8), and collagenase-3 (MMP-13). Both MMP-1 and MMP-8 are commonly thought to be the only MMPs capable of cleaving native fibrillar collagens. They have the distinctive ability to cleave alpha chains of type I, II and III at a single site, producing fragments approximately ¾ and ¼ the size of the original molecule.(170-173) MMP-13 has been identified more recently and appears to be critical in bone metabolism and homeostasis.(174)

The second member of the substrate subclass is the gelatinases, being made up of gelatinase A (MMP-2) and gelatinase B (MMP-9). These enzymes have substrate specificity for denatured collagens or gelatins(170;175)- the fragments produced due to collagen degradation by MMP-1. MMP-2 and –9 can also degrade

elastin.(150;176) It is also generally thought that the gelatinases have substrate specificity for intact collagen type IV basement membrane although some reports suggest that type IV collagen is a poor substrate for these enzymes.(177;178) Although it is widely reported that only the collagenase subgroup are capable of degrading fibrillar collagen, MMP-2 has been shown to cleave fibrillar collagen and generate the collagen fragments characteristic of interstitial collagenases.(179) As MMP-2 (and MMP-9) are known to degrade these fragments, this essentially means that MMP-2 may perform both the actions of both an interstitial collagenase and a gelatinase.The stromelysins, the third subclass of the MMP family, have broad substrate specificity. The subclass comprises stromelysin-1, -2 and –3 or MMP-3, MMP-10 and MMP-11 respectively. MMP-3 has a strong homology to MMP-10 and both enzymes have the ability to degrade collagens type IV, V, IX and X, elastin, gelatin, laminins, fibronectin and proteoglycan proteins. In addition, MMP-3 activates other MMPs including MMP-1 and MMP-9(180;181). Matrilysin or MMP-7 also belongs to the stromelysin subgroup and has similar substrate affinity as MMP-2.

The membrane type or MT-MMPs are the most recent subgroup to be added to the MMP family. These MMPs are involved in the activation of other MMPs.

Table 1.3 Principle members of the MMP family.

MMP	Name	Latent/active MW	Substrates
MMP-1	Interstitial Collagenase-1	55 / 45,000	Collagens type I, II, III*, gelatins, proteoglycans
MMP-8	Neutrophil collagenase	75 / 58,000	As above.
MMP-13	Collagenase-3	60 / 48,000	Collagens, gelatin, aggrecan.
MMP-3	Stromelysin-1	57 / 45,000	Collagens IV, V, IX, X, elastin, gelatin casein etc.
MMP-10	Stromelysin-2	57 / 45,000	As above. Proteoglycans*, gelatin, elastin
MMP-7	Matrilysin	28 / 19,000	etc.
MMP-2	72 kDa gelatinase 92 kDa gelatinase	72 / 66,000	Gelatin, elastin
MMP-9	MT-MMP-1	92 / 86,000	Gelatin, elastin
MMP-14	MT-MMP-2	66 / 56,000	Collagens, casein, fibronectin
MMP-15	Macrophage metalloelastase	72/60,000	Gelatin, fibronectin Collagen, gelatin, elastin.
MMP-12		54 / 45,000	

Latent/active MW = latent or active molecular weight. MT-MMP= membrane type MMP. Components marked with an asterix (*) are exceptionally good substrates. Table modified from matrix metalloproteinases- www.biotrak.apbiotech.com

1.4.3 Regulation of MMPs

The regulation of the MMPs is complex and occurs at three different levels. The first is transcriptional or gene regulation. The synthesis of MMPs may be induced or stimulated by a number of cytokines, growth factors and chemical agents. These include platelet-derived growth factor (PDGF), interleukin-1 (IL-1) and tumour necrosis factor-α (TNF- α). Others such as heparin, corticosteriods and transforming growth factor-β (TGF- β) have an inhibitory effect.

The second level of control of the MMPs is through activation of the latent pro-enzymes. MMPs are secreted in an inactive or zymogen state and require activation before exerting their proteolytic activity. Plasmin is a potent activator of most MMPs (although plasmin-independent pathways do exist).(178;182;183) When plasmin cleaves and therefore activates stromelysin, the active form of this enzyme can activate other enzymes forming a positive-feedback loop. The cleavage of collagenases by stromelysin results in a fivefold to eightfold increase in proteolytic activity. Non-proteolytic agents such as mercurial compounds and reactive oxygen can also activate secreted proMMPs to their active state.(184) It is thought that the activation of most MMPs by proteolytic and non-proteolytic agents occurs via the disruption of the Cys-Zn^{2+} (cysteine switch), with the removal of the propeptide occurring in a stepwise manner.(185) While this activation occurs in the ECM for most MMPs, the activation of latent MMP-2 occurs on the cell surface, where the major proMMP-2 pathway is through MT-MMP.(186)

The third level of MMP control occurs through the inhibition of proteolytic activity by a naturally occurring family of specific inhibitors. These are known as the tissue inhibitors of metalloproteinases (TIMPs), four of which have been purified and characterised.(187) TIMPs are secreted (but may also be found at the cell surface) multifunctional proteins that are essential in the regulation of ECM breakdown. All four TIMPs inhibit active forms of the MMPs although with varying affinities, by binding with the catalytic site of the enzymes.(188;189) Although TIMP-1 acts against all members of the collagenase, stromelysin and gelatinase subgroups of the enzyme, it is a poor inhibitor of MMP-19 and a number of MT-MMPs. It forms high affinity, irreversible (*in vivo*) non-covalent complexes with the active forms of the enzyme and is highly expressed in actively resorbing tissue. TIMP-2 acts to directly inhibit MMP-2 activation(190) while on the other hand, TIMP-2 present at low concentrations, bound to MT-MMP is required for MMP-2 activation.(191) Activated MMP-9 is also preferentially inhibited by TIMP-2, although it exists in complex with TIMP-1, which slows activation of the latent pro-form.(188;192) In addition to regulating MMPs, TIMPs have effects on cell growth and survival independent of MMP inhibitory activity. TIMP-1 has been shown to stimulate the growth of human keratinocytes(193) while TIMP-2 stimulates the growth of lymphoma cells.(194) It has also been demonstrated that TIMP-2 stimulates the proliferation of fibrosarcoma cells(195) and dermal fibroblasts(196) and may therefore be related to vascular cell proliferation and vascular matrix production.

1.4.4 MMPs and the Vessel Wall

MMPs play a fundamental role in physiological and pathological vascular matrix degradation and collectively are capable of degrading the entire ECM. Under normal conditions the metabolic turnover of mature collagen and elastin is relatively slow and only small amounts of these proteins are degraded. Collagenolytic and elastolytic enzymes are mainly MMPs (although serine, aspartic and cysteine proteinases are also involved in elastin degradation).(197;198)

Vascular collagenases include MMP-1 (responsible for the degradation of native collagens type I, II, and III), which is expressed by endothelial cells, while MMP-8 is stored in polymorphonuclearneutrophils (PMN).(199;200) The gelatinases, MMP-2 and –9 are expressed in macrophages, PMN and vascular endothelial and SMCs.(199;201) The vascular elastases include MMP-7, expressed at a low level in the vascular wall and MMP-12, which is synthesized during the differentiation of monocytes into macrophages. Stromelysins and MT-MMPs are also present in the vessel wall (Table 1.4).

Table 1.4 MMPs in the vessel wall

MMPs produced by vascular cells	MMPs produced by inflammatory cells	Inhibitors
Collagenases: MMP-1	Collagenases: MMP-1, MMP-8, MMP-13	}TIMPs, α2-macroglobulin
Gelatinases: MMP-2, MMP-9	Gelatinases: MMP-2, MMP-9	
Elastases: MMP-7	Elastases: MMP-7, MMP-12	
Stromelysins: MMP-3	Stromelysins: MMP-3	
MT-MMPs: MMP-14		
Other proteinases		
Serine proteinases: SMC elastase	Serine proteinases: Leukocyte elastase, cathepsin G	α1-Antitrypsin, α2-macroglobulin, elafin
Cysteine proteases: Cathepsin S	Cysteine proteases: Cathepsin S, Cathepsin K.	Cystatin

Modified from: Extracellular matrix remodelling and matrix metalloproteinases in the vascular wall during aging and in pathological conditions. Jacob P.J, 2003.

1.4.5 MMP Activity and Ageing

As MMP-2 and –9 degrade elastin(150;176) and non-fibrillar collagens such as those found in basement membranes,(177;178;202) these enzymes are likely candidates of mediators of vascular extracellular remodelling in health and disease. Therefore, the expression and activity of MMP-2 has been investigated in rats in relation to ageing.(203) A strain of rat was used, in which isolated systolic arterial pressure develops with ageing, as in humans. It was demonstrated that the aortic intima of young rats consisted only of a single layer of endothelial cells. The aortic intima of old rats exhibited a five-fold increase in width by comparison and was mainly composed of matrix molecules – collagen, proteoglycans, and SMCs. The intima also contained markedly higher levels of MMP-2. The study demonstrated increased levels of both the inactive and active forms of MMP-2 in the aortae of older rats, although the active levels of MMP-2 seemed to localize to the intima and elastic lamellae.

Within the aged aorta there was also evidence of discontinuities of the IEL. In addition, it was demonstrated that rat VSMCs are a potential source of the age-related increase in aortic MMP-2, as the VSMCs from the old aortae secrete more MMP-2 than VSMCs from young aortae. Increased MMP-2 activation has also been demonstrated in the thickened aortic intima of the older non-human primates (204). The authors speculated that the intimal MMP-2 is likely to result from secretions from endothelial and VSMCs, as macrophage cells could not be detected. The aortic intima of the old animals also displayed increased staining

for angiotensin II, a potent vasoconstrictor and also activator of MMP-2.(205) Angiotensin II has previously been linked with age-associated vascular remodelling, as long term ACE inhibition prevents or delays age-related vascular remodelling and endothelial dysfunction.(164;206)

In addition to the relationship between MMP activity and ageing, alterations in MMP and TIMP activity has also been explored in relation to gender and the effects of exercise (207). TIMP-1 and TIMP-2 were significantly altered after exercise but not MMP-2 and MMP-9. Both MMP-2 and TIMP-1 and TIMP-2 were not related to gender or ethnic origin, although MMP-9 was significantly lower in subjects of Far Eastern/Chinese origin. (207)

1.4.6 MMPs and Cardiovascular Disease

The fundamental role played by MMPs in maintaining vessel wall integrity is reflected in the fact that MMP activity is altered in virtually all cardiovascular disease. Inappropriate vascular remodelling is thought to be the cause of most prevalent vascular pathologies. MMPs therefore play a crucial role in vascular diseases including hypertension,(208-210) aortic aneurysms(211;212) and atherosclerotic remodelling.(213-215)

Adaptive restructuring of the vessel wall in hypertension leads to changes in collagen and elastin concentrations and composition to allow the vessel to withstand increases in pressure. The remodelling process is however ultimately detrimental, as the restructured ECM is never fully optimal. This reorganisation occurs through altered protein synthesis and degradation and is thought to involve MMP-9 in early hypertensive remodelling.(208) It has been shown that MMP-9

(which was activated when vessels were placed under high pressure) is critical for initial vessel adaptation to increases in pressure. As circulating MMP-9 levels have been reported as lower in hypertensive patients compared with normotensive subjects,(216;217) the authors speculated that increased MMP-9 and vessel distensibility are hallmarks of early hypertension as vessels adapt to the increase in pressure. Mice with a deficiency for the MMP-9 gene were used to investigate the role of MMP-9 in vessel remodelling through ligation of the carotid artery to induce remodelling.(218) The MMP-9 deficient mice showed a significant accumulation of interstitial collagen compared to their wild-type counterparts. These results suggest that although MMP-9 is known to degrade short collagens and elastin, it may also participate directly or indirectly to the metabolism of interstitial collagen *in vivo*. Circulating MMP-2 levels have also been reported as depressed in hypertensive patients(216) although more recently both MMP-2 and MMP-9 levels have been reported as increased in patients with isolated systolic hypertension compared to normotensive controls, with MMP-9 being related to increased arterial stiffness.(219) It has also been reported that increased MMP-2 and MMP-9 activity contribute to renal fibrosis in hypertensive rats.(209) Circulating levels of TIMP-1, known to inhibit MMP-2 and particularly MMP-9 have also been investigated in patients with essential hypertension and are reported as both depressed(217) and elevated(220) in hypertensive versus normotensive subjects.

MMP-2 also seems to play an important role in the transition from left ventricular hypertrophy to heart failure in hypertensive rats.(221) It is suggested that the

increased MMP-2 activation may be the determining factor for left ventricular enlargement and dysfunction. This may occur through degradation or damage of the fibrillar collagen network that connects myocytes. Increased concentrations of MMP-3 and MMP-9 have been reported in patients with LVH, the levels of which also correlated with left posterior wall thickness, suggesting these enzymes contribute to left ventricular diastolic dysfunction (222). In addition, plasma TIMP-1 levels have been shown to correlate with markers of LV diastolic filling, and are predictive of LV dysfunction (223). Another study has demonstrated that in an animal model of heart failure, MMP-2 and –9 are significantly increased, undenatured collagen is reduced and total collagen increased,(224) while MMP-9 and MMP-3 activity are reported as increased in the failing human heart.(225;226) It is proposed that the increased levels of these enzymes in the myocardium may contribute to ventricular dilatation and wall thinning. It is also reported that heart failure patients have increased circulating levels of MMP-2 and MMP-9.(227)

Human abdominal aortic aneurysms (AAA) are characterised by progressive dilatation of the aortic wall.(228;229) The ECM remodelling in AAA is characterised by structural disorganisation and disappearance of the elastic lamellae and the main MMPs detected in aneurysmal aortas have elastolytic qualities. It has been demonstrated that the principal MMP in small aneurysms is MMP-2, while it is suggested that MMP-9 is involved in aneurysm expansion at a later stage.(230) It has also been shown that cultured VSMCs derived from aneurysmal aorta produce three-fold higher levels of MMP-2 than from age-

standardised atherosclerotic tissue.(231) Elevated MMP-2 levels are also present in the vasculature of patients with AAA distinct from the aorta due to increased MMP-2 expression from VSMCs, illustrating the systemic nature of the disease.(212) Using a rat model of aneurysm associated with arterial dilatation, TIMP-1 levels were over-expressed by seeding syngeneic rat SMCs transfected with TIMP-1 onto the luminal surface of the vessels.(232) This led to decreased MMP-2 and MMP-9 activity, preserved elastin in the media and prevented aneurysmal rupture. MMP-2, MMP-9, MMP-3 and MMP-12 genes have also been analysed for possible associations with coronary aneurysms (CA). The MMP-9 and MMP-12 were not associated with the occurrence of CA, while a polymorphism of MMP-2 tended to be more frequent in CA cases than controls, although this was not statistically significant. However the MMP-3 5A allele genotype was an independent predictor of CA. Because the 5A allele is associated with high promoter activity,(233) it may be that increased proteolysis in the vessel wall may act as a susceptibility factor for the development of CA. The first process in the formation of an atherosclerotic plaque is the adherence of circulating monocytes to the vascular endothelium, through which they gain access to the sub-intimal tissue. A series of complex cell to cell interactions then take place involving the secretion of growth factors and cytokines such as TNF-α, IL-1 and PDGF.(234) These cytokines and growth factors are known to stimulate MMP synthesis in human aortic VSMCs and vascular endothelial cells.(202;235;236) Such changes in MMP expression lead to the migration and proliferation of cells and the deposition of ECM components in the same manner

as intimal formation with ageing. In a model of hamster atherosclerosis, activated MMP-1, -2, -3 and –9 was observed in endothelial cells, medial VSMCs, foam cells and within the adventitia with the exception of MMP-1. The content and activity of these MMPs increased as the atherosclerotic lesions progressed. Although MMP-2, -3 and –9 was also detected in control aortic tissue, these enzymes were not activated. In addition to increased MMP activity throughout the vessel wall, MMPs play a fundamental role in plaque destabilisation in atherosclerosis. Advanced atherosclerosis is characterised not just by a thickened intima but also a lesion that encroaches into the lumen. The ECM in atherosclerotic lesions consists largely of proteoglycan with loosely scattered collagen fibrils.(237) The cells found in these lesions are predominantly macrophages and VSMCs, although foam cells (macrophages with high lipid content) are also common. The stability of the fibrous cap of the lesion (rupture of which triggers most of the clinical manifestations of atherosclerosis such as myocardial infarction) depends primarily upon the content of interstitial collagen type I. Over-expression of MMPs promotes destabilisation of plaques in regions prone to rupture.(214;215;238;239) This is due to the breakdown of collagen and thinning of the fibrous cap rendering these enzymes an important determinant of the vulnerability of atherosclerosis.

There is accumulating evidence that MMPs also play an important role in angiographic restenosis (renarrowing of the vessel) that occurs after coronary angioplasty and coronary artery bypass grafting (CABG). The determinants of

restenosis include constrictive remodelling and neointimal formation, processes characterised by altered ECM turnover.

Intimal hyperplasia causes stenoses in the saphenous vein, which is commonly used as a bypass during CABG. The proliferation and migration of VSMCs, a process requiring the degradation of basement membrane, play a key role in the development of intimal hyperplasia, along with excessive degradation and accumulation of ECM. Neointimal formation in these veins is accompanied by increased MMP-9 production.(240) In addition, when sections of intact saphenous vein were cultured with a therapeutic concentration of doxycycline (a potent MMP inhibitor) neointimal thickness and MMP-9 production was significantly reduced. It has also been shown that intimal formation in organ-cultured human saphenous vein segments is inhibited by simvastatin, an effect that is associated with reduced MMP-9 activity.(241) Surgical preparation in saphenous veins also increased proMMP-2, active MMP-2 and proMMP-9 secretion, with increased expression of MMP-9 in highly proliferative neointimal SMCs.(242)

Increased MMP-2 expression has been demonstrated in the saphenous vein versus the internal mammary artery (IMA), two vessels used as grafting conduits during CABG.(243) This increased expression may, in part, contribute to pathological remodelling in the saphenous vein, compared to the IMA following CABG.

In human restenotic carotid arteries following carotid endarterectomy, early restenotic lesions contained abundant type I procollagen mRNA compared to normal arteries and some immunoreactive MMP-1. In late restenotic lesions

abundant type I procollagen mRNA is also present along with increased immunoreactive MMP-1 and TIMP-1.(244)

In addition, altered MMP/TIMP ratios have been demonstrated during Gestational Hypertension (GH). The MMP/TIMP ratio was lower in subjects with GH compared to that in normal pregnancy. It is suggested that the normally high MMP/TIMP ratio in pregnancy may be required for the development and maintenance of placental arteries, and therefore that the altered MMP/TIMP ratio displayed may be central to the pathophysiology of GH. (245)

1.4.7 MMPs and Arterial Stiffening

It has recently been reported that the MMP-9 genotype influences large artery stiffness. MMP-9 genotype was identified in the aortic tissue of patients following measurement of aortic stiffness. Carriers of the T-allele (T/T as opposed to C/T), which is associated with greater MMP-9 mRNA and protein levels, had stiffer arteries (246). So although ageing results in stiffer arteries, it is likely that arteries will become stiffer in certain individuals compared to others irrespective of age.

1.4.8 MMPs as a Therapeutic Target

The potential for using MMP inhibitors as therapeutic targets to address the imbalance between the MMP and their inhibitors in various disease states such as cancer, cardiovascular disease and arthritis has led to intensive research.

Early attempts at MMP inhibition to prevent connective tissue destruction in diseases such as arthritis focused on hydroxamate derivatives which chelate the zinc containing active site in MMPs.(247-249) These hydroxamic acid based compounds were the basis of first and second generation MMP inhibitors such as Batimastat and Marimastat. Preclinical animal studies involving these drugs demonstrated promising results in various connective tissue disorders including cardiovascular disease. Both Batimastat and Marimastat have been shown to have a profound effect on constrictive vessel remodelling after angioplasty in pigs, although no effect was seen on neointima formation.(250;251) It has also been reported that Batimastat reduced neointima formation 7 days after balloon injury in rat arteries but not beyond 14 days.(252) A subsequent study did however report a reduction in neointimal formation due to Batimastat administration in balloon injured rat carotid arteries up to 75 days after arterial injury.(253) Hydroxamate compounds drugs however have failed in clinical trials due to low oral availability and poor pharmacokinetics and side effects, such as severe musculoskeletal pain. This failure is thought to be due to the broad substrate spectrum of the enzymes and the poor stability of hydroxamate *in vivo*. The third generation 'deep pocket' MMP inhibitors such as Tanomastat had higher substrate selectivity, with affinity for the deep active sites in MMP-2 and MMP-9, but not for the shallow sites in MMP-1 catalytic sites. However two clinical trials in subjects with end-stage pancreatic cancer and small-cell lung cancer were halted as patients receiving the drug showed significantly poorer survival than patients receiving placebo.(254) Although, it has been demonstrated that the

activity of different MMPs is altered at various stages of tumour progression, regulated by various growth factors, (255)the normal physiological function of MMPs has yielded unsatisfactory results in clinical trials. (256)

However recently, ONO-4817 which belongs to a different class of drugs-tetracycline derived compounds, has been shown to suppress neointimal formation in hypercholesterolemic hamsters. ONO-4817 prevented vascular stenosis in hamsters by inhibiting both SMC proliferation and migration.(257)

Despite intense research, the only currently approved MMP inhibitor, also a tetracycline derived compound is the third generation Periostat. Furthermore this drug is only licensed for the treatment of periodontitis. Present studies aimed at developing MMP inhibitors are therefore aimed at producing drugs with improved pharmacokinetic properties and improved selectivity for specific MMPs.(258)

1.5.1 ADVANCED GLYCATION END-PRODUCTS

Glucose and proteins react non-enzymatically under physiological temperature and pH to form advanced glycation end-products (AGEs) in proteins with long half-life. Such reactions between carbohydrates and proteins are known as the Maillard reaction, after the French chemist L.C. Maillard, reported the formation of yellow-brown substances after heating of amino acids with sugar. It is the end products of the Maillard reaction that are referred to as AGEs. AGEs are generated during cooking when food is 'browned', retarding digestion and reducing the nutritional value. AGEs are also produced in the body, the reaction depending principally on the body's glucose concentration.(259) Therefore under normal glycemic conditions, AGE accumulation in tissue proteins is low.(260) However increased AGE formation under hyperglycemic conditions that characterize diabetes mellitus is considered an important factor in the pathogenesis of late diabetic complications.(261;262)

1.5.2 AGE Formation

The Maillard reaction involves two stages, early and advanced. In the early stage, the reactive amino groups of proteins react with glucose to form relatively unstable Schiff bases. This formation of early glycosylation products is relatively fast and highly reversible(263) in which the amount of Schiff base formed is dictated by glucose concentration.(264) The removal or lowering of glucose causes reversal of the Schiff bases within minutes.

The more stable Amadori products are then formed over a period a days by the rearrangement of the unstable Schiff bases. Although the formation of Amadori products from Schiff bases is slow, it is faster than the reverse reaction which leads to the formation of Amadori products on proteins. The equilibrium levels of Amadori products are reached in weeks(265) and the amount formed is also related to the glucose concentration.(264) Amadori product formation is called glycation(263) and proteins bearing Amadori products are referred to glycated proteins and are distinguished from enzymatically glycoslyated proteins. On long-lived proteins, such as vessel wall collagen, Amadori products continue to undergo a series of complex chemical reactions over a period of weeks to months, forming compounds and cross-links known as AGEs.(263;265) In contrast to Amadori products, AGE-protein adducts are stable and virtually irreversible. As AGE formation, which is relatively slow, follows the formation of Amadori products, the degree of AGE formation is determined by the time of exposure and glucose concentration. AGEs comprise a large number of chemical structures including, 2-(2-furoyl)-4(5)-furanyl-1*H*-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrroles (AFGPs), *N*-ε-carboxy-methyl-lysine (CML), pyrraline and pentosidine,(261) although biochemical and immunohistochemical studies have shown that CML modifications of proteins are the predominant AGEs that accumulate *in vivo*.(266-268)

1.5.3 AGEs in Diabetes

Hyperglycaemia leads to the increased formation of AGEs in the blood and tissue of diabetics.(267;269-272) Furthermore, animal studies have demonstrated an increase in AGE concentration in the kidneys, skin and vascular tissue within several weeks after the animal is rendered diabetic.(273-275) This increased AGE formation is implicated in the pathogenesis of many of the complications occurring in this disease.(269;276;277) The accumulation of AGE-modified proteins is associated with macrovascular complications in diabetes as demonstrated by the association of AGE concentrations and the severity of these complications.(278-280) Serum AGEs have been shown to reflect the severity of coronary arteriosclerosis, independent of other cardiovascular risk factors in diabetic patients.(281) In addition, typical microvascular complications develop following AGE-modification of proteins in non-diabetic animals.(282) Numerous studies have provided evidence of the involvement of AGEs in the pathogenesis of atherosclerosis, particularly in diabetes-associated atherosclerosis. AGE modification of apolipoprotein-B prevents the normal uptake of LDL by tissue LDL receptors and therefore increases circulating LDL cholesterol levels.(283;284) It has also been demonstrated that AGE content is related to circulating LDL and the vascular complications that lead to the development of atherosclerosis in type-2 diabetes.(285) Endothelial dysfunction in patients with type-2 diabetes is also associated with increased serum AGE concentration, independent of other cardiovascular risk factors.(269) The underlying mechanism by which AGEs induce endothelial dysfunction is not

fully understood. AGEs are known to quench nitric oxide leading to impaired endothelium dilatation in experimental diabetes. Inhibition of advanced glycosylation with aminoguanidine prevented nitric oxide quenching and restored vasodilation.(286) In isolated rabbit aorta, endothelium dependent, but not endothelium independent vasodilation, was inhibited by AGE-modified glucose, an effect that was reversible.(287) However, it has more recently been suggested that mechanisms additional to the quenching of nitric oxide may be responsible for the vasoactive properties of AGEs. AGEs cause a reduction in nitric oxide synthesis,(288) reduce the expression of endothelial nitric oxide synthase RNA, while promoting the expression of the cytokine, TNF-α in cultured endothelial cells.(289) In addition, AGEs induce the expression of the vasoconstrictor endothelin-1 by endothelial cells.(290) Also, as serum AGE concentration in diabetics is an independent determinant of plasma C-reactive protein levels, an acute phase protein, sub-clinical inflammation in diabetic patients may be partly due to the activation of an inflammatory response by AGEs.(272)

1.5.4 AGEs and Arterial Stiffening

Animal studies have shown that the formation of cross-links on long-lived proteins such as collagen and elastin within the vessel wall stiffen arteries, and arterial compliance can be increased by decreasing these cross-links. In rats, a diabetes-induced increase in large artery stiffness was reversed by treatment with a thiazolium derived compound, Alagebrium (formerly ALT-711), that has been shown to break AGEs *in vitro* and *in vivo*.(291) Glucose induced damage is not

unique to diabetic patients and as AGEs accumulate slowly over time at normal levels of blood glucose, an AGEs cross-link breaker may have implications in age-associated vascular stiffening. In old non-diabetic rhesus monkeys, arterial compliance measured by PWV and AIx, improved to 74% and 41% of baseline values respectively, following treatment with Alagebrium.(292) More recently the effects of Alagebrium have been investigated in older human subjects with stiff arteries.(293) The drug reduced PWV and PP without a reduction in mean pressure, cardiac output or heart rate. Alagebrium, which was effective even during concomitant use of ACE inhibitor/angiotensin II receptor blocker or calcium channel blocker, most likely exerts additive effects by a different mechanism than these drugs, by interacting with the structural cross-links in the vessel wall.

Animal studies have also demonstrated positive effects of AGE cross-link formation in hypertension. In genetic hypertensive rats, long term administration of an AGE inhibitor reduced both BP and oxidative damage.(294)

AGE accumulation within the human aorta has been studied. An immunohistochemical study of atherosclerotic aortas obtained at autopsy revealed intracellular AGE deposition in 12 out 22 specimens of aortic intima, while AGE deposition in the diffuse intimal thickening occurred in 3 out of 22 cases.(295) Extracellular accumulation of AGE was demonstrated in 20 of 22 aortic medial specimens while AGE deposition was not detected in aortic specimens from the 3 autopsy cases aged 10 years or less.

1.5.5 AGEs and Myocardial Stiffening

AGEs form cross-links not only on vascular but also on myocardial collagen. The development of stiff myocardium in diabetic rats was not associated with a change in myocardial collagen content, but an increase in myocardial collagen age-associated fluorescence.(296) This increase in myocardial collagen fluorescence and left ventricular diastolic stiffness was prevented by aminoguanidine, an AGE cross-link inhibitor, but not with ACE inhibition. The AGEs cross-link breaker Alagebrium also reduces not only age-related vascular, but also myocardial stiffness. In old, non-diabetic dogs, Alagebrium administration resulted in a significant increase in left ventricular diastolic compliance and improved left ventricular systolic function.(297) In old monkeys, the reduction in arterial stiffness achieved through Alagebrium administration was coupled with an increase in left ventricular stroke volume and an improvement in the coupling between the heart and the vasculature.(292) As the improvement in stroke volume occurred without an increase in blood pressure or a reduction in heart rate, it may be that Alagebrium alters the baroreceptor responses by modifying mechanical properties of the carotid sinus.

1.5.6 AGEs and Smoking

Glycotoxins are tobacco derived reactive glycation products, identified in tobacco smoke, that promote AGE formation *in vitro* and *in vivo*.(298) It has been suggested that these glycotoxins are a product of the rearrangement of products formed during the Maillard reaction, which is initiated during the tobacco curing

process. Glycotoxins, present in cigarette smoke, readily cross the cell membrane of mammalian cells, are absorbed through the lungs. Whereas glucose induces AGE formation over a period of weeks,(264;265) glycotoxins can induce AGE formation in hours.(298) Once in the blood stream, glycotoxins may induce the formation of AGEs on serum proteins such as apolipoprotein B, vascular wall proteins and lens proteins of the eye.(298-300) As AGE modifications of apolipoprotein B increase circulating LDL cholesterol levels(283;284) and modifications of vascular collagen trap serum proteins such as lipoproteins,(301) increased levels of circulating AGEs may therefore in part explain the increased incidence of atherosclerosis in smokers.

1.5.7 AGEs as a Therapeutic Target

The structural and physiological changes associated with AGE accumulation have been implicated in virtually all cardiovascular disease. Pharmacological approaches are being developed to reduce the pathophysiological burden of AGEs. Animal studies have shown that drugs such as aminoguanidine can prevent the formation of new AGE cross-links which has been shown to limit the development of the main diabetes-associated complications in animals. However clinical trials of aminoguanidine in diabetic patients were halted due to adverse effects such as vitamin B_6 deficiency and neurotoxicity. Currently, Pimagedine (Alteon), an orally active inhibitor of AGE formation that exerts its action through irreversibly binding with an AGE moiety on the surface of structural proteins, has completed a phase 2/3 clinical trial as a treatment for diabetic

nephropathy. Treatment with the drug resulted in reduction of urinary protein excretion, triglyceride levels and the progression of retinopathy. Pyridorin (Biostratum) which also inhibits the formation of AGEs has recently completed Phase II clinical trials for the treatment of diabetic kidney disease.

As previously mentioned, a thiazolium derived compound- Alagebrium (formerlyALT-711), breaks existing cross-links and the drug is currently being investigated in clinical trials in relation to various cardiovascular diseases. In March 2004, Alteon, the pharmaceutical company responsible for the manufacture of Alagebrium, initiated SPECTRA (Systolic Pressure Efficacy and Safety Trial of Alagebrium), a Phase 2b clinical trial of Alagebrium in patients with systolic hypertension. PEDESTAL (Patients with Impaired Ejection Fraction and Diastolic Dysfunction: Efficacy and Safety Trial of Alagebrium) began in April 2004, a Phase 2 clinical trial of Alagebrium evaluating the effect of the drug on diastolic function and ventricular mass in patients with significant heart failure. Phase 2 trials are also currently ongoing to investigate the effect of this drug on endothelial function and left ventricular and vascular stiffness.

Chapter 2

Aims and Methods

VASCULAR EXTRACELLULAR MATRIX REMODELLING IN RELATION TO AGE AND LOCATION IN THE VASCULATURE

2.1 HYPOTHESIS

Ageing of the human vasculature is associated with altered expression of the matrix-degrading enzymes, MMP-2 and MMP-9. This altered expression is differential depending upon location within the vasculature.

2.2 AIMS

As increased MMP-2 activity has been associated with age-related ECM remodelling in the aorta of rats and non-human primates, the principal aim of this study is to investigate the relationship between age and aortic MMP-2 activity in humans. Using human aortic tissue, MMP-2, MMP-9 and TIMP-2 activity was semi-quantified to establish if similar alterations occur with age in humans, using aortic tissue collected from 15 patients undergoing CABG. This was achieved by analyzing protein-standardized, homogenates of aortic tissue obtained from the anterior aspect of the ascending aorta from each patient, using gelatin zymography and Western blotting.

It has been demonstrated that in rat aorta, both the latent and activated forms of MMP-2 increase with ageing,(203) whereas only increased activation of latent MMP-2 is reported in non-human primates.(204) As gelatin zymography allows for quantification of both the latent and active forms of the enzyme, the

relationship between age and aortic MMP-2 and MMP-9 activation was also investigated.

As it is currently unclear if age-related vascular remodelling occurs only in centrally located elastic arteries, parallel investigation of MMP-2 and MMP-9 activity was carried out for each patient as for the aorta, using the muscular artery- the IMA.

The activity of MMP-2 and -9 was also localized within aortic and IMA tissue using *in situ* zymography.

2.3 STUDY DESIGN

Aortic and IMA tissue was collected from patients (n=15) undergoing CABG. Tissue was collected and processed depending upon the experimental method that was subsequently carried out. Homogenates of whole tissue sections were used to test for MMP-2, MMP-9 and TIMP-2 activity in relation to age in both the aorta and IMA. Gelatinolytic activity within tissue sections of the aorta and IMA was localised using *in situ* zymography.

2.4 STUDY GROUP CHARACTERISTICS

The study population consisted of 15 patients (12 male, 3 female, aged 46-72 years, mean age 60.9±9 years) undergoing CABG (Cardiac Surgery Unit, St James Hospital, Dublin) for coronary heart disease. Exclusion criteria included patients suffering from inflammatory diseases, cancer, infection or other major medical illness. There were 8 smokers and 9 patients suffered from hypertension.

11 patients were receiving a beta-blocker at the time of haemodymanic measurement and arterial tissue removal while 6 patients were being treated with ACE inhibitors. In addition, all patients were also receiving lipid lowering therapy. Patient characteristics and haemodynamic data are listed in table 2.1. Patient consent was obtained and all procedures were in accordance with the principles of the Declaration of Helsinki; 1975 (revised 1983).

Table 2.1: Characteristics of study population (n=15).

Characteristic	Study population (n=15)
Age	60.9±9
Male/female	12 /3
Smokers	n=8 (53%)
BMI	28.6±4
Hypertension	n=9 (60%)
Double vessel disease	n=4 (27%)
Triple vessel disease	n=11 (73%)
Previous myocardial infarction	n=8 (53%)
Brachial systolic BP(mm Hg)	139
Brachial diastolic BP(mm Hg)	79
Brachial pulse	56

2.5 METHODS

2.5.1 Tissue Collection

Surplus arterial tissue was collected from patients undergoing CABG. The nature of the study was explained to patients and consent was obtained. Aortic 'punches' removed from the anterior aspect of the ascending aorta were collected, along with excess sections of the graft artery- the IMA. After removal from the body, samples of IMA were carefully cleaned of surrounding connective tissue and blood while immersed in ice-cold Krebs buffer. Aortic tissue was also rinsed in ice-cold Krebs buffer and samples that contained atherosclerotic lesions were discarded. Tissue that was used for zymography was snap-frozen in liquid nitrogen and then stored at -80˚C until use. Tissue that was used for *in situ* zymography and histology was frozen in isopentane cooled in liquid nitrogen and stored at -80˚C until use.

2.5.2 Preparation and Standardisation of Arterial Homogenates

Samples of frozen arterial tissue samples, weighing around 80mg were kept frozen at all times during homogenization. After tissue had been ground to a fine powder using a pestle and mortar, 200µl extraction buffer (Iodoacetamide 5mM, EDTA10mM, Trasylol 200 KIU/ml, Tris HCl 1M pH 7.4, Triton X-100 0.2%) was added for every 50mg of ground tissue sample. The tissue was then further ground in the frozen state in the presence of the extraction buffer. The tissue and the extraction buffer were then allowed to come to room temperature and the suspension was placed in an eppendorf and homogenized using a hand-held Pellet

Pestle® motor (Kontes, Vineland, New Jersey USA) and then spun in a microcentrifuge (Hettich Zentrifugan, Tuttlingen, Germany) at 2000g for 5 minutes at 4˚C. The supernatent was then removed, placed in a clean eppendorf tube and kept on ice, while a further 100µl extraction buffer was added to pellet. Following further homogenization with the hand-held Pellet, the mixture was spun again in the microcentrifuge at 2000g for 5 minutes at 4˚C. The resulting supernatant was combined with the original supernatant and mixed thoroughly. The protein concentration of the resulting arterial homogenate was then determined by the method of Bradford(302) as previously described. The samples were then standardized to a concentration of 2mg/ml using the extraction buffer.

2.5.3 Gelatin Zymography

Tissue homogenates containing 12µg protein were mixed 3:1 with sample loading buffer (40% Glycerol, 0.4% Bromophenol blue, 12% SDS, 200mmol/L Tris-HCl pH6.8), mixed gently and kept on ice until loaded onto gels.

10% SDS-PAGE gels-containing gelatin (1mg/ml) was constructed from a lower gel (27% bis/acrylamide, tris-HCl pH8.8 375mmol/L, 0.1% SDS, 0.1% APS, 0.06% TEMED, gelatin 1mg/ml), poured between glass plates assembled in a casting frame after which 1-butanol was pipetted along the top to prevent oxidation while the gel polymerized. After approximately 30 minutes, the 1-butanol was removed and an upper gel (0.83% bis/acrylamide, tris-HCl pH6.8 125mmol/L, 0.1% SDS, 0.1% APS, 0.1% TEMED) was constructed and poured

between the glass plates. Comb teeth were then added in order to create wells for sample loading. The upper gel was allowed to polymerize for approximately 30 minutes, after which the comb teeth were removed. The gel cassette was then removed and placed into the electrode assembly. The electrode assembly was placed in a mini tank, which was subsequently filled with running buffer (Trisma base 25mM, Glycine 192mM, SDS 0.1%) before sample loading.

A current of 100V was applied until the blue dye incorporated into samples was approximately 0.5cm from the bottom of the glass plates. Following electrophoresis, the upper gel was cut off and the lower gel was renatured by incubation in Triton-X 100 (2.5%) for 30 minutes. Gels were then placed in incubating buffer (Tris-HCl 50mmol/L, $CaCl_2$ 10mmol/L, NaCl 50mmol/L, pH7.6) for 18 hours at 37°C. Subsequently gels were stained in 0.25% Coomassie blue followed by destaining (30% methanol, 10% glacial acetic acid) to visualise the proteolytic bands. Recombinant latent MMP-2 and MMP-9 (Chemicon International) were included on the gel to serve as standards, and all gels were carried out in duplicate. Gelatinolytic activity, an area of digestion on a blue background was analysed by densitometry (Gene Genius Gel Documentation System with Genetools Software, Syngene). Intra- and interassay variation was <10%.

2.5.4 Western blotting for TIMP-2

A 12% lower gel was constructed (40% Bis/Acrylamide, 0.375mM tris-HCl pH8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED) and poured between glass plates assembled in a casting frame after which 1-butanol was pipetted along the top to prevent oxidation while the gel polymerized. After approximately 30 minutes, the 1-butanol was removed and an upper gel (16.75% Bis/Acrylamide, 0.125mM tris-HCl pH6.8, 0.1% SDS, 0.1% APS, 0.01% TEMED) was constructed and poured between the glass plates. Comb teeth were then added in order to create wells for sample loading. The upper gel was allowed to polymerize for approximately 30 minutes, after which the comb teeth were removed. The gel cassette was then removed and placed into the electrode assembly. The electrode assembly was placed in a mini tank, which was subsequently filled with running buffer (Trisma base 25mM, Glycine 192mM, SDS 0.1%), before sample loading. Arterial tissue homogenates was prepared for Western blotting as for gelatin zymography with minor modifications. Samples were standardised to 26μgs protein containing 5% β-mercaptoethanol and 20% sample buffer (0.4% bromophenol blue, 12% SDS, 40% glycerol, 200mM Tris-HCl pH7). Samples were then boiled for 10 minutes and cooled on ice before loading. Samples and standards were then separated by electrophoresis by applying a current of 100 volts.

When the blue dye reached approximately 1cm before the bottom of the gel, electrophoresis was stopped and the gel placed in transfer buffer (Trisma Base 50mM, Glycine 40mM, Methanol 20%, SDS 0.037%) for 15 minutes. The

Hybond-P PVDF membranes (Amersham Biosciences) used for protein transfer were also placed in transfer paper for 15 minutes. Fibre pads and filter paper required for protein transfer were also equilibrated in transfer buffer for the same period of time. A gel cassette was constructed by placing the membrane on top of the gel, carefully removing air bubbles. The membrane and gel were then placed between the pre-soaked fibre pads and filter paper. The gel cassette was then placed in a transfer module together with a cooling unit. A stir bar added to the module which was filled with transfer buffer and placed on a magnetic plate. The protein transfer from gel to membrane was carried out by applying a current of 100 volts to the transfer module for 1 hour. Following protein transfer, poinceau red was added to membranes to confirm protein transfer. Membranes were then blocked in 5% dried milk (fat-free) in TBS (Trisma base 10mM, NaCl 100mM, HCl 3.8mM pH7.5) containing Tween-20 (0.1%) for 1 hour. Membranes were then incubated in the presence of rabbit anti-TIMP-2 polyclonal antibody (1:1000) as a primary antibody (Chemicon International) and subsequently washed (TBS containing Tween-20 0.1%) to remove excess antibody. Following a further incubation in the presence of a HRP-conjugated swine anti-rabbit as a secondary antibody at a suitable dilution, membranes were washed prior to protein detection and visualization. ECL+plus Western Blotting Detection System (Amersham Biosciences) was used to detect protein bands which were then exposed to X-Omat™ X-ray film (Kodak). A positive control for the protein to be detected was also loaded onto the gel along with molecular weight markers (Chemiblot™, Chemicon International) for molecular weight determination.

Bands) corresponding to the positive control were analysed by densitometry (Gene Genius Gel Documentation System with Genetools Software, Syngene). Intra- and interassay variation was <10%.

2.5.5 Preparation of Arterial Tissue for Sectioning

Cross-sectional samples of aortic tissue (1-2mm) and samples of the IMA (3-5mm tubes), that had previously been frozen in isopentane cooled with liquid nitrogen, were prepared for tissue sectioning. A drop of optimum cutting temperature solution, (OCT, RA Lamb, Eastboune, England) was placed onto a cork disk, which was then chilled until the OCT was turned opaque and became semi-solid. Frozen sections of the aorta and IMA were then placed onto the OCT, orientated so that subsequent sectioning would result in a cross-section of the vessel wall. The disk was then re-cooled until the OCT turned solid and held the tissue sample firmly in place, after which another layer of OCT was added and re-cooled. This process was continued until the OCT had been completely built up around the tissue, keeping the tissue frozen at all times. Samples, embedded in OCT were stored at -80°C until sectioning.

2.5.6 Arterial Tissue Sectioning

Tissue sectioning was carried out using the Bright 20 Cryostat, (Bright, Cambridgeshire, England). Frozen samples, embedded in OCT were placed in the chamber of the cryostat the temperature of which was maintained at -20°C. Samples were allowed to equilibrate with the chamber temperature before

sectioning. Frozen serial sections (10μm) were then cut and placed onto Superfrost plus microscope slides (Fisher Scientific). Sections were then stored at -20°C until use.

2.5.7 In Situ Zymography

Serial cryostat sections (10μm) of the aorta and IMA, embedded on OCT were cut and placed onto Superfrost plus slides (Fisher Scientific). Sections were allowed to thaw for approximately 10 minutes after which a circle was drawn around sections using a PAP pen (Super PAP pen, Daido Sangyo Co.) to ensure that substrate remained in place. Sections were then incubated with fluoresceine-labelled DQ Gelatin (Molecular Probes), diluted 1:10 in substrate buffer (Tris-HCl 50mM, PMSF 5mM, Brij 35 0.05%, $CaCl_2$ 10mM, pH 7.4). DQ gelatine is gelatin so heavily labelled with fluoresceine that all fluorescence is quenched until gelatin is digested. Sections were then incubated for 3 hours at 37°C in a humidified chamber, in the dark. Following rinsing in dH_20 (x3), Triton-X 100 (1%) and again in dH_20 (x3), sections were mounted with Vectashield mounting medium (Vector Laboratories).

Sections were viewed by fluorescence microscopy (Leica DMIRB, Leica Microsystems Inc) using 10X magnification and photographed using the Openlab Image Analysis package (Improvision). Adjacent sections containing 20mM EDTA in the substrate buffer served as negative controls.

Gelatinolytic activity was observed as areas of green fluorescence on a dark background. Regions of interest (ROI) were identified within each layer of the

vascular wall. A ROI of a standard surface area was placed randomly within each layer a total of 6 times. The amount of gelatinolytic activity within ROIs was evaluated by analysing the raw pixel intensities (Openlab Image Analysis package, Improvision) corresponding to bright areas on a dark background. For each layer of the vessel wall, the mean pixel intensity was calculated based on the pixel intensities for all ROIs in that layer.

2.5.8 Statistical Analysis

Statistical analysis was performed using Prism version 3.0 (GraphPad Software Inc) and JMP (JMP IN, version 5, SAS Institute Inc). Data were analysed using paired t-tests where protein expression is compared between matched vessels, and unpaired t-tests for analysis of protein expression between subjects. JMP IN was used to identify associations between variables (i.e. age and protein expression) that were carried out, adjusted for other variables. Data are expressed as mean ±SD and $P<0.05$ was considered statistically significant.

VASCULAR EXTRACELLULAR MATRIX REMODELLING IN RELATION TO ARTERIAL STIFFENING

2.6 HYPOTHESIS

Alterations in collagen accumulation and elastin architecture are related to stiffening of the human vasculature. These alterations are related specifically to vascular stiffening, independent of the ageing process.

2.7 AIMS

Vascular remodelling has been investigated in terms of age or various cardiovascular diseases. Increasing evidence suggests that in addition to vascular ageing, independent mechanisms may contribute to arterial stiffening. Despite this, little is known regarding vascular remodelling in human tissue in relation to arterial stiffening. Whether a change in the amount of collagen in the arterial wall of human subjects is related to stiffer arteries is unclear and was determined. In addition, while intimal thickening and elastic tissue fragmentation is known to occur in old arteries, it is unclear if these processes are specifically related to ageing or arterial stiffening, and was therefore be investigated. The relationship between gelatinase activity and arterial stiffness was also determined.

2.8 STUDY DESIGN

Aortic and IMA tissue was collected from patients (n=10) undergoing CABG following the measurement of arterial stiffness by PWV. Tissue was collected and processed depending upon the experimental method subsequently carried out. Collagen content was assessed in tissue sections of the aorta of these patients using Sirius red staining. Elastic tissue within sections of aortic tissue was stained so that intimal thickness and elastic fragmentation could be examined in these patients in relation to arterial stiffness. Using gelatin zymography, MMP-2 and MMP-9 activity was semi-quantified in whole tissue homogenates of the aorta, to establish if a relationship exists between gelatinase activity and arterial stiffness. Investigations carried out in the aorta in relation to arterial stiffening were also carried out in the IMA (with the exception of gelatinase activity) to investigate vascular remodelling in arterial stiffening in elastic versus muscular arteries.

2.9 STUDY GROUP CHARACTERISTICS

The study population consisted of 10 patients (8 male, 2 female, aged 48-76 years, mean age 61.8±9 years) undergoing CABG (Cardiac Surgery Unit, St James Hospital, Dublin) for coronary heart disease for vascular MMP quantification. Exclusion criteria included patients suffering from inflammatory diseases, cancer, infection or other major medical illness. There were 4 smokers and 4 patients suffered from hypertension. 7 patients were receiving a beta-blocker at the time of haemodymanic measurement and arterial tissue removal while 2 patients were being treated with ace inhibitors. In addition, 8 of the 10

patients were also receiving lipid lowering therapy. Collagen and elastin staining was carried out for 8 of the 10 due to the limited amount of vascular tissue available. Patient consent was obtained and all procedures were in accordance with the principles of the Declaration of Helsinki; 1975 (revised 1983). Patient characteristics are listed in Table 2.2.

Table 2.2: Characteristics of Study Population (n=10).

Characteristic	**Study population (n=10)**
Age	61.8±9
Male/female	8 /2
Smokers	n=4
BMI	27.4±4
Hypertension	n=4
Previous myocardial infarction	n=3
Brachial systolic BP(mm Hg)	134
Brachial diastolic BP(mm Hg)	73
Brachial pulse pressure(mmHg)	61

2.10 METHODS

2.10.1 Measurement of PWV

Carotid-femoral PWV was determined with the foot-to-foot method (Complior, Colson, Dupont Medical) as previously validated.(277) The simultaneous recordings by two pressure-sensitive transducers of the carotid and femoral waveform and measurement of the time delay of successive records from the foot of each wave divided by the distance, measured over the body surface, between the transducers allows calculation of PWV in metres/second (m/sec).

2.10.2 Tissue Collection

Surplus arterial tissue was collected from patients undergoing CABG as described in section 2.5.1.

2.10.4 Preparation of Arterial Tissue for Sectioning

Cross-sectional samples of aortic tissue and samples of the IMA were prepared for sectioning as described in section 2.5.5.

2.10.5 Arterial Tissue Sectioning

Tissue sectioning was carried out as described in section 2.5.6.

2.10.6 Sirius Red Staining for Collagen

Serial cryostat sections (10μm) of the aorta and IMA, embedded in OCT (RA Lamb, Eastbourne England) were cut and placed onto Superfrost plus slides

(Fisher Scientific). Sections were allowed to thaw for approximately 10 minutes before being rinsed in dH_2O. Sections were then placed in coplin jars containing 0.1% direct red 80 (0.1% direct red 80 in saturated picric acid) for 1 hour 30 minutes. Sections were then removed and placed into a coplin jar containing 0.01M HCl for 2 minutes to differentiate samples and remove picric acid. Sections were then dehydrated in a series of increasing concentrations of ethanols (75% methanol, 95% methanol, 100% methanol), cleared with xylene and mounted in DPX mounting medium (Sigma, St Louis, Missourri, USA).

Sirius red stained sections were examined by microscopy (Leica DMIRB, Leica Microsystems Inc) using 10X magnification and photographed using the Openlab Image Analysis package (Improvision). Collagen content was determined by colour threshold masking (Openlab Image Analysis package, Improvision) of Sirius red stained sections. A Sirius red stained tissue image was magnified so that individual pixels could be identified. The threshold mask was determined by selecting all pixels that were contained within the stained areas of tissue. This mask was then used to identify collagen stained areas in all tissue sections. For each area within the tissue section to be assessed, ROIs of known surface area were randomly placed within that area a total of 6 times. The mean percentage area covered by collagen in an area of interest was then calculated using the values obtained from the ROIs.

2.10.7 Elastin Staining

Serial cryostat sections (10μm) of the aorta and IMA, embedded on OCT were cut and placed onto Superfrost plus slides (Fisher Scientific). Elastin in tissue sections was identified using the ACCUSTAIN® elastic (Sigma, St Louis, Missourri, USA). Slides were rinsed in dH_2O before being placed in a jar containing working elastic stain solution (55% Hematoxylin Solution, 8.3% ferric chloride solution, 22.2% Weigert's iodine solution) for 10 minutes. Samples were then rinsed in dH_2O for 2 minutes before differentiation of elastic fibres for 2 minutes using working ferric chloride solution. Samples were then rinsed in tap water before being inspected using the microscope at X4 magnification to ensure that tissue samples were not over differentiated. If elastic fibres were over differentiated, the tissue section was returned to the working elastic solution for 30 seconds. Tissue sections were then rinsed in 95% methanol and then placed in a coplin jar containing Van Gieson solution for 2 minutes. Samples were then rinsed in 95% methanol, dehydrated in xylene and mounted using DPX mounting medium (Sigma, St Louis, Missourri, USA).

Elastic fibre morphology was visualized by microscopy (Leica DMIRB, Leica Microsystems Inc) using 10X magnification and photographed using the Openlab Image Analysis package (Improvision). The intimal thickness was determined as the area between the IEL and the endothelium. Using a calibrated line measuring tool (Openlab Image Analysis package, Improvision) a mean of 10 distances was calculated between the IEL and the luminal surface as the intima thickness.

Elastic fibres were examined for evidence of fragmentation. The degree of elastic

fibre fragmentation was assessed using a simple four point grading system depending on the appearances of the elastic fibres in the tunica media of each tissue section as previously described.(303) The grade was allocated depending on the most advanced fragmentation present in the tissue section being assessed, as listed in Table 2.3. Grading was carried out on three occasions, with no reference to previous allocated grades.

Table 2.3; Classification of various grades of elastin fibre fragmentation

Grade1	No appreciable elastic fragmentation with a normal lamellar structure
Grade2	Early focal fragmentation but with a preserved lamellar structure
Grade3	Established, moderately advanced fragmentation but with a largely preserved lamellar structure
Grade4	Severe fragmentation often with complete disruption of the lamellar structure in whole segments of the section.

Modified from Johnson CO *et al.* Age related changes in the tunica media of the vertebral artery: implications for the assessment of vessels injured by trauma. Journal of Clinical Pathology 2001;54:139-145.

2.10.8 Gelatin Zymography

MMP-2 and MMP-9 was semi-quantified in the tissue homogenates as described in section 2.5.3.

2.10.9 Statistical Analysis

Associations between variables (i.e. arterial stiffness and collagen content) were assessed by Spearman rank correlation analysis. Data are expressed as mean ±SD and $P<0.05$ was considered statistically significant. Where * is indicated $P<0.05$, ** $P<0.01$, ***$P<0.001$. Statistical analysis was performed using Prism version 3.0 (GraphPad Software Inc, San Diego, CA).

THE RELATIONSHIP BETWEEN AGE, ARTERIAL STIFFNESS AND CIRCULATING MARKERS OF EXTRACELLULAR MATRIX TURNOVER

2.11 HYPOTHESIS

Circulating markers of extracellular matrix turnover are related to measures of stiffness of the human vasculature in both normotensive and hypertensive subjects.

2.12 AIMS

The relationship between circulating markers of vascular remodelling and both hypertension and vascular stiffening is conflicting, in terms of the metabolites of collagen turnover and MMPs. Previous studies have not quantified the circulating markers of matrix turnover that may be related to vascular remodelling in a single study. This chapter investigated the relationship between arterial stiffness and markers of ECM remodelling. Arterial stiffness was assessed in subjects by PWV. As this study will examine circulating markers, as opposed to protein and enzyme expression in aortic tissue, AIx will also be measured in subjects in addition to PWV. Whereas PWV is a measure of aortic stiffness, AIx is a global estimate of arterial stiffness and depends upon PWV and the magnitude of reflected waves from the periphery and pattern of left ventricular ejection. Plasma measurements of collagen type I metabolites and matrix degrading enzymes were determined in order to determine if a relationship

exists between collagen type-I turnover (the main collagen type of the vascular wall), ECM degradation, PWV, AIx and age. The relationship between vascular stiffening and circulating metabolites and enzymes was analysed in both normotensive and hypertensive subjects and the markers of vascular remodelling compared between these two groups.

2.13 STUDY DESIGN

Fasting blood samples were collected from subjects following the measurement of PWV, AIx and mean arterial pressure (MAP). Plasma was collected and stored at -80°C until use. Commercially available immunoassays were used to measure plasma levels of PIP (Takara Bio Inc.), ICTP (Orion Diagnostica), total MMP-1- free and bound to TIMP-1 (Amersham Biosciences), TIMP-1 (Chemicon) and TIMP-2 (R and D Systems) according to the manufacturers instructions. Using gelatin zymography, MMP-2 and MMP-9 expression was also semi-quantified in plasma samples.

2.14 STUDY GROUP CHARACTERISTICS

The study population (Table 2.4) consisted of a group of never-treated patients (n=46, 48.7±2 years, 22 female). Subjects (n=32) were classed as hypertensive if clinic systolic BP was ≥140mmHg and diastolic BP was ≥90mmHg on 3 occasions and >135/80mmHg on the daytime reading of the 24 hour ambulatory BP monitor. Exclusion criteria included inflammatory states and conditions known to affect bone and collagen turnover. The subjects were studied fasting,

having abstained from caffeine, alcohol and smoking in the previous 12 hours. Baseline heamodynamic measurements were recorded in each subject after a supine rest for 15 minutes in a quiet room at 22°C.

Table 2.4; Characteristics of study population (n=46).

	Hypertensive (n=32)	**Normotensive (n=14)**
Age (years)	49±2.	44±3
Brachial systolic BP(mm Hg)	160±3	125±2***
Brachial diastolic BP(mm Hg)	96±1	76±1***
Brachial pulse pressure(mmHg)	69±2	64±2***
Aortic systolic BP(mm Hg)	149±3	115±3***
Aortic diastolic BP(mm Hg)	96±1	77±2***
Aortic pulse pressure(mm Hg)	53±3	38±3***
MAP	116±2	93±2***
Male, female	20/12	4/10*
Smokers(n)	7	4

2.15 METHODS

2.15.1 Haemodynamic Measurements

Haemodynamic measurements were carried out by clinicians at the Hypertension Clinic, St James's Hospital, Dublin.

2.15.2 Blood Pressure Measurement

Brachial BP and heart rate were measured with an automated digital oscillometric monitor (Omron model HEM 705-CP, Omron Healthcare Inc., Vernon Hills, IL), in the right arm, after a supine rest of 15 minutes and three readings at 1 minute intervals were taken, and the mean used for data analysis. Brachial PP was calculated as the difference between systolic and diastolic BP.

2.15.3 Measurement of PWV

Carotid-femoral PWV was determined as described in section 2.10.1.

2.15.4 Measurement of Augmentation Index

The technique of pulse wave analysis was used. A high-fidelity micromanometer (SPC-301, Millar Instruments, Houston, Texas) was used to flatten the radial artery, and the radial pulse was continuously recorded. The aortic pressure waveform was derived from radial tonometry using a previously validated transfer function, relating radial to aortic pressure waveform within the system software (SphygmoCor, Atecor Medical, version 7.0, West Ryde, Australia), as

previously described(304) and ascending aortic pressures and the AIx were derived from the aortic pressure waveform.

2.15.5 Blood Sample Collection and Processing

Blood samples were obtained from patients attending the Hypertension Clinic, St James's Hospital, Dublin. Samples were taken from the anticubital vein of subjects, following an overnight fast. Blood was collected into 10ml Ethylenediaminetetraacetic acid (EDTA) and 5ml lithium heparin (LH) coated tubes and transported to the laboratory immediately for centrifugation. In addition, standard haematological (full blood count) and biochemical (renal function test, glucose and lipids) were measured by routine automated techniques within the hospital laboratory.

Blood samples were centrifuged at 2500g, for 10 minutes at 4°C using a microcentrifuge (Hettich Zentrifugan, Tuttlingen, Germany). LH plasma was then separated into 150ul aliquots. TTI (150ul) was added to one LH plasma sample for zymographic analysis. EDTA plasma was separated into 500µl aliquots. Plasma samples were stored at -80˚C until use.

2.15.6 ELISAs

Commercially available ELISA kit were used to quantify plasma enzyme and plasma samples were diluted to fall within the detection range of the assay according to the manufacturer's instructions. Plates were read at the specified absorbance using an automated plate reader. Plasma from blood samples

collected in either EDTA or lithium heparin coated tubes were used, depending on the manufacturer's instructions.

Plasma PIP was measured in the range 10-640ng/ml and inter-assay and intra-assay variation for the assay was <7% and <6% respectively, using the Takara Procollagen Type-I C-Peptide EIA kit. Plasma ICTP levels were measured in the range 1-50ng/ml using the Orion ICTP EIA Type I Telopeptide Enzyme Immunoassay Kit. Although inter- and intra-assay variation data were not supplied, the intra-assay variation obtained in this study was <4%. MMP-1 was measured in the range 6.25-100ng/ml, using the MMP-1 Human Biotrak Elisa System (Amersham Biosciences). It has previously been demonstrated that this range is suitable for the measurement of MMP-1 in human plasma samples collected with lithium heparin.(305) Inter-assay and intra-assay variation for the assay was <13% and <7% respectively. Using the Human TIMP-1 Immunoassay Kit (Chemicon International), circulating TIMP-1 levels were measured in the range 1.2-49ng/ml, inter-assay and intra-assay variation was not specified, and the intra-assay variation obtained in this study was <7%. Plasma TIMP-2 levels were quantified using the Human TIMP-2 Quantikine® Kit (R and D Systems) in the range 0.156-10ng/ml, and inter- and intra-assay variation was given at <7% and <4% respectively.

2.15.7 Plasma Protein Quantification (Bradford Assay)

The protein concentration of the plasma was determined prior to zymographic analysis and western blotting according to the method of Bradford, (302) using

bovine serum albumin (BSA-2mg/ml) as the assay standard. Serial dilutions of BSA were used to generate a standard curve ranging from 1500μg/ml to 93.75μg/ml and plasma samples of unknown protein concentrations were diluted 1 in 50. Bradford reagent (Sigma, St Louis, Missourri, USA) was then added to the standards and plasma samples to produce a colour change proportional to the protein concentration. Plasma protein concentration was then determined from the standard curve and standardised to 4mg/ml using TTI solution. Spectrophotometric determinations were obtained using the UV visible spectrophotometer UV-1601 (Shimadzu, Kyoto, Japan).

2.15.8 Gelatin Zymography

MMP-2 and MMP-9 was semi-quantified in plasma samples containing 16μg protein, mixed 3:1 with sample loading buffer (40% Glycerol, 0.4% Bromophenol blue, 12% SDS, 200mmol/L Tris-HCl pH6.8) as described in section 2.5.3. Intra- and interassay variation was <10%.

2.15.9 STATISTICAL ANALYSIS

Data were analysed with JMP (JMP IN, version 5.0, SAS Institute Inc). Where data were not distributed normally, non-parametric methods (Wilcoxon Rank Sums Test) were used. Correlations between the haemodynamic parameters and plasma makers were derived using multivariate correlations (Spearman's Rho). As both the markers of collagen turnover and arterial stiffness are strongly related to age and BP, the relationship between these parameters was examined using a

stepwise regression model. In the first model, PWV was the dependent variable and AIx was the dependent variable in the second model, with age and MAP as independent variables in both models. All the results are expressed as mean ±SEM and $P<0.05$ was considered significant. Where * is indicated P<0.05, ** P<0.01, ***P<0.001.

ARTERIAL STIFFNESS AND ADVANCED GLYCATION END PRODUCTS

2.16 HYPOTHESIS

Circulating AGEs are related to measures of vascular stiffness in normotensive and hypertensive subjects.

2.17 AIMS

It has been demonstrated that circulating AGEs are a marker of various vascular pathologies. Despite the improvement in arterial compliance following treatment with an AGE crosslink breaker, it has not been determined whether circulating levels of AGEs are a marker of vascular stiffening. Plasma AGE levels were measured in normotensive and hypertensive subjects following the determination of arterial stiffness to determine if circulating AGEs are related to aortic stiffening in both populations. As there is disagreement regarding the effect of an AGE crosslink breaker on the vasculature, other than the aorta, it was also assessed whether circulating AGEs are related to the stiffness of the smaller muscular arteries.

2.18 STUDY DESIGN

Fasting blood samples were collected from subjects following the measurement of PWV and AIx. Plasma was collected and stored at -80°C until use. Following proteinase K digestion of plasma samples to reveal hidden epitopes,(306) plasma

AGEs were measured using a non-competitive immunoassay which was developed based on methods previously described.(307-309) An anti-AGE monoclonal antibody (CD12, NBS Biological , Cambrex, UK) was used that recognizes both N(epsilon)-carboxymethyl lysine (CML) and N(epsilon)-carboxyethyl lysine (CEL) products. Results are expressed as arbitrary units- 1 unit of AGE (uAGE) is equal to 1μg AGE-BSA.

2.19 STUDY GROUP CHARACTERISTICS

The study population consisted of a group of never-treated, non-diabetic patients (n=41, 48.6±2 years, 20 female) of which 27 were classed as hypertensive (clinic BP>140/90 mm Hg and ambulatory daytime BP>135/85 mmHg), and the remaining 14 as normotensive (clinic BP<120/80 mmHg). The subjects were studied fasting, having abstained from caffeine, alcohol and smoking in the previous 12 hours. Baseline haemodynamic measurements were recorded in each subject after a supine rest for 15 minutes in a quiet room at 22°C (Table 2.5).

Table 2.5. Baseline demographic and haemodynamic data (n=41) (mean±SEM)

Characteristic	Hypertensive (n=27)	Normotensive (n=14)
Age (years)	52±3	42±3
Male, female	14/13	7/7
BMI	29.7±1	29.8±1
Waist (cm)	95±3	93±5
Hip (cm)	105±3	100±5
Waist/hip ratio	0.90±0.01	0.93±0.02
Smokers(n)	8	4
Cholesterol (mmol/L)	5.4±0.2	5.1±0.3
HDL (mmol/L)	1.4±0.3	1.3±0.3
Triglycerides (mmol/L)	1.6±0.23	1.2±0.17
Glucose (mmol/L)	5.6±0.1	5.1±0.1
Creatinine (μmol/L)	85±2	81±2
AGEs (uAGE/ml)	8±1	3±1
Brachial systolic BP(mmHg)	159±3	28±2
Brachial diastolic BP(mmHg)	92±1	78±2
Brachial PP (mmHg)	68±3	50±2
MAP (mmHg)	117±2	96±2
PWV (m/sec)	11±0.3	9.5±0.5
AIx (%)	28±2	22±6
Heart rate	69±2	69±4
Aortic pulse pressure (mmHg)	55±3	37±3
Aortic SBP (mmHg)	149±3	117±3
Aortic DBP (mmHg)	94±2	80±2
T_R	133±2	133±5

2.20 METHODS

2.20.1 Blood Sample Collection and Processing

Blood samples were obtained from patients attending the Hypertension Clinic, St James's Hospital, Dublin as described in section 2.15.5.

2.20.2 Haemodynamic Measurements

Haemodynamic measurements were carried out by clinicians at the Hypertension Clinic, St James's Hospital, Dublin. Routine biochemistry measurements were carried out by technicians at the Biochemistry Dept., St James's Hospital, Dublin using automated analysers.

2.20.3. Blood Pressure Measurement

Brachial BP and heart rate were measured with an automated digital oscillometric monitor (Omron model HEM 705-CP, Omron Healthcare Inc., Vernon Hills, IL) as described in section 2.15.2.

2.20.4 Measurement of PWV

Carotid-femoral PWV was determined with the foot-to-foot method (Complior, Colson, Dupont Medical) as described in section 2.10.1.

2.20.5 Measurement of Augmentation Index

The technique of pulse wave analysis was used as described in section 2.15.4.

2.20.6 Sample Preparation for AGE ELISA

Prior to AGE quantification, LH plasma was subject to proteinase K digestion to reveal hidden epitopes.(306) An enzyme stock solution (ESS) containing proteinase K (Sigma, St Louis, Missourri, USA) was prepared in Tris buffer (0.8mg/ml proteinase K, trisma base 50mM, pH 8) and stored at -20°C until use. ESS was diluted 1:20 in a sodium phosphate buffer (0.02M di-sodium hydrogen orthophosphate, 0.02% sodium azide, pH7.4) to make the enzyme working solution (EWS). Fifty microlitres of plasma sample was placed in an eppendorf tube with 100µl EWS, vortexed and incubated at 37°C for 18 hours. The samples were then heated to 70°C for 1 hour to denature the enzyme. Samples were then subject to microcentrifugation at 13,500 RPM for 10 minutes. The resulting supernatant was collected for AGE quantification.

2.20.7 AGE ELISA

The measurement of plasma AGEs was based on non-competitive ELISA methods previously described.(307-309)

A standard curve was constructed from synthetic AGE-BSA (AGE-Bovine serum albumin, MBL) produced from the reaction of BSA with glycoaldehyde. The standard curve (0-1µg/ml) was constructed by serially diluting AGE-BSA in carbonate buffer (50mM sodium carbonate, 0.06% sodium azide, pH 9.6). Proteinase K digested plasma samples were diluted (1:50) to fall within the range of the standard curve. Each well of a 96 well microtitre plate (NUNC®-immuno maxisorp plates, Sigma, St Louis, Missourri, USA) was coated with 100µl

standard or plasma sample and incubated for 18 hours at 4°C. All other steps were carried out at room temperature. Wells were then washed 3 times with wash buffer (10mM phosphate buffered saline, 0.05% tween-20, pH 7.4) before the addition of 200µl blocking buffer (2% skimmed milk, 0.5% BSA, 0.05% gelatin in 50mM carbonate buffer). After 1 hour, wells were washed 3 times with washing buffer. An anti-AGE monoclonal antibody (clone no. 6D12, Trans Genie Inc.) was then diluted 1:1000 in antibody diluent (wash buffer + 0.3% BSA) and 50µl added to each well of the microtitre plate for 2 hours. Samples were then washed for 3 times with wash buffer before the addition of 100µl HRP-conjugated goat anti-mouse immunoglobulins diluted 1:100 in antibody diluent for 1 hour. Following 3 washes, colour development was carried out using 50µl 0.4 mg/ml 1,2-phenylendiamine dihydrochloride in phosphate citrate buffer (50mM phosphate citrate, pH 5). The reaction was stopped by the addition of 100µl 1M H_2SO_4. The absorbance was read at 492 using an ELISA plate reader. Units are expressed as arbitrary units- 1 unit of AGE (uAGE) is equal to 1µg AGE-BSA. Plasma AGEs present in samples ranged from 0.5-21.5 uAGE/ml, mean 6.1±1 uAGE/ml. Intra- and inter-assay variation was <7% and <10% respectively.

2.20.8 STATISTICAL ANALYSIS

Data were analysed with JMP (JMP IN, version 5.0, SAS Institute Inc). Where data were not distributed normally, non-parametric methods (Wilcoxon Rank Sums Test) were used. Correlations between the haemodynamic parameters and

plasma AGEs were derived using multivariate correlations (Spearman's Rho). As arterial stiffening is closely associated with both age and BP, correlations were carried out following correction for these variables. AGEs and biochemical parameters were also examined in relation to vascular stiffening, using a stepwise regression model with PWV as the dependent variable in the first model and AIx as the dependent variable in the second model. All the results are expressed as mean ±SEM and $P<0.05$ was considered significant. Where * is indicated $P<0.05$, ** $P<0.01$, ***$P<0.001$.

Chapter 3

Results

VASCULAR EXTRACELLULAR MATRIX REMODELLING IN RELATION TO AGE AND LOCATION IN THE VASCULATURE

3.1 Aortic MMP-2 Activity and Age

Levels of active MMP-2 (Arbitrary units- Au) in the aorta displayed a positive correlation with age ($r=0.65$; $P<0.001$; Figure 3.1.A). In subjects over 60 years, aortic MMP-2 showed an increase of 46% compared to those less than 60 years (Figure 3.1.B; Table 3.1)

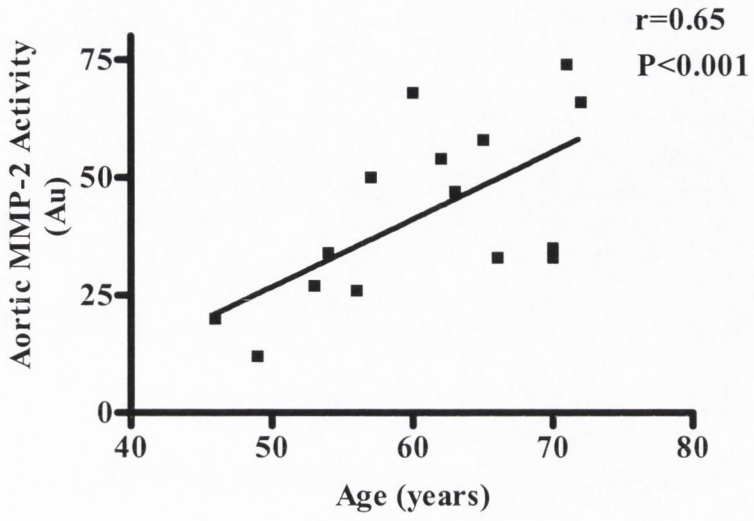
The level of latent MMP-2 was not related to age in the aorta, and there were no differences in aortic latent MMP-2 levels from subjects under and over 60 years (Table 3.1). The regulator of MMP-2 activity, TIMP-2 was not related to age in the aorta

The latent or active forms of MMP-9 were not related to age in the aorta (Table 3.1)

Table 3.1: Expression of Latent and Active MMP-2 and -9 in the Aorta

Enzyme:	Aortic Enzyme Expression (Au) Subjects <60 Years (n=6)	Aortic Enzyme Expression (Au) Subjects≥60 Years (n=9)
Latent MMP-2	110±7	119.7±8
Active MMP-2	28.2±5	52.0±5*
Latent MMP-9	59.2±17.5	62.7±9
Active MMP-9	7±2	11±3

Figure 3.1
A



B

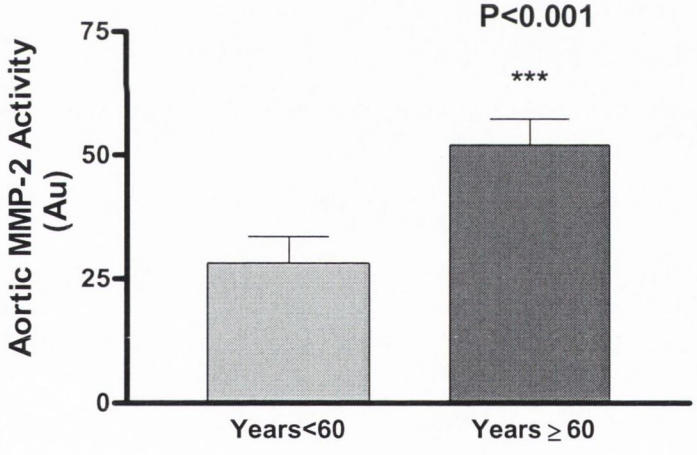


Figure 3.1: (A) Activated MMP-2 (Au) in the aorta and age (years) and (B) MMP-2 activity (Au) in aortic tissue from subjects undergoing coronary artery bypass grafting under 60 years (n=6) and over 60 years (n=9).

3.1.2 Vascular MMP-2 Activation and Age in the Aorta

An activation ratio was calculated as the percentage of the activated enzyme present in relation to the latent form. The ratio of MMP-2 activation from the latent enzyme in the aorta also displayed a positive correlation with age (r=0.63; P<0.05; Figure 3.2).

Figure 3.2

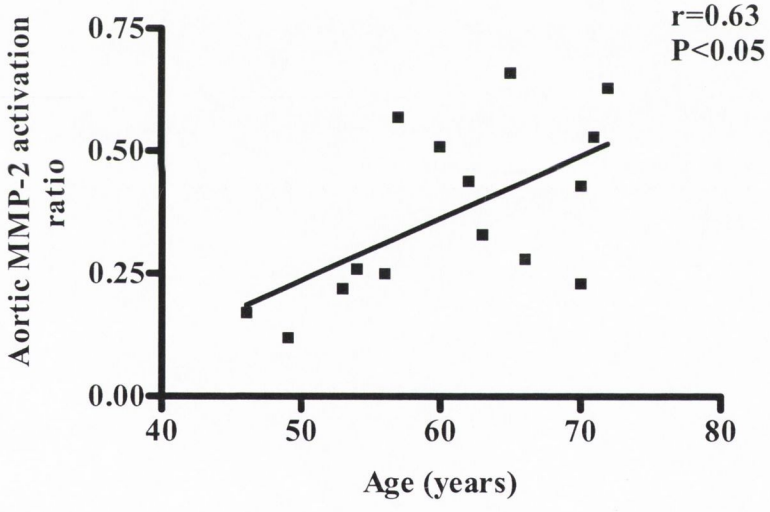


Figure 3.2: MMP-2 activation ratio in the aorta and age (years) of patients undergoing coronary artery bypass grafting (n=15).

3.1.3 *Localization of Gelatinase Activity Within the Aorta*

In the aorta gelatinolytic activity was observed in all layers of the vessel wall (Figure 3.3A). Gelatinolytic activity, visible as fluorescence on a dark background, was greatest in the intimal layer (122±8 pixels) and was also present in the media (56±20 pixels) and adventitia (91±5 pixels). EDTA a potent inhibitor of MMP activity was added to the substrate buffer to test for confounding protease activity (Figure 3.3).

Figure 3.3

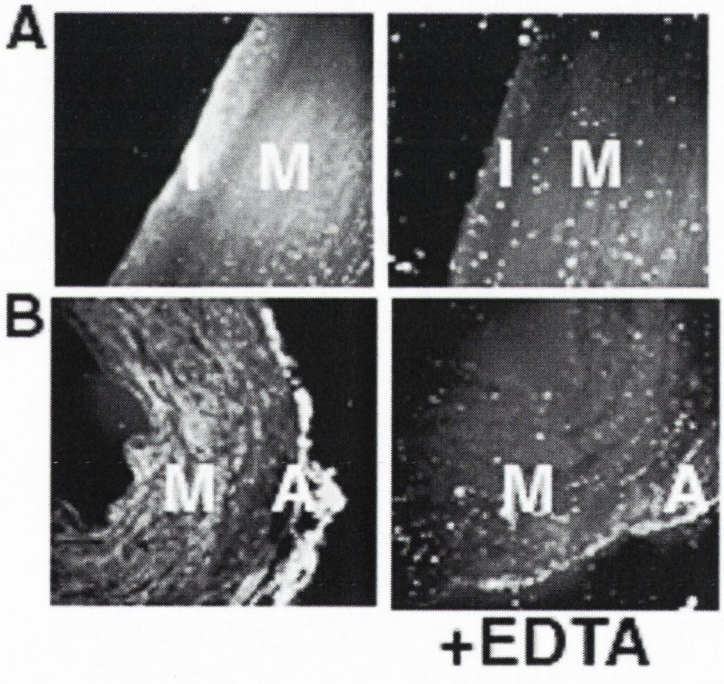


Figure 3.3: Localization of gelatinase activity within the (A) aorta and (B) IMA (internal mammary artery) of patients undergoing coronary artery bypass grafting (n=15). *I=intima, M=media, A=adventitia.*

3.1.4 Vascular MMP Activity and Age in the IMA

In contrast to aortic tissue, active MMP-2 was not related to age in the IMA. Levels of latent MMP-2 and latent and active MMP–9 also showed no correlation with age in the IMA. There were no differences between latent and active MMP-2 and -9 levels in the aortic tissue from subjects under and over 60 years (Table 3.2). The regulator of MMP-2 activity, TIMP-2 was not related to age in the IMA, and was similar in the aorta of subjects under 60 years and over 60 years.

Table 3.2: Expression of latent and active MMP-2 and -9 in the IMA

Enzyme:	IMA Enzyme Expression (Au) Subjects <60 Years	IMA Enzyme Expression (Au) Subjects≥60 Years
Latent MMP-2	106.8±12	109.0±6
Active MMP-2	30.6±7.4	28.9±5
Latent MMP-9	77.0±13	72.5±11
Active MMP-9	8.4±3	6.9±2.4

3.1.5 Localization of Gelatinase Activity Within the IMA

Diffuse gelatinolytic activity could also be seen in all layers of the IMA (Figure 3.3.B). Fluorescence intensity was present to an almost equal extent in the intima (31±13 pixels) and media (31±15 pixels). Stronger gelatinolytic activity was present in the adventitia (58.7±41 pixels) of the IMA compared to the intima and media layers.

3.1.6 Comparison of MMP-2, -9 and TIMP-2 in the Aorta and IMA

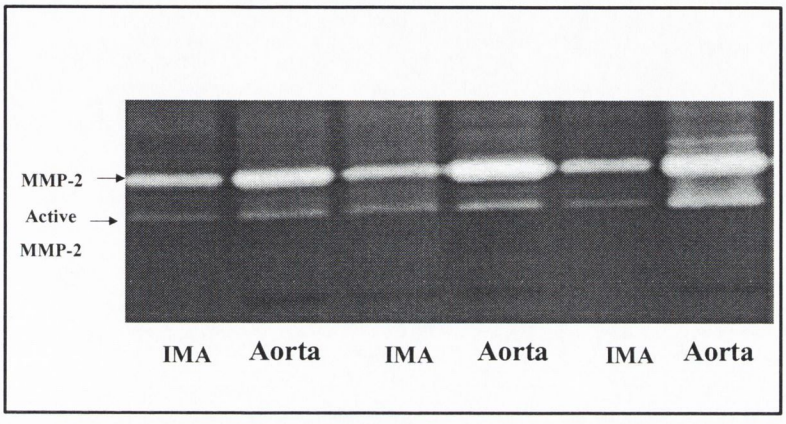
MMP-2 activity, which was positively correlated with age in the aorta, was 36% higher in this artery compared to matched IMA samples (42.5±2 v 27.2±1Au; P<0.001; Figure 3.4). Latent MMP-2 expression and latent and active MMP-9 (which was not related to age in either vessel) was similar in the aorta and IMA (Figure 3.5 A, B and C respectively). TIMP-2 was detected in 14 out of 15 aortic tissue samples, but in only 4 out of 15 IMA samples. TIMP-2 levels were higher in the aorta compared to matched IMA samples (33.2± 42 v 6.5±15Au; P<0.001; Figure 3.6).

3.1.7 Disease State and Vascular MMP-2 Activity

Patients were grouped according to the presence of hypertension, number of vessels affected by coronary artery disease, history of a coronary event and smoking status to assess a possible pathological contribution to increased MMP-2 activity in relation to age. There was no relationship between any of these risk factors and MMP activity in the aorta or IMA (Table 3.3). Furthermore, the relationship between aortic MMP-2 activity and age remained statistically significant ($P<0.05$) after adjustment for disease state (the presence of hypertension and the number of vessels affected by coronary artery disease) and other cardiovascular risk factors (smoking status and history of a coronary event).

Figure 3.4

A



B

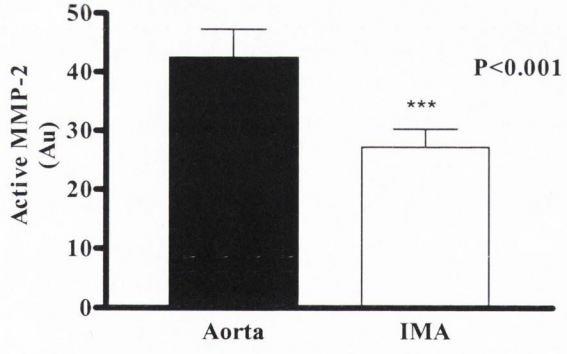
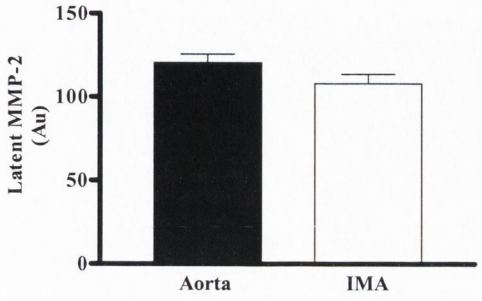


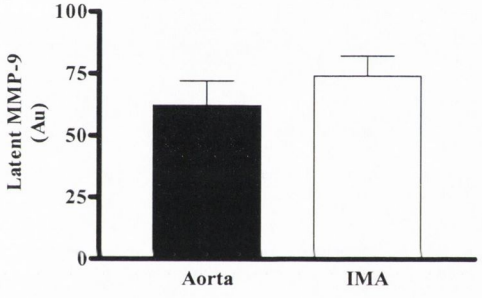
Figure 3.4: (A) Typical zymogram illustrating MMP-2 activity in whole tissue samples of the aorta and IMA (Internal mammary artery). (B) Comparison of active MMP-2 (Au) in the aorta and IMA (internal mammary artery) in patients undergoing coronary artery bypass grafting (n=15).

Figure 3.5

A



B



C

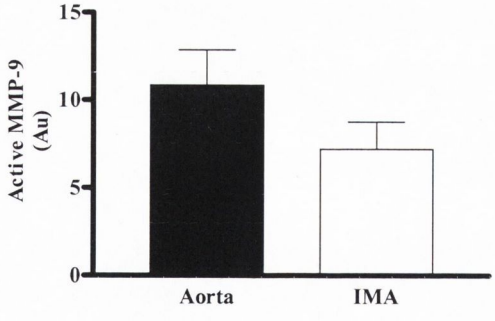


Figure 3.5: Comparison of (A) latent MMP-2 (Au) (B) latent MMP-9 (Au) and (C) active MMP-9 (Au) in the aorta and IMA (internal mammary artery) in patients undergoing coronary artery bypass grafting (n=15).

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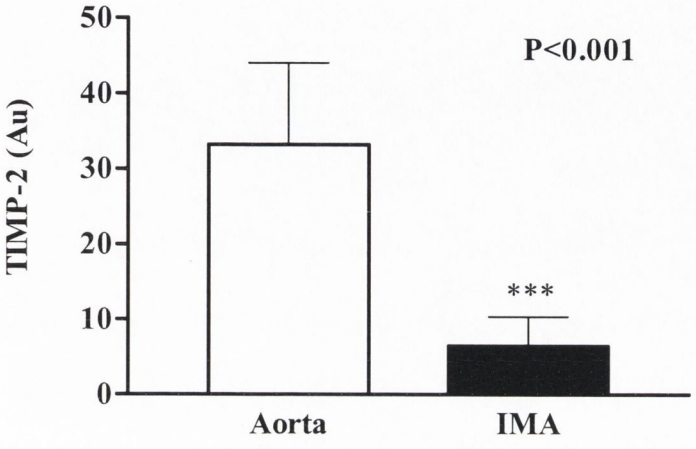
Figure 3.6

A



B

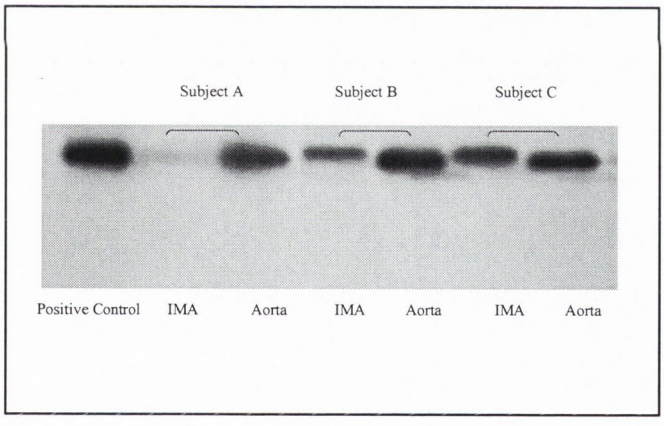


Figure 3.6: A. Comparison of TIMP-2 expression (Au) in the aorta and IMA (internal mammary artery) and B. Example Western blot showing TIMP-2 expression in the aorta and IMA (internal mammary artery) in patients undergoing coronary artery bypass grafting (n=15).

Table 3.3: Aortic MMP-2 (Au) Activity in Relation to Disease and Smoking Status.

Characteristic	Aortic MMP-2
Normotensive v hypertensive	45.8±8 v 40.2±2
No coronary event v coronary event	45.4±2 v 39.9±2
Non-smoker v smoker	47.1±2 v 38.4±2

VASCULAR EXTRACELLULAR MATRIX REMODELLING IN RELATION TO ARTERIAL STIFFENING

3.2.1 Aortic Collagen Content

Mean collagen concentration was higher in the aortic intima (50.16±5.0%) compared to the aortic media (42.9±8%; Figure 3.7). Intimal collagen ranged from 45.0% to 57.0%, while medial collagen showed greater variation (33.0% to 55.2%). As expected collagen concentration was highest in the adventitia (76.01±7.7%) and ranged from 69.4% to 84.2%.

Figure 3.7

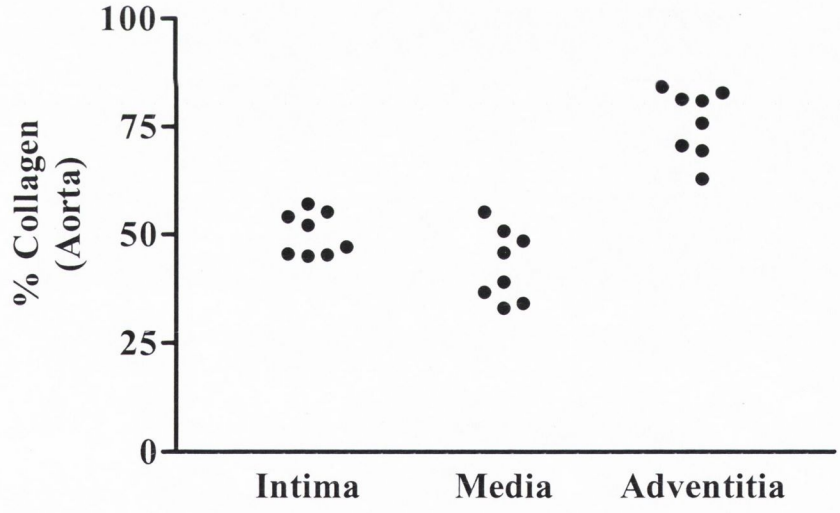


Figure 3.7: Comparison of collagen concentration (%) in the aortic intima, media and adventitia in patients undergoing coronary artery bypass grafting (n=8).

Figure 3.8

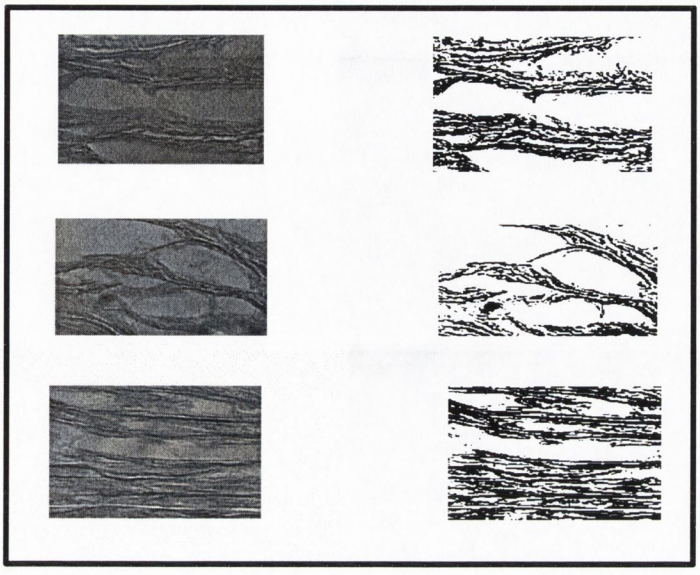


Figure 3.8: Left hand column: arterial medial tissue sections from 3 subjects stained with Sirius red and in right hand column corresponding masks created to measure area covered by collagen (X10 magnification).

3.2.2 IMA Collagen Content

Small amounts of collagen were only occasionally detected within the small intima of the IMA (mean 6.2±4.3μm). Within the media of IMA, the mean collagen concentration was 29.2±6%, ranging from 19.8% to 36.9%. The mean

collagen content in the adventitia of the IMA was 66.3±10, which ranged from 49.7% to 75.5%.

3.2.3 Aortic Collagen, Arterial Stiffness and Age

Neither intimal nor adventitial collagen concentration was related to arterial stiffness. Medial collagen concentration increased with increased arterial stiffness although this did not reach statistical significance (r=0.67; P=0.08; Figure 3.9). Collagen content in the intima, media or adventitia was not related to age.

Figure 3.9

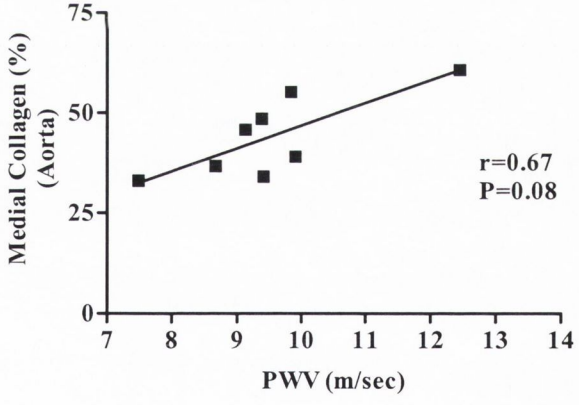


Figure 3.9: Collagen content (%) in the aortic media in relation to arterial stiffness (PWV m/sec) in patients undergoing coronary artery bypass grafting (n=8).

3.2.4 IMA Collagen, Arterial stiffness and Age

Neither the collagen content of the IMA media or adventitia was related to arterial stiffness or age.

3.2.5 Intimal Thickness, Arterial Stiffness and Age

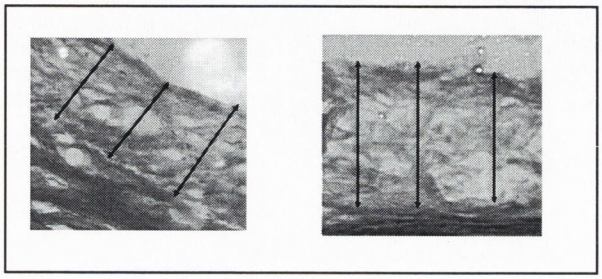
The mean intimal thickness of the aortic samples ranged from 105.3μm to 287.4μm. Aortic intimal thickness was not related to arterial stiffness but displayed a positive correlation with age (Figure 3.10). In the IMA, the mean intimal thickness was 6.2±4.3μm, and was undetectable in 1 tissue sample. The intimal thickness of the IMA was not related to age or arterial stiffness.

3.2.6 Aortic Elastin Architecture

In the aorta, elastin was abundant only in the medial layer, and showed fragmentation to various degrees in all tissue samples. As is typical of large elastic arteries there was no clearly defined IEL or EEL, and the elastic lamellae adjacent to the aortic intima were fragmented in all tissue sections. Small elastic fibres were visible throughout the aortic intima and adventitia.

Figure 3.10

A.



B.

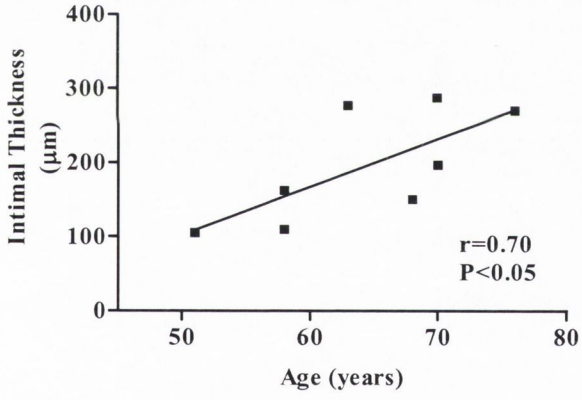


Figure 3.10: A. Diagram illustrating the measurement of intimal thickness in aortic tissue samples. B. Relationship between intimal thickness (µm) and age (years) in patients undergoing coronary artery bypass grafting (n=10).

Figure 3.11

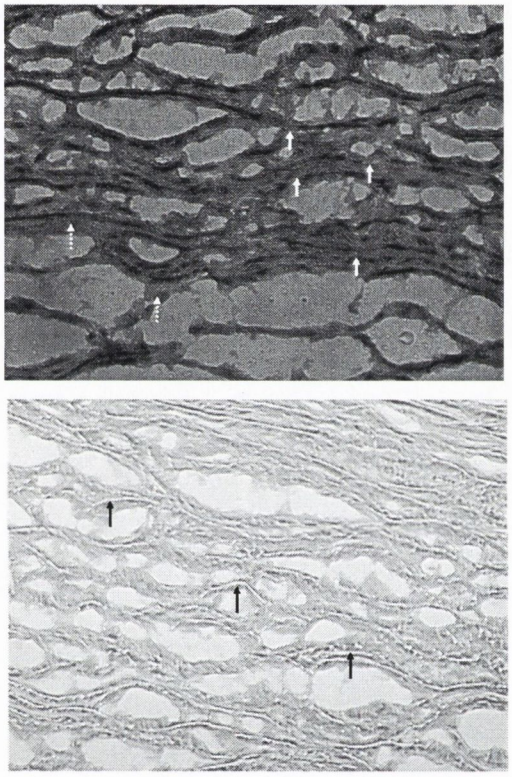


Figure 3.11: Elastin & Collagen Fibre Architecture in the Aorta. In the upper panel, aortic elastin staining reveals areas of elastin fibre fragmentation, indicated by arrows, broken arrows show lighter stained collagen fibres. The lower panel shows collagen staining in aortic tissue for the same subject. The arrows indicate clear areas within collagen fibres, where elastic fibres lie.

3.2.7 IMA Elastin Architecture

As is typical of muscular arteries, elastin staining in IMA tissue sections revealed a clearly defined IEL and EEL. Smaller elastic fibres were present throughout the IMA media, between the IEL and EEL. A large amount of small elastic fibres were also present in the adventitia of the IMA sections. Elastic fibre fragmentation was not present in tissue sections of the IMA

Figure 3.12

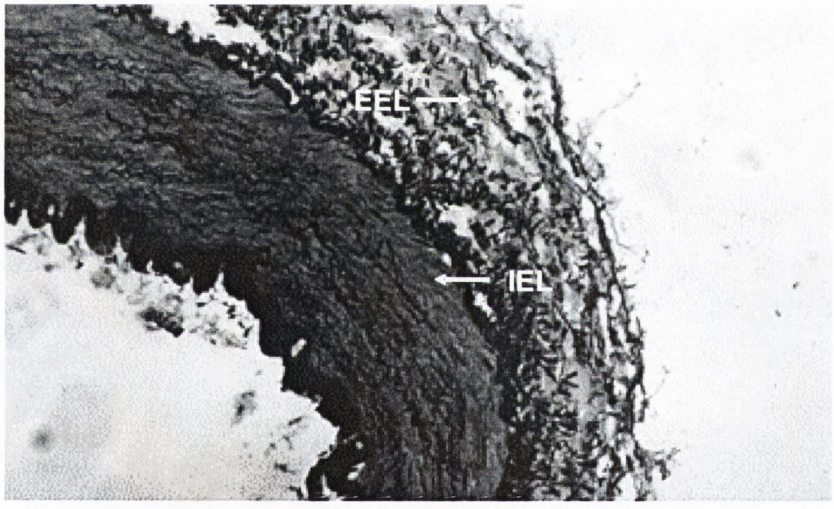


Figure 3.12: Typical Cross Section of Internal Mammary Artery Stained to Illustrated Elastic Fibres (stained black). *IEL= internal elastic lamina, EEL=external elastic lamina.*

3.2.8 Elastin Fragmentation, Arterial Stiffness and Age

The degree of elastin degeneration was considered within the aortic media, based on the disruption of the elastic lamellar structure. While it was evident that there was a trend towards increased elastin fragmentation and lamellar disruption in subjects with stiffer arteries, this did not reach statistical significance (r=0.6, *P*=0.09). Elastin fragmentation was not related to age. As elastin fragmentation was not detected in the IMA, this was not considered in relation to arterial stiffness or age.

Figure 3.13

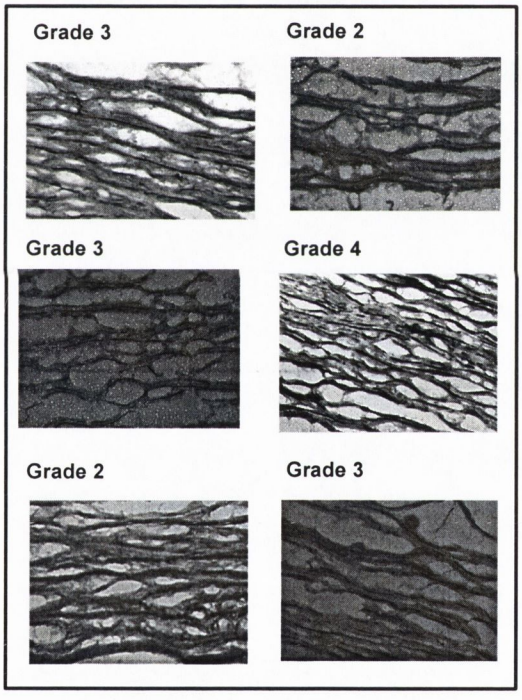


Figure 3.13: Sections of aortic media with elastic fibres stained black, and grade of elastin fragmentation allocated.

3.2.9 *Aortic MMP Activity and Arterial Stiffness*

Neither latent nor activated MMP-2 activity in the aorta was related to arterial stiffness (r=-0.43; P=0.22 and r=-0.17; P=0.63 respectively; Figure 3.15). Latent MMP-9 expression in aortic tissue displayed a positive correlation with PWV (r=0.66, $P<0.05$; Figure 3.15). Latent MMP-2 and MMP-9 expression was not related to age in the aorta. Although activated MMP-2 in aortic tissue was related to age, this did not reach statistical significance (r=0.60; P=0.07).

Figure 3.14

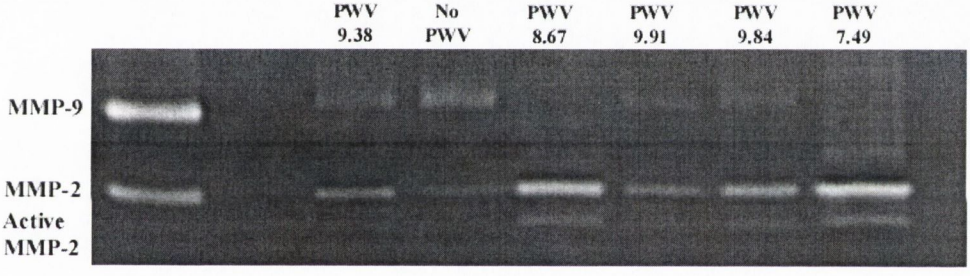
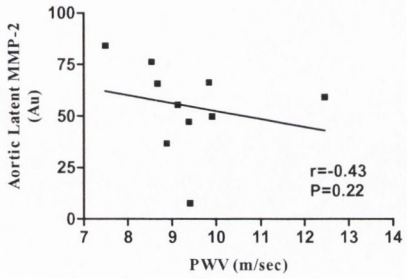


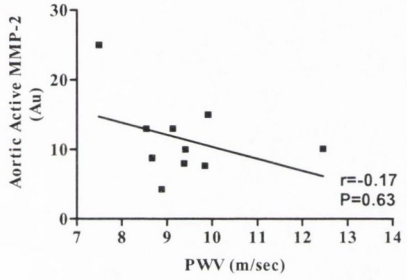
Figure 3.14: Zymogram illustrating MMP activity (Au) in aortic tissue homogenates in relation to PWV (m/sec).

Figure 3.15

A



B



C

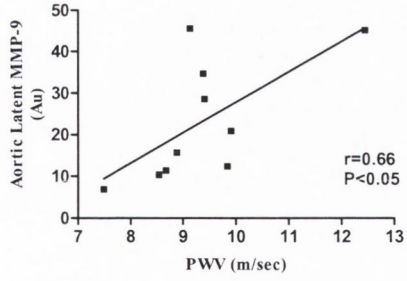


Figure 3.15: A. Aortic latent MMP-2 expression (Au) and PWV (m/sec); B. Aortic activated MMP-2 (Au) and PWV (m/sec); C. Aortic latent MMP-9 expression (Au) and PWV (m/sec) in patients undergoing coronary artery bypass grafting (n=10).

THE RELATIONSHIP BEWTEEN AGE, ARTERIAL STIFFNESS AND CIRCULATING MARKERS OF EXTRACELLULAR MATRIX TURNOVER

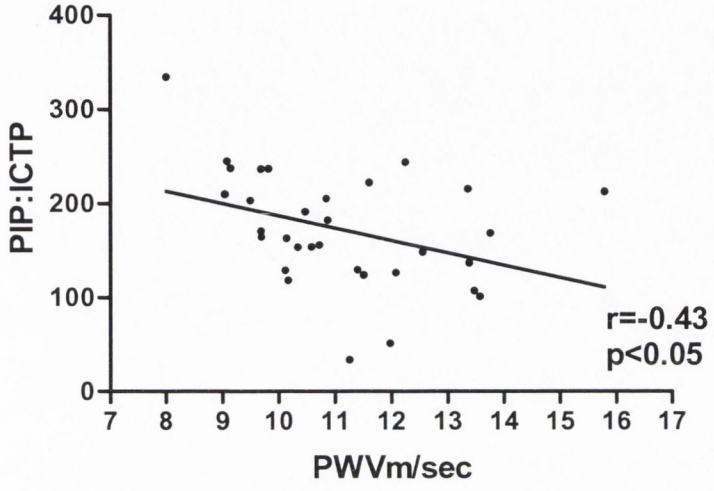
3.3.1 Circulating Metabolites of Collagen Type I, Arterial Stiffness and Age

The PIP:ICTP ratio (a measure of collagen type I turnover-synthesis/degradation) although unrelated to age, displayed a significant relationship with PWV ($r=-0.36$; $P<0.05$). When the relationship was examined separately in the hypertensive and normotensive groups, the relationship between PIP: ICTP ratio and PWV remained in the hypertensive group ($r=-0.43$, $P<0.05$; Figure 3.16A), although was weaker in the normotensive group and did not reach statistical significance ($r=-0.36$; $P=0.13$; Figure 3.16B). The relationship between PIP:ICTP and PWV was not present in the whole group, or in either of the subgroups, when corrected for age MAP.

Plasma PIP (marker of collagen type I synthesis) levels were not related to PWV whereas plasma ICTP levels (marker of collagen type I degradation) displayed a positive correlation with PWV ($r=0.51$;$P<0.001$) which remained significant after adjustment for age and MAP ($P<0.01$). This relationship was not present when the groups were divided into hypertensive and normotensive subgroups. Plasma PIP or ICTP was not related to age.

Figure 3.16

A



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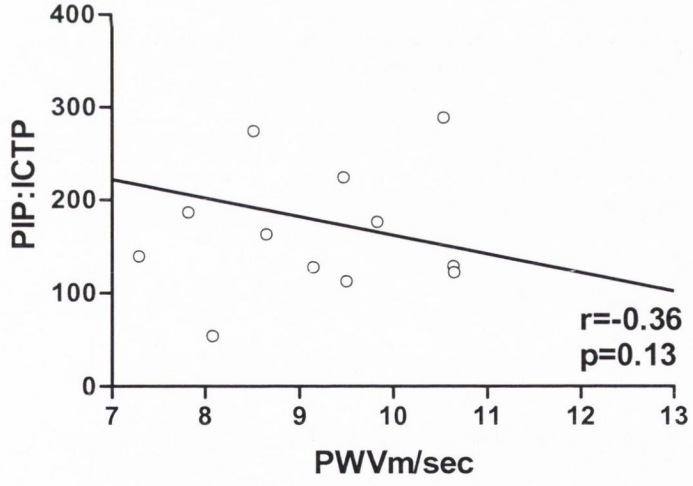


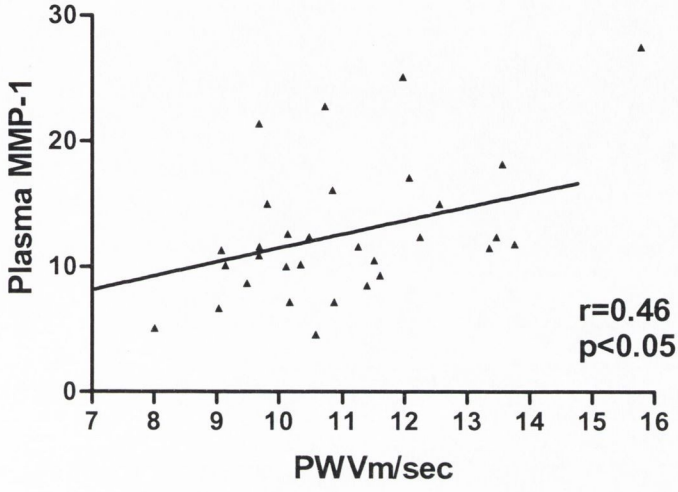
Figure 3.16: Relationship between PWV (m/sec) and PIP:ICTP in (A) hypertensive subjects (n=32) and (B) normotensive subjects (n=14).

3.3.2 Plasma MMP-1, Arterial Stiffness and Age

Plasma MMP-1, the enzyme responsible for native collagen type I degradation, displayed a positive relationship with PWV ($r=0.50$;$P<0.001$) which remained significant after adjustment for age and MAP ($P<0.01$). This relationship was also similar when both hypertensive and normotensive subjects where analysed individually ($r=0.46$;$P<0.05$; Figure 3.17A, $r=0.67$;$P<0.01$; Figure 3.17B respectively). In a stepwise regression model with PWV as the dependent variable, only age and MMP-1 (but not PIP:ICTP, ICTP or PIP) independently predicted PWV ($R^2=63$; $P<0.0001$). MMP-1 also displayed a weak positive relationship with both age ($r=0.33$;$P<0.05$) and plasma ICTP levels ($r=0.38$;$P<0.05$). There was a negative relationship with the TIMP-1/MMP-1 ratio and PWV ($r=-0.29$;$P<0.05$). TIMP-1 levels were not related to PWV or age.

Figure 3.17

A



B

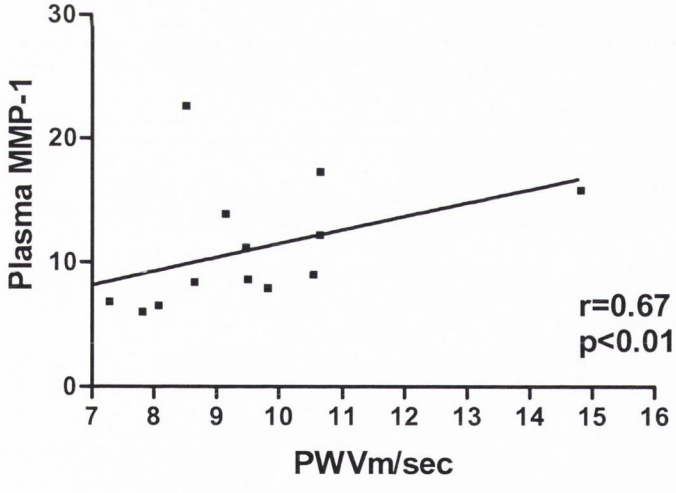


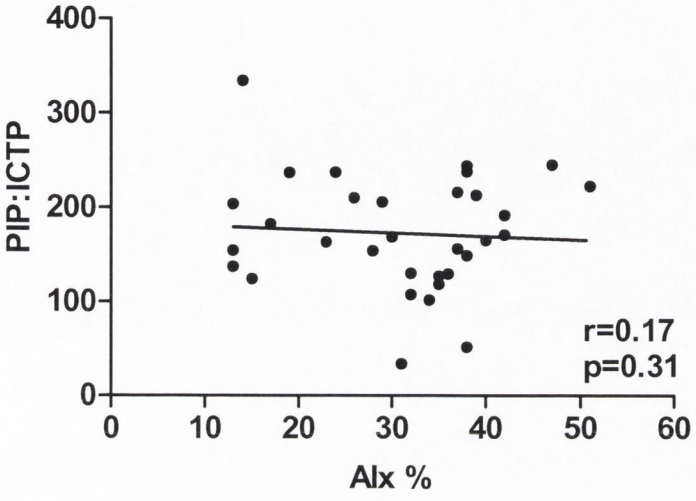
Figure 3.17: Relationship between PWV (m/sec) and MMP-1 (ng/ml) in (A) hypertensive(n=32) and (B) normotensive subjects (n=14).

3.3.3 Circulating Metabolites of Collagen Type I and AIx

The PIP:ICTP ratio also displayed a significant negative relationship with AIx ($r=-0.29$; $P<0.05$) although this was weaker than that for PIP:ICTP and PWV, and was not present when corrected for age and blood pressure. The relationship between PIP:ICTP and AIx was not present when the hypertensives and normotensives were analysed as separate groups (Figures 3.18A, 3.18B). As for PWV, plasma ICTP levels displayed a positive correlation with AIx ($r=0.35$;$P<0.05$) and the relationship was not present when the groups were divided into hypertensive and normotensive subjects.

Figure 3.18

A



B

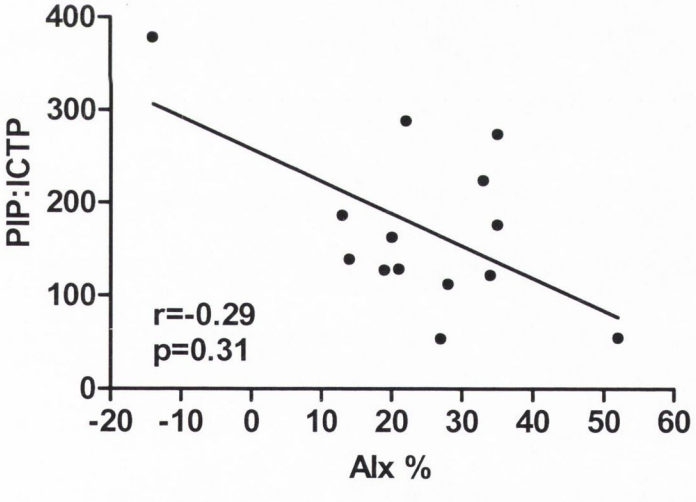


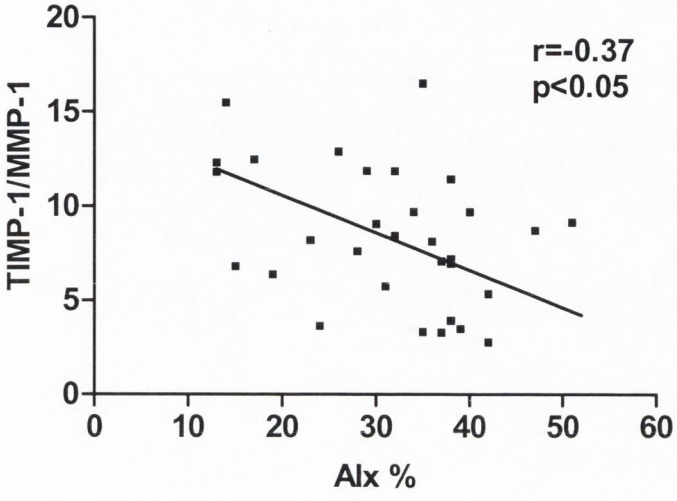
Figure 3.18: Relationship between AIx and PIP:ICTP in (A) hypertensive (n=32) and (B) normotensive subjects (n=14).

3.3.4 Plasma MMP-1 and AIx

Plasma MMP-1 levels displayed a positive relationship with AIx ($r=0.51;P<0.001$) which remained significant after adjustment for age and MAP ($P<0.01$). This relationship was also similar when both normotensive and hypertensive subjects were analysed with AIx individually ($r=0.64;P<0.05$, $r=0.43;P<0.05$ respectively). In a stepwise regression model with AIx as the dependent variable, only age, heart rate and MMP-1 (but not PIP:ICTP, ICTP or PIP) independently predicted AIx ($R^2=0.52;P<0.001$). The TIMP-1/MMP-1 displayed a negative correlation with AIx ($r=-0.42;P<0.05$), which remained when the groups were analysed as hypertensive ($r=-0.37;P<0.05$;figure 3.19A) and normotensive ($r=-0.40;P<0.01$;figure 3.19B) groups. As for PWV, TIMP-1 levels were not related to AIx.

Figure 3.19

A



B

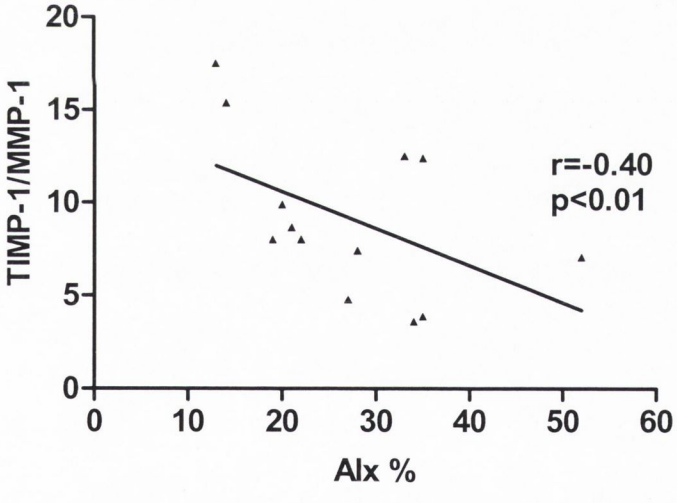


Figure 3.19: Relationship between augmentation index (AIx %) and TIMP-1/MMP-1 in (A) hypertensive (n=32) and (B) normotensive subjects (n=14).

3.3.5 Collagen Type I Turnover in Hypertensives Versus Normotensives

Plasma PIP levels (collagen type I synthesis) were significantly higher in the hypertensive group versus the normotensive group (839 ± 50 versus 648 ± 57ng/ml, $P<0.05$; figure 3.20) although this was unrelated to either PWV or AIx. Plasma ICTP levels (collagen type I degradation) were similar in both hypertensives and normotensives (4.8 ± 0.2 ng/ml versus 4.2 ± 0.4). There was also no statistical difference in the plasma MMP-1 levels between the hypertensive and normotensive subjects (12.7 ± 1 versus 10.8 ±1 ng/ml) respectively. TIMP-1 levels were similar between the hypertensive (93 ± 7 ng/ml) and normotensive group (92 ± 6 ng/ml), as was the TIMP-1/MMP-1 ratio (9 ± 1 versus 8 ± 1).

Figure 3.20

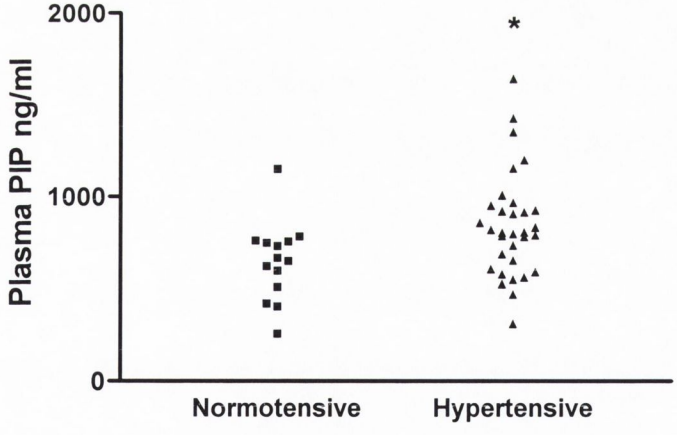


Figure 3.20: Comparison of plasma PIP in hypertensive and normotensive subjects. *P<0.05. (Normotensive n=14, Hypertensive n=32)

3.3.6 Plasma MMP-2 and MMP-9 Levels, Arterial Stiffness and Age

Latent MMP-2 displayed a weak correlation with PWV ($r=0.33;P<0.05$), although the relationship did not remain when corrected for age and MAP, and was not present when the hypertensive and normotensive subgroups were analysed with PWV individually. As expected, activated MMP-2 was not detected in plasma. Latent MMP-9 was not related to PWV, whereas activated MMP-9 was significantly correlated with PWV ($r=0.36;P<0.05$), which remained significant following correction for age and MAP ($P<0.05$). This relationship was not present when the subgroups were analysed individually. TIMP-2 did not display a relationship with PWV, MMP-2 or MMP-9.

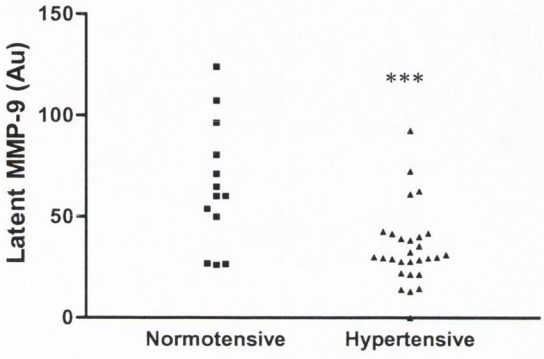
Circulating latent MMP-2 and latent and active MMP-9 and TIMP-2 were not related to AIx or age.

3.3.7 Plasma MMP-2 and MMP-9 in Hypertensives Versus Normotensives

Plasma levels of level MMP-2 were similar in hypertensive and normotensive subjects (53.7±6 versus 50.1±7 Au). Latent MMP-9 levels were depressed in hypertensive subjects compared to normotensive subjects (34.7±4 versus 65.3±9 Au;$P<0.001$;figure 3.21A) whereas active MMP-9 levels were increased in hypertensives compared to normotensive subjects (6.0±1 versus 3.3±1 Au;$P<0.01$;figure 3.21B). TIMP-2 levels were not different between hypertensives (82.2±2 versus 85.5±6 ng/ml) and normotensives.

Figure 3.21

A



B

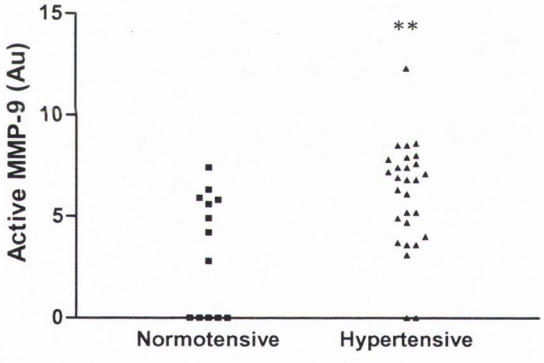


Figure 3.21: Comparison of plasma (A) Latent (Au) and (B) Active MMP-9 (Au) expression in normotensives (n=14) and hypertensives (n=32).

3.4.1 Plasma AGEs, PWV and Age

Plasma AGEs were positively related to PWV ($r=0.48$;$P<0.01$). This relationship remained significant following adjustment for both age ($P<0.01$) and MAP ($P<0.05$). When the relationship between PWV and plasma AGEs was examined in the hypertensive subjects only, the relationship between PWV and plasma AGEs was stronger ($r=0.63$;$P<0.001$;figure 3.22) and was not present in the normotensive subjects alone ($r=0.19$;$P=0.54$). When the relationship between plasma AGEs and arterial stiffness was analysed in the hypertensive subjects using a stepwise regression model, with PWV as the independent variable, only age and plasma AGE levels predicted PWV ($r^2=0.72$; $p<0.0001$) with no contribution from BP, gender or heart rate. Plasma AGE levels also displayed a correlation with age ($r=0.33$;$P<0.05$).

Figure 3.22

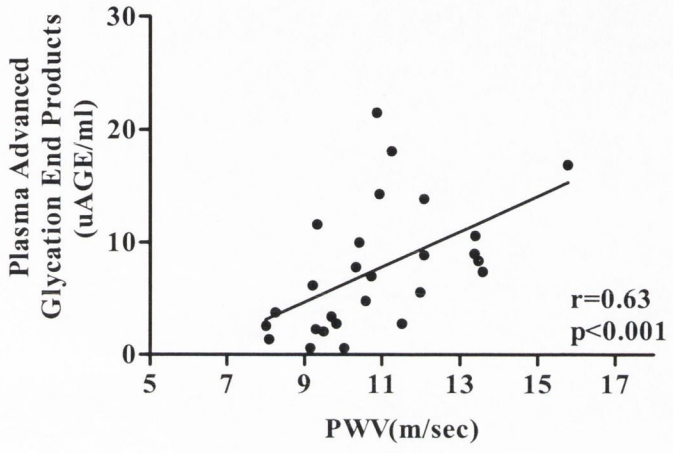


Figure 3.22: Relationship between PWV (m/sec) and plasma AGEs (uAGE/ml) in hypertensive subjects (n=27).

3.4.2 Plasma AGEs and AIx

There was no relationship between AIx and plasma AGE levels when all subjects were analysed in the same group (r=0.21; P=0.24) and also when normotensive subjects (r=-0.10; P=0.77) and hypertensive subjects (r=0.13; P=0.52) were analysed individually. There was however a weak positive correlation between MAP and plasma AGE levels (r=0.33;P<0.05)

3.4.3 Relationship Between Plasma AGEs, PWV, and Biochemical Markers

In the entire group, there was no relationship between plasma AGE levels and glucose (r=0.29;P=0.14). There was no relationship between AGEs and renin, aldosterone, or the aldosterone/renin ratio. Although plasma AGE levels were not related to HDL or triglycerides, there was a significant positive correlation between plasma AGE levels and total cholesterol (r=0.50;P<0.01;figure3.23). There was also no relationship between glucose and PWV or AIx (r=0.27; P<0.10, r=0.09; P=0.06 respectively) and there was also no relationship between PWV and AIx and cholersterol, renin, aldosterone, or the aldosterone/renin ratio.

Figure 3.23

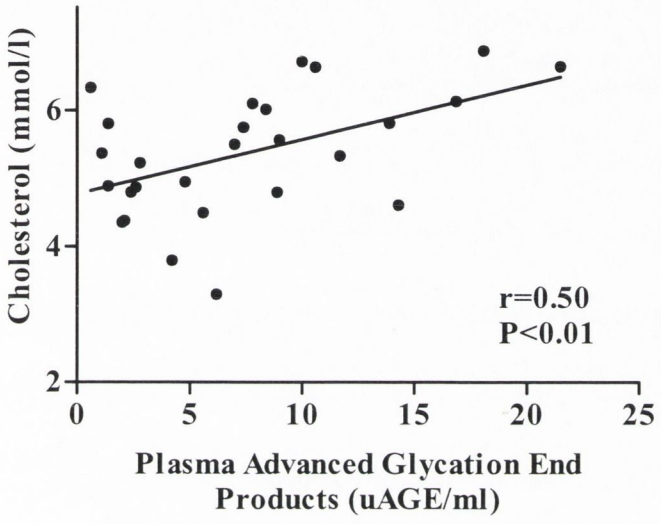


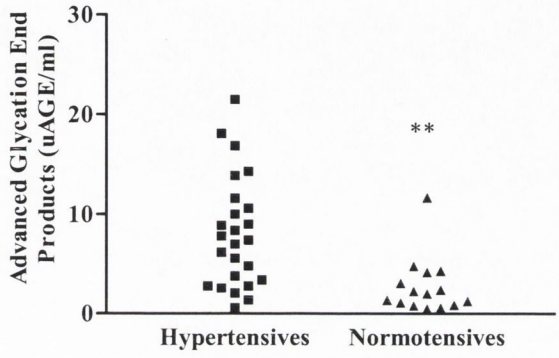
Figure 3.23: Relationship between cholesterol (mmol/l) and plasma AGEs (uAGE/ml) (n=41).

3.4.4 Plasma AGEs in Hypertensives versus Normotensives

Plasma AGE levels were increased in the hypertensives compared to the normotensive subjects (7.6±1.1 versus 3.0±1.0 uAGE/ml; $P<0.01$; figure 3.24A), which remained significant when corrected for age (6.6±0.3 versus 5.2±0.4 uAGE/ml; $P<0.05$; figure 3.24B). There was no difference between smokers (n=12) and non-smokers (n=29) in the group as whole (P=0.81) and when smokers were compared to non-smokers in the hypertensive (P=0.056) and normotensive groups (P=0.71).

Figure 3.24

A



B

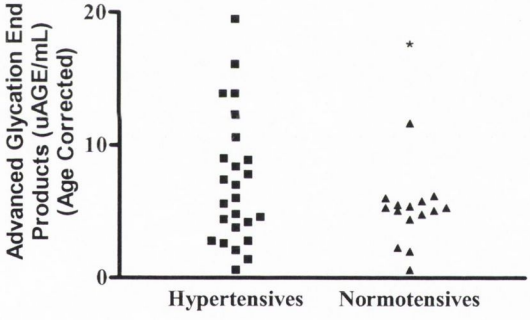


Figure 3.24: (A) Comparison of plasma AGEs (uAGE/ml) in hypertensive (n=27) and normotensive subjects (n=14) and (B) age-corrected plasma AGEs (uAGE/ml) in hypertensive (n=27) and normotensive (n=14) subjects.

Chapter 4

Discussion

VASCULAR EXTRACELLULAR MATRIX REMODELLING IN RELATION TO AGE AND LOCATION IN THE VASCULATURE

4.1 Aortic MMP-2 Activity and Age

The data from this study provides evidence that ageing is associated with increased MMP-2 activity in the human aorta. Levels of the active, but not the latent enzyme displayed a highly significant correlation with age ($r=0.65$; $P<0.001$), and in addition, in subjects over 60 years, there was an increase of 46% in aortic MMP-2 activity compared to those less than 60 years. Studies using animal-models of ageing that have been used to investigate age-related changes in the vasculature (203;204;310) have yielded similar findings. MMP-2 has been shown to be up-regulated in the aorta of old rats and non-human primates (203;204). Increased levels of activated MMP-2 could impact upon various aspects of vascular remodeling. As with all members of the MMP family, MMP-2 has broad substrate specificity, but is reported to have increased affinity for native collagen type IV, V, VII and X as well as denatured collagens (311) and elastin (176).

Tissue inhibitors of MMPs (TIMPS), particularly TIMP-2, regulates MMP-2 activity through direct inhibition (188) or in contrast, through the involvement of MMP-2 activation together with MT-MMP (190;191). This data demonstrates that TIMP-2 levels are not related to age, or MMP-2 expression in the aorta.

4.1.2 Vascular MMP-2 Activation and Age in the Aorta

The age-related increase in aortic MMP-2 activity in humans is also accompanied by an increase in the MMP-2 activation ratio. Therefore while there is an overall change in the quantity of activated MMP-2 in the aorta, the rate of MMP-2 activation from the latent enzyme is directly related to age.

Previously animal studies have yielded inconsistent results with regards to the activation of MMP-2 in relation to age. In age-related vascular remodelling in rats both the latent and activated forms of MMP-2 were up-regulated. (203) However, in non-human primates no significant differences in the latent form of MMP-2 were observed between young and old aortae, whereas there was a three-fold increase in activated MMP-2 in old aortae accompanied by an increase in MMP-2 mRNA (204). Therefore, as in non-human primates, increased activation of the latent enzyme occurs in the human aorta with ageing.

Two main mechanisms control the enzymatic activity of MMPs (180). Before exerting proteolytic activity the latent form of the enzyme must undergo activation. MMP-2 is not activated by serine proteases such as plasmin, as for other MMPs. The activation of MMP-2 is dependent upon other MMPs, namely MT-MMP (191). It has also recently been demonstrated that MT-MMP is associated with the activation of latent MMP-2 in the rat neointima (312), and is increased in the intima of old non-human primates (204).

4.1.3 Localization of Gelatinase Activity Within the Aorta

In situ zymography revealed that although gelatinase activity was present in all layers of the aortic wall, activity was highest in the aortic intima. Increased MMP-2 expression in the intima may affect vascular remodelling in several ways. MMP-2 activity may be in part responsible for the disruption of the IEL (which divides the intima and media into two distinct areas), as it has a high affinity for components of elastic fibres. Increased MMP-2 activity has also been demonstrated in the intima of balloon injured rat carotid arteries. As localisation of MMP-2 to the rat neointima persists beyond the period during which cell recruitment to the neointima is thought to occur, it is suggested MMP-2 may be involved in the control of collagen accumulation in the developing neointima (312).

MMP-2 activity was also present to a lesser extent in the aortic media, where it may also participate in age-related vascular remodelling in various ways. MMP-2 accumulates around the elastic fibres in the aortic media in rats (203) that become fragmented with the age-associated increase in arterial stiffness in humans (313). Another likely possibility is the involvement of MMP-2 in VSMC migration within the human aorta. It has previously been shown that MMP-2 is involved in age-related VSMC migration from the media to the intima. Medial VSMCs are embedded in extracellular matrices that provide barriers to VSMC movement. It has been demonstrated *in vitro* that activation of MMP-2 is a critical step in the migration of VSMCs through a reconstituted basement membrane similar to that which surrounds VSMCs *in vivo* (314). Furthermore, the degradation of not only

basement membranes, but also elastic fibres by MMP-2, could facilitate VSMC migration from the media to the thickened intima.

High gelatinase activity was also present in the adventitia in aortic sections. Although it has been proposed that adventitial staining for MMP-2 in the aorta of young rats is non-specific (203), a high proportion of adventitial gelatinase activity in the present study was abolished when EDTA (a MMP inhibitor) was added to the substrate buffer. It is therefore possible that collagen and elastin may also be subject to degradation by MMPs in the adventitia. In addition, it has also been suggested that adventitial fibroblasts may be involved in neointimal formation and vessel remodelling (315).

4.1.4 Vascular MMP Activity and Age in the IMA

No age-related changes in vascular MMP or TIMP activity were observed in the IMA. As expected, the age-related increase in MMP-2 activity that occurred in the aorta, did not occur in the IMA.

4.1.5 Localization of Gelatinase Activity Within the IMA

Diffuse gelatinolytic activity was present in all layers of the IMA, to a similar extent in the intima and media, with stronger gelatinolytic activity in the adventitia of IMA. As for aortic adventitial gelatinase activity, adventitial gelatinase activity in the IMA was abolished by MMP inhibition, suggesting collagen and elastin degradation by MMPs.

4.1.6 Comparison of MMP-2, -9 and TIMP-2 in the Aorta and IMA

There were no age-related increases in MMP-9 and TIMP-2 activity in the aorta or the IMA. The age-related increase in MMP-2 activity observed in the aorta but not in the IMA is consistent with the knowledge that age-related changes are non-uniform throughout the vascular tree and affect large elastic arteries such as the aorta to a greater extent than muscular arteries (316). However it must also be considered that the aorta is more susceptible to disease than the IMA, which remains relatively free of atherosclerosis (317;318) and is therefore commonly used as a conduit graft vessel. It could therefore be that increased enzyme activity in the aorta may have a pathological basis.

4.1.7 Disease State and Vascular MMP-2 Activity

Patients in this study were therefore classified into groups according to the number of vessels affected by coronary artery disease, hypertension or smoking history to observe possible relationships with MMP activity and other cardiovascular risk factors. MMP-2 activity in the aorta was not related to any of the other risk factors and the relationship between aortic MMP-2 activity and age remained statistically significant when adjusted for all the above cardiovascular risk factors.

4.1.8 Summary

Ageing is associated with large artery remodelling resulting in increased intima-media thickness and vascular stiffness (10;319). Increased concentrations of

MMP-2 have recently been associated with this age-associated vascular remodelling in the aorta of rats and non-human primates. This study demonstrates that an age-associated increase in MMP-2 activity occurs in the human aorta. The increase in MMP-2 activity, which does not occur in the IMA, is consistent with the knowledge that age-related changes occur in a non-uniform manner depending upon location in the vascular tree. MMP-9 which belongs to the same subgroup of the MMPs as MMP-2 does not increase with age in the human aorta.

4.1.9 Study Limitations

Due to time constraints and the difficulty in obtaining vascular tissue from human subjects the number of patients involved in this study is relatively small (n=15). Although the results obtained in the study were highly significant in relation to MMP-2 activity and age, a study with a greater number of subjects would be of more benefit. A larger study would allow the lack of a relationship between MMP-9 and TIMP-2 and ageing to be confirmed.

Although exclusion criteria included patients suffering from inflammatory diseases, cancer, infection or other major medical illness, 9 patients in this study were suffering from hypertension, a condition known to influence MMP activity (208;216;217). Patients in this study were classified into groups according to the number of vessels affected by coronary artery disease, hypertension or smoking history to observe possible relationships with MMP activity, which was not

observed, however vessels obtained from patients not suffering from cardiovascular disease would be of more benefit.

11 patients were receiving a beta-blocker at the time arterial tissue removal while 6 patients were being treated with ACE inhibitors. All patients were also receiving lipid lowering therapy. These drugs have been shown to the have various effects on the cardiovascular system. Beta blockade has been shown reduce cardiovascular protein synthesis (320) and ACE inhibition has also been shown to reduce medial cross sectional area, which could potentially result in altered MMP expression implications in this study (321). As mentioned all patients in this study were also receiving lipid lowering therapy which has been shown to produce various structural changes within the vessel wall. Although most studies have examined the effects of lipid lowering therapy in relationship to coronary plaque stabilization, it has also been shown that such therapy induces direct effects on the arterial wall such as the prevention of aortic SMC proliferation (322). Such arterial effects could again have consequences for alterations in MMP expression for the subjects involved in this study.

4.1.10 Future Studies

The activation of MMP-2 is dependent upon other MMPs, namely MT-MMP (191) which has recently been demonstrated to be associated with the activation of latent MMP-2 in the rat neointima (312). MT-MMP has also been shown to be increased in the intima of old non-human primates (204). MT-MMP may therefore play an important role in age-related MMP-2 activity in the human aorta

and warrants further investigation, in conjunction with the investigation of aortic MMP-2 activity.

The investigation of aortic and IMA MMP-2 activity provided information regarding MMP-2 activity in the vessel wall as a whole. Although *in situ* zymography localised gelatinase (MMP-2 & MMP-9) activity within specific aortic compartments, the use of antibodies directed only at MMP-2 would provide additional information as to the specific regions of the aorta where the age-related increase in MMP-2 activity occurs. Such information would also provide further insight into the consequences of aortic MMP-2 up-regulation. The specific localisation of these enzymes within aortic compartments in a subsequent study would provide valuable additional information as to the role of these enzymes in age related remodelling.

The present data, which is statistically significant, despite the small numbers involved in the study, warrants a similar study being carried out on a larger scale. Larger subject numbers are needed to confirm the lack of a relationship between MMP-9 and TIMP-2 and ageing in the aorta. In addition, TIMP-1 expression, which also regulates MMP-9 activity should also be explored.

Although potentially difficult to obtain, it would be optimal to carryout this study using tissue from subjects not suffering from cardiovascular disease as discussed in section 4.1.9. In addition, a greater age range of subjects (i.e. children to the very elderly) included in the study would provide a greater insight into the relationship between vascular MMP activity and age.

4.1.11 Conclusion

Ageing is associated with increased aortic MMP-2 expression and activation. The relationship is not present in the smaller muscular artery, the IMA. Vascular MMP-9 and TIMP expression are not related to ageing in the human aorta or IMA.

4.2.1 Aortic Collagen Content

The results of this study show that the mean collagen concentration in the media of the ascending aorta is 42.9%. There is remarkably little previous data available detailing collagen distribution in human aortae. This is in part due to the huge variation in the proportion of structural components, depending upon the specific region of the aorta. The proportions of collagen and elastin vary at different aortic locations because the physiological requirements of the vessel are different depending upon proximity to the heart (323). In the thoracic aorta collagen concentration has been estimated at around 37%. In this study, aortic tissue samples were removed from the ascending aorta, which begins at the aortic valve and connects to the left ventricle. The ascending aorta therefore directly receives the force of blood ejection from the heart. It has been shown that medial thickness and the amount of elastic lamellae are greatest where the aorta is in closest proximity to the heart (324). In keeping with this, the lower medial collagen content previously demonstrated in the thoracic aorta, is most likely related to the increased proximity of the ascending aorta to the heart.

The mean collagen concentration within the intima of the ascending aorta was 50%. This high proportion of intimal collagen in this region of the aorta may also be a response to the high pulsatile force received from the heart. Arterial intimae are reported to be composed of collagenous bundles, VSMCs and some elastic

fibres. However there is no previous data detailing collagen content within the aortic intima.

4.2.2 IMA Collagen Content

As expected the mean collagen content of the IMA, a smaller muscular artery, was significantly lower than that of the aorta, ranging 19.8% to 36.9%, being higher in the adventitia which lends the vessel structural support.

4.2.3 Aortic Collagen, Arterial Stiffness and Age

Aortic collagen content, intimal thickness, elastic fibre architecture and matrix-degrading activity were examined in relation to arterial stiffness. These measurements were also related to age. The results demonstrate that while various aspects of vascular structural alterations are related to age, others are related only to measurements of vessel stiffness. Although aortic intimal or adventitial collagen content was not related to PWV, medial collagen concentration showed a relationship with arterial stiffness but not with age. This relationship between medial collagen concentration and arterial stiffness did not reach statistical significance although this is most likely related to the small group size. It is difficult to compare these results directly to those carried out in the vasculature of rats, as these studies have often been carried out in relation to hypertension and not directly to arterial stiffening. However, as arterial stiffening occurs during hypertension (11), changes occurring in the hypertensive aorta are of relevance to aortic stiffening. Aortic collagen accumulation is reported in the

aorta of SHRs as opposed to their normotensive counterparts WKY rats (325). Aortic collagen accumulation in the hypertensive rats was prevented by treatment with the aldosterone antagonist spironolactone. This is of interest to the present study because it is now recognized that subjects with hyperaldosteronism have increased arterial stiffness (326).

The results of the work carried out in this chapter, demonstrate a direct relationship between aortic medial collagen accumulation and arterial stiffness. In addition, aortic collagen concentration was related specifically to arterial stiffness and not to age, for which exist conflicting reports in relation to vascular collagen accumulation. This lends support to the possibility that aldosterone antagonism reduces arterial stiffness in humans through the prevention of collagen accumulation as demonstrated in rats, in addition to possible short-term functional alterations.

4.2.4 IMA Collagen, Arterial stiffness and Age

As expected, the collagen content of the IMA media or adventitia was not related to PWV, which is a measure of the stiffness of the aorta. Neither was the collagen content of the IMA media or adventitia related to age

4.2.5 Intimal Thickness, Arterial Stiffness and Age

Despite the relationship between medial collagen content and arterial stiffness, no relationship was found between intimal collagen and arterial stiffness. Neither was there a relationship between arterial stiffness and aortic intimal thickness. Aortic intimal thickness was however positively related to age. Although not all

species form an intima unless their arteries are injured, in humans, the intima develops spontaneously after birth (29). These alterations occur in the absence of cardiovascular disease, although intimal thickening is known to progress at greater rate in atherosclerotic arteries (30;327). In the thickened aortic intima of aged rats, the intima is composed of VSMCs, collagen, fibronectin, proteoglycans and interstitial cell adhesion molecule (ICAM-1) (328). In addition, increased MMP-2 activity was present in the thickened intima of aged rats (328). In the results discussed in section 4.1, increased MMP-2 activity was also demonstrated in aged human aorta. This activity was highest in the thickened aortic intima, which as demonstrated, also increases with age but not with arterial stiffness. The increased MMP-2 activity may be directly involved in the subsequent thickening of the aortic intima with age, through the degradation of the IEL and the mediation of VSMC migration. Despite the lack of an association between intimal thickness and arterial stiffness in this study, previous non-invasive studies has correlated carotid intimal-medial thickening (which is closely related to aortic intimal-medial thickening (329)) with arterial stiffness in elderly individuals (330). This positive relationship between arterial stiffness and carotid intimal-medial thickness may be explained on the basis that the thickness of the intimal + medial layers is considered. Therefore increased thickness of the medial layer, which represents the main basis of the mechanical properties of arteries and increases with increased arterial stiffness, may be more important in determining arterial stiffness than intimal thickness. Although the number of subjects in this study is small and came from a homogenous population with coronary artery

disease, the measurement of intimal thickness in the aorta was extremely precise, and still showed no relationship with measures of aortic stiffness. In addition, a recent study concluded that carotid intima-media thickness and arterial stiffness are not related and represent two separate entities of vascular damage, i.e. subclinical atherosclerosis and vascular compliance respectively (331).

4.2.6 Aortic Elastin Architecture

As expected, elastin was abundant in the aortic medial layer, and showed no clearly defined IEL or EEL. Fragmentation was present to various degrees in all tissue samples as expected for subjects in this age range, as the ageing process can be visualised microscopically as fragmentation and loss of regularity in elastic layers of the medial layer.

4.2.7 IMA Elastin Architecture

A clearly defined IEL and EEL was present in all IMA sections, as is typical of muscular arteries, and did not show elastin fibre fragmentation.

4.2.8 Elastin Fragmentation, Arterial Stiffness and Age

While intimal thickness does not seem to be directly related to arterial stiffness, these results highlight the importance of elastin fragmentation in aortic stiffening. Although this did not reach statistical significance, the relationship between elastin fragmentation and arterial stiffness was stronger than that for age and elastin fragmentation. Degradation of elastic fibres has obvious implications for

arterial stiffness and has previously been demonstrated in old rats (310), although arterial stiffness was not quantified.

4.2.9 Aortic MMP Activity and Arterial Stiffness

Latent aortic MMP-9 displayed a positive correlation with PWV, suggesting that MMP-9 activity may be directly involved in elastin fragmentation also displayed in stiff arteries. In addition to the evidence from this study, MMP-9 has previously been related to arterial stiffness. As previously discussed, the MMP-9 genotype has been shown to influence large artery stiffness. The genotype associated with carriers of the T-allele which results in greater MMP-9 mRNA and protein levels, was quantified in vascular tissue and was related to increased arterial stiffness as assessed by Doppler velocimetry (246). In addition, a relationship between plasma MMP-9 levels and PWV was demonstrated in a population with isolated systolic hypertension in a recent study (219). In addition, while MMP-9 levels were increased in the hypertensives, there was also a relationship between MMP-9 levels and PWV in the healthy control population. As with the results from section 4.1, activated MMP-2 displayed a positive relationship with age, although this did not reach statistical significance.

4.2.10 Summary

Increased medial collagen content of the human aorta is related to increased aortic stiffness. Subjects with stiffer arteries also display increased fragmentation of the

aortic elastic lamellae and aortic latent MMP-9 expression. MMP-2 was not related to vascular stiffness.

Increased aortic intimal size was related to age but not to measurements of vascular stiffness. In the IMA, collagen and elastin morphology and intimal thickness was not related to vascular stiffness or age.

4.2.11 Study Limitations

As for section 4.1.9, increasing the number of subjects in this study would be of benefit. This highlighted by the strong, yet statistically insignificant correlation coefficients (elastin fragmentation and PWV; aortic MMP-2 and age).

Also as in section 4.1.9, both smokers (n=4) and hypertensive subjects (n=4) were included in the study, again a homogenous population of healthy non-smokers would be more beneficial in this study.

7 patients were receiving a beta-blocker at the time of haemodymanic measurement and arterial tissue removal, while 2 patients were being treated with ACE inhibitors. In addition, 8 of the 10 patients were also receiving lipid lowering therapy. As beta blockade has been shown reduce cardiovascular protein synthesis (320), treatment may result in alterations in the parameters measured in this study, such as intimal thickness. ACE inhibition has also been shown to reduce medial cross sectional area, which could have obvious implications in this study, such as altered collagen content measurements (321). In addition, ACE inhibition has been shown to reduce vascular wall elastin content in SHRs (332). Aside from the direct implications in relation to the measurement of elastin and

collagen in the vascular wall in this study, such changes could result in altered MMP activity. As all patients in this study were also receiving lipid lowering therapy, which have been shown to produce various structural changes within the vessel wall (322), arterial effects could have consequences for alterations in measurements of vascular collagen, elastin and MMP expression.

Due to the limited amount of vascular tissue available, collagen and elastin staining could only be carried out for 8 of the 10 subjects as stated in section 2.9.

4.2.12 Future Studies

The strong but statistically insignificant correlations in this study, highlight the need for a further study to be carried out with greater subject numbers. Previous studies have not directly compared measurements of vascular stiffness in humans with direct examination of human arteries. While restrictions with sample tissue size allowed for a limited number of measurements to be carried out, various histological studies in aortic tissue following the measurement of PWV would be of great benefit. Perhaps of greatest interest, would be the simultaneous investigation of aortic aldosterone receptor expression. Aldosterone is a hormone capable of inducing collagen synthesis (333;334) has receptors in the aorta (335), which is also capable of endogenous aldosterone synthesis {553;554;555). As discussed, aortic collagen accumulation in hypertensive rats was prevented by treatment with the aldosterone antagonist spironolactone, and more recently, the role of aldosterone has been investigated not only in hypertension, but in relation to collagen accumulation and arterial stiffness in

normotensive animals. Aldosterone antagonism has been shown to reduce both aortic collagen accumulation and arterial stiffness in old normotensive rats (336). In addition, aortic immunostaining for MMP-9, in addition to the elastin staining carried out, would provide additional evidence as the the role of MMP-9 in aortic elastin fragmentation in subjects with stiff arteries.

4.2.13 Conclusion

Increased aortic stiffness is related to increased aortic medial collagen content, increased fragmentation of aortic medial elastic lamellae and latent MMP-9 expression.

THE RELATIONSHIP BEWTEEN AGE, ARTERIAL STIFFNESS AND CIRCULATING MARKERS OF EXTRACELLULAR MATRIX TURNOVER

4.3.1 Circulating Metabolites of Collagen Type I, Arterial Stiffness and Age

The work carried out in section 4.2 demonstrated a relationship between aortic medial collagen concentration and aortic stiffness. However the aorta is comprised of distinct collagen subtypes, the majority of which are made up by collagen type I, with smaller contributions from types III and V. Therefore, while aortic medial concentration increases in subjects with stiffer arteries, the contribution of collagen subtypes is not known. The ratio of collagen type I synthesis in relation to collagen type I degradation (PIP:ICTP) in this study displayed a negative correlation with arterial stiffness. This relationship remained in the hypertensive subgroup but not in the normotensive. As the results also show that ICTP levels (marker of collagen type-I degradation) increase with increasing PWV, increased collagen type I degradation is likely to be responsible for the reduced PIP:ICTP in subjects with stiffer arteries. The relationship between PWV and ICTP, not present when subjects were analysed in the hypertensive subgroup, may be related to insufficient study subject numbers. There are a number of possible explanations for the relationship between collagen type I degradation in relation to collagen type I synthesis and increased arterial stiffness.

A change in the proportions of collagen subtypes can affect the mechanical properties of the vessel wall due to the diversity of the structure and function of the different subtypes. Although collagen type I comprises the majority of aortic collagen (70% to 75%), studies have shown that increases in collagen type III and type V occur during vascular stiffening in hypertension. Several animal studies have demonstrated that hypertension and arterial stiffening is associated with increased proportions of collagen types III and V.(98;99) Stiffer arteries in hypertensive rats were specifically related to increased collagen type III but not type I.(98) Decreased amounts of collagen type I have also been reported in hypertensive rats, accompanied by an increase in collagen type V.(99) Although the authors did not find a direct relationship between collagen type V and arterial stiffness, the hypertensive rats did display increased arterial stiffness. It has also been proposed that the loss of collagen from the vessel wall may be accompanied by a greater loss of other vascular components leading to an overall increase in collagen concentration.(114) The inhibition of collagen degradation via MMPs after arterial injury and sustained blood flow changes resulted in diminished arterial remodelling,(251;337) which suggests that collagen degradation may be involved in the arterial response to injury.

It is also likely that only small changes in the proportions of collagen subtypes may affect vascular stiffening. Although ICTP was significantly related to measures of vascular stiffness, the absolute circulating levels were very low. Therefore, low levels of collagen type-I degradation, and possibly small increases

in collagens type III and/or type V may have a profound influence on arterial stiffening.

No relationship was present between circulating markers of collagen type I turnover and age in this study, which agree with the results discussed in section 4.2 where aortic collagen accumulation was related to PWV, but not to age.

4.3.2 Plasma MMP-1, Arterial stiffness and Age

The results of this study show that the PIP:ICTP ratio decreases with increasing PWV, while ICTP levels (marker of collagen type-I degradation) increase with increasing PWV, suggesting increased collagen type I degradation is responsible for the negative relationship. This is strengthened by the positive relationship displayed between MMP-1 levels (responsible for the degradation of native collagen type I) and PWV, in both hypertensive and normotensive subjects. Furthermore, the finding that MMP-1 is an independent determinant of PWV highlights the importance of matrix degradation and specifically, collagen type I degradation in arterial stiffness. In addition, the fact that MMP-1 is related to PWV in both healthy people and in hypertension, suggests that arterial stiffness occurs by similar mechanisms in health and disease. Redmond *et al* have shown that MMP-1 expression is significantly up-regulated in VSMCs exposed to pulsatile stress as opposed to static pressures.(338) Also, as increased MMP-1 expression is associated with the phenotypic modulation of the VSMC cell to a 'synthetic state' following injury to saphenous vein bypass grafts, this enzyme may be involved not only in collagen degradation, but may contribute to intimal

thickening.(339) This is also of interest as this study also demonstrated a weak correlation between plasma MMP-1 expression and age, which coincides with the finding discussed in section 4.2.5, that intimal thickening is associated with age.

4.3.3 Circulating Metabolites of Collagen Type I and AIx

The relationships between markers of collagen type I turnover and AIx display a similar relationship as that between collagen type I turnover and PWV. However the relationships with both PIP:ICTP and ICTP were stronger for PWV than for AIx, and were present only when the hypertensive and normotensive subjects were analysed as a single group. This may suggest that collagen turnover is more important in determining PWV, a marker of aortic stiffness, than AIx, which is largely determined by wave reflections and represents much more the vasomotor tone in the small medium-sized muscular vessels downstream in the circulation.

4.3.4 Plasma MMP-1 and AIx

Although the metabolites of collagen type I display a stronger relationship with PWV than AIx, AIx is also significantly related to increased plasma MMP-1 levels, also reflective of increased collagen type I degradation. As for PWV, MMP-1 is also an independent determinant of AIx. Therefore, as for PWV, these results correspond with the reduced PIP:ICTP ratio, and increased ICTP levels in subjects with increased AIx.

4.3.5 Collagen Type I Turnover in Hypertensives Versus Normotensives

Hypertensives displayed higher levels of circulating PIP levels compared to normotensives, indicative of increased collagen type I synthesis. This increase, which was not related to PWV or AIx has previously been related to anatomic and functional alterations of the hypertensive left ventricle(95) and myocardial fibrosis in hypertensive heart disease.(96) Another study has also related increased markers of collagen type I synthesis in hypertension with vessel fibrosis,(100) which is in disagreement with the results of animal studies and those from this chapter. This study has also demonstrated that although ICTP levels are slightly higher in hypertensives versus normotensives, this was not statistically different, confirming other studies.(95;96) Although MMP-1 levels have previously been reported as depressed in hypertensive patients,(340) particularly in patients with left ventricular hypertrophy, the present study demonstrated no difference in plasma MMP-1 levels between hypertensives and normotensives.

While the absolute plasma PIP values obtained in this study are comparable with those obtained by others using the Takara ELISA kit(257;341) and an immunoenzymatic method(342) they are somewhat higher than values obtained in some recently published studies.(95;96) These differences may be explained by variation in the PIP standard but do not detract from the findings of an association between levels and arterial stiffness.

Although PIP:ICTP displayed a similar relationship with PWV in normotensives as in hypertensives, this did not reach statistical significance, which may be

explained by the smaller group size. Therefore in both hypertensive and normotensive subjects with stiffer arteries, collagen type I turnover is altered, favouring either reduced collagen type I synthesis or increased collagen type I degradation.

4.3.6 Plasma MMP-2 and MMP-9 Levels, Arterial Stiffness and Age

Plasma MMP-2 displayed a weak positive relationship with PWV, although there was no relationship between aortic MMP-2 expression and PWV in the results discussed in section 4.2. However, PWV is closely associated with both age and BP, and when corrected for these variables, MMP-2 was no longer related to PWV, although there was no direct relationship between age and plasma MMP-2. In view of the recent evidence in support of increased aortic MMP-2 with age, and that discussed so far in this study, the lack of association between plasma MMP-2 and age, may be related to the non-specificity of plasma MMP-2. Activated MMP-9, however, displayed a positive relationship with PWV, which remained significant after adjustment for age and BP. As discussed in section 4.2.9, aortic latent MMP-9 expression was also related to PWV, although activated MMP-9 was not detected in vascular tissue. The differential expression of the latent and activated forms of MMP-9 in vascular tissue and plasma may be related to decreased detection of MMPs in the tissue homogenates compared to plasma, where activated MMP-9 was detected in small concentrations. The two recent studies which have related MMP-9 to arterial stiffness have related both arterial MMP-9 gene expression with arterial stiffness(246) and also increased

elastase activity and circulating MMP-9 mass with arterial stiffness.(219) In the latter study, circulating MMP-9 was identified as an independent predictor of PWV in both hypertensive and control subjects. Although MMP-2 was also related to PWV, the relationship was not as strong as that for PWV and MMP-9. The mechanisms by which MMP-9 may affect vascular stiffening is most likely by aortic elastin fragmentation, as previously discussed. In addition, MMP-9 and MMP-2 degrade collagens that have been denatured by other enzymes such as MMP-1.(170;175)

4.3.7 Plasma MMP-2 and MMP-9 in Hypertensives Versus Normotensives

The relationship between MMP-9 and arterial stiffness was present when both the normotensives and hypertensives were analysed as a single group and does not provide evidence of altered involvement of MMP-9 in arterial stiffening in hypertensives compared to normotensives, in agreement with a recently published study.(219) However there were differences in circulating MMP-9 levels- decreased latent MMP-9 and increased activated MMP-9 in hypertensives versus normotensives. These differences were not directly related to arterial stiffness in this study, and may be related not only to vascular, but also to cardiac alterations in the hypertensive subgroup.(343;344)

4.3.8 Summary

Alterations in collagen type I are related not only to large artery stiffness but also but also to the modulation of the smaller-medium sized vessels downstream, not

only in patients with hypertension, but also in those with healthy arteries. Increased elastin degeneration in arterial stiffness is reflected by increased levels of circulating activated MMP-9 in both normotensive and hypertensive subjects with stiffer arteries.

4.3.9 Study Limitations

It would be beneficial to carry out a similar study with more equally matched study group sizes (this study comprised of 32 hypertensive subjects and 14 normotensive subjects) to increase the power of the study, especially in relation to correlations displayed in the normotensive subgroup.

4.3.10 Future Work

Through the measurement of circulating collagen type I metabolites, this study has demonstrated the importance of collagen type I degradation in relation to arterial stiffness. It would therefore be of great interest to analyse collagen subtype expression within the human aorta in relation to vascular stiffening. Results from animal studies have also highlighted the potential importance of alterations in collagen subtypes in vascular remodelling. The identification and quantification of collagen types I, II and V within the aortic wall, together with measures of vascular stiffness, is required to provide a more complete understanding of the relationship between collagen turnover and arterial stiffening. The benefit of such investigation would also be increased by the localisation of MMP-1 expression within the aorta and internal mammary artery,

considering the strong relationship demonstrated between MMP-1 and both PWV and AIx.

In addition to the correlation of collagen type I metabolites and matrix degrading enzymes with measures of vascular stiffness, these markers should also be correlated to measures of myocardial stiffness in the study subjects, especially when considering the increased concentrations of plasma PIP levels measured in the hypertensive subjects. As previously discussed (section 4.3.5), these increased levels in hypertensive subjects have previously been related to measures of myocardial fibrosis.

4.3.11 Conclusion

Collagen type I degradation in related to the stiffness of large and smaller medium size arteries in both hypertensive and normotensive subjects.

ARTERIAL STIFFNESS AND ADVANCED GLYCATION END PRODUCTS

4.4.1 Plasma AGEs, PWV and Age

The results of this study demonstrate a positive relationship between aortic stiffness and plasma AGE levels, which remains significant following correction for the major determinants of PWV, age and MAP. The relationship between AGEs and aortic stiffness is most likely related to increased mechanical stiffness of vascular collagen. As AGEs have also been shown to stimulate the potent vasoconstrictor, endothelin-1 and reduce the bioavailability of the vasodilator-nitric oxide, it is also possible that AGEs increase aortic stiffness through the modulation of vascular tone.

The relationship between AGEs and PWV is specifically between PWV and the CML and CEL AGE structures, as the anti-AGE antibody (CD12) used in this study has specificity for a common epitope on these structures. Anti-age antibodies have been used previous studies (281;309) where the specificity of the antibody is not defined, while other studies have also used antibodies specifically targeted only at CML, or at other AGE structures such pentosidine and pyrraline. The role of different types of AGEs in vascular pathology is still unclear, although CML is one of the major AGEs *in vivo*(345) with increased levels present in the skin collagen of diabetic patients.(346) It has also been shown that both CML and CEL levels are increased in the kidneys of hypertensive versus

normotensive rats.(347) Interestingly, a recent study investigating CML levels in identical healthy twins has also demonstrated that CML levels and the subsequent influence on vascular pathology are predominantly genetically determined.(348) Plasma AGE levels also displayed a positive relationship with age, although this was weaker than that between AGEs and PWV. The relationship between increased AGE formation with ageing is well documented as AGEs accumulate slowly over time under physiological conditions, and are involved in age-related arterial stiffening.(292;293;349;350)

4.4.2 Plasma AGEs and AIx

This study also demonstrates while circulating AGEs are related to aortic stiffness, as demonstrated by the relationship with PWV, they are not related to the stiffness of the smaller muscular arteries as no relationship was seen with AIx. This is in agreement with the results of the study which evaluated changes in aortic and systemic arterial compliance following treatment with Alagebrium, an AGE cross-link breaker. Administration of the drug in humans reduced aortic stiffness,(293) as measured by PWV, but did not affect systemic arterial resistance, which had previously been reported in animal studies.(292)

4.4.3 Relationship Between Plasma AGEs, PWV, and Biochemical Markers

The results of this study also demonstrate a relationship between plasma AGEs and cholesterol, but not with glucose, plasma HDL or triglycerides. The relationship between circulating AGE levels and cholesterol has not previously been reported, although inhibiting advanced glycation in diabetic patients reduced total cholesterol by 19%.(351) The authors speculate that dyslipidemia in diabetes may be related to AGE modification of LDL. LDL, the major transporter of plasma cholesterol, can undergo modification by circulating AGE products, resulting in decreased plasma clearance of LDL. Although the relationship between LDL and plasma AGEs was not determined in this study, it is possible that the increased levels of cholesterol in relation to increased plasma AGEs demonstrated in this study may be related to AGE modification of plasma LDL.

4.4.4 Plasma AGEs in Hypertensives versus Normotensives

Although the study group consisted of both hypertensive and normotensive subjects, the relationship between circulating AGEs and PWV was stronger when the normotensive subjects were removed from the group, suggesting that AGE formation is of greater functional importance in hypertension-associated vascular stiffening, than in normotensive subjects. In addition, circulating AGEs are increased in the hypertensive compared to the normotensive subjects. Animal studies have previously described the relationship between increased AGE formation and hypertension. In aged SHR, therapy with Alagebrium lead to a reduction in SBP, left ventricular and aortic mass and myocardial collagen

content.(352) In addition, the drug also exerted beneficial renal effects in these animals, where increased kidney AGE formation has also been demonstrated.(347) The increased aortic stiffness of stroke-prone SHR (in comparison to SHR and WKY rats) has been related in part to increased formation of collagen cross-links.(353) It has also been demonstrated that following the administration of methylglyoxal (a reactive dicarbonyl compound which gives rise to AGE formation, including CML and CEL) to normotensive rats, there was a significant increase in SPB which was accompanied by microvascular damage.(354) Subsequently, it was demonstrated that methylglyoxal content is increased in VSMCs from hypertensive compared to normotensive rats, which was linked to significantly increased AGE formation in the hypertensive animals.(355) In addition, increased methylglyoxal in SHRs has also been related to increased oxidative damage. Increased oxidative damage has previously been shown to contribute to the pathology of hypertension by decreasing cGMP in VSMCs,(356) and thereby causing significant vasoconstriction.(357) In addition methylglyoxal and subsequent AGE formation may also play a role in the association between inflammation and hypertension. Increased methylglyoxal was related to increased activation of the transcription factor NF-κB, which is associated with a vascular inflammatory response.(358) The increased activation of NF-κB is also associated with increased ICAM-1 expression in VSMCs of hypertensive rats.(355) AGES also stimulate endothelin-1, which is not only a vasoconstrictor, but also stimulates the expression of pro-inflammatory molecules and is considered an important

mediator of chronic inflammation in the vascular wall of hypertensive animals.(359;360) Increased AGE formation in hypertension may therefore be responsible in part for increased aortic wall stiffness, but may also play a role in hypertension-related oxidative damage and in the activation of inflammatory markers.

4.4.5 Summary

Circulating levels of AGEs are related to aortic but not systemic vascular stiffening. The relationship between plasma AGE levels and vascular stiffening was seen only in hypertensive subjects, where increased AGE levels may contribute to oxidative damage, vascular inflammation and vascular stiffening. Plasma AGE levels are also related to both age and cholesterol levels.

4.4.6 Study Limitations

As for the previous study, the study would benefit from equally matched study subgroups, i.e. a larger normotensive study population. In addition it would be beneficial to carry out the analysis of plasma AGE levels in relation to HbA1c levels in addition to glucose levels. As blood glucose levels are subject to a high degree of fluctuation, whereas as HbA1c levels change slowly, over a period of up to 10 weeks, this could provide a more reliable index of blood glucose levels.

4.4.7 Future Work

The relationship between circulating AGEs and arterial stiffness should also be investigated further, by quantifying AGE formation within vascular tissue. This would not only provide additional evidence for the relationship between AGEs and aortic PWV, but allow for localisation of the structures within the vessel wall. In addition, by identifying AGEs in both the aorta and the IMA, the extent of AGE accumulation in muscular in addition to elastic arteries could also be more fully addressed.

Drug treatments which break AGE crosslinks have been shown to reduce not only vascular but also myocardial stiffness (297;361). The measurement of circulating AGEs should therefore also be investigated in relation to myocardial stiffness.

Circulating AGE levels have previously been measured and related to impaired endothelium-dependent vasodilation in diabetic subjects (269). Considering the important role of AGE formation in the pathogenesis of diabetic complications, the measurements carried out in this study should also be carried out in a subgroup of diabetic subjects and related to measures of vascular stiffness.

4.4.8 Conclusion

Circulating levels of AGEs are related to aortic but not systemic vascular stiffening, in hypertensive subjects, where levels are increased.

4.5 SUMMARY OF STUDIES

4.5.1 Differential Remodelling of the ECM during Ageing and Arterial Stiffening

Vascular remodelling, a general term used to describe structural alterations occurring in the vessel wall, is not only of relevance in cardiovascular disease, but also in ageing. Remodelling, or alteration of the vascular ECM may begin initially as an adaptive process, as a response to increased pressure, but is however, ultimately detrimental. It has been hypothesized that alterations in old 'healthy' arteries provides an environment where vascular disease can flourish. In addition, intrinsic alterations of vascular structural components in subjects with or without evidence of cardiovascular disease, may lead to an increase in vascular stiffness, a well established independent cardiovascular risk factor.(1;2) Vascular ageing and vascular stiffening may be to a large extent independent, and it is plausible that both vascular ageing and vascular stiffening occur through separate mechanisms of vascular remodelling. However the age-related changes that occur in the vasculature, most likely not only increase the susceptibility to cardiovascular disease such as atherosclerosis, but also to increased vessel stiffness.

Recently, the results of several studies have suggested that arterial ageing occurs independently of both cardiovascular disease and arterial stiffening. It is known that increased degradation occurs in the aortic ECM of ageing animals, as a result of intimal MMP-2 up-regulation.(203;204) The results of these studies demonstrate that this increase also occurs in the human aorta, and that no

relationship exists between vascular MMP-2 up-regulation and arterial stiffness. Although circulating levels of MMP-2 displayed a weak correlation with PWV, this relationship was not present following adjustment for age and BP. In addition, aortic intimal thickness also displayed a strong positive relationship with ageing, but not with arterial stiffening.

As altered vascular remodelling has been related specifically to ageing, altered expression of matrix-degrading enzymes has also been related specifically to vascular stiffening. Recently, it has been demonstrated that the progression of vascular stiffening is in part genetically determined. Specifically, the MMP-9 genotype influences large artery stiffness. Carriers of the T-allele (T/T as opposed to C/T), which is associated with greater MMP-9 mRNA and protein levels, had stiffer arteries.(246) So although ageing results in stiffer arteries, it is likely that arteries will become stiffer in certain individuals compared to others, irrespective of age. The results of previous studies and the results of this work have also related MMP-9 expression to arterial stiffening,(219) but not to ageing. In addition, increased MMP-9 expression in relation to increased aortic stiffness was accompanied by increased aortic elastin fragmentation in this study, also related to arterial stiffness, but not to age.

4.5.2 Vascular Remodelling and Ageing

MMP-2, a matrix-degrading enzyme which has broad substrate specificity is up-regulated with ageing in the human aorta. Gelatinolytic activity was present in the aortic intima, media and adventitia. It has been demonstrated that MMP-2

can degrade arterial elastin,(176) collagens types-IV, V, VII, X and denatured collagens.(311) Increased degradation of these substrates can affect various aspects of vascular remodelling.

One of the most likely effects of increased aortic MMP-2 expression is a resultant increase in VSMC migration from the media to the intima. MMP-2 has previously been shown to degrade various components of the vessel wall in the 'path' of the migrating VSMCs.(314) Not only was activated MMP-2 increased in the human aorta in this study, but an increase in MMP-2 activation from the latent enzyme was also demonstrated. In addition, migrating VSMCs must travel through a media containing elastic lamellae and pass across the IEL, areas where increased MMP-2 activity has been specifically located in animal studies.(203;204)

An increase in VSMC migration to the aortic intima, as a result of increased MMP-2 activity with ageing, would in part explain the relationship that was observed specifically between aortic intimal thickening and ageing. Unlike VSMCs that reside in the media that are normally quiescent and contractile, VSMCs that migrate to the intima exhibit a 'synthetic migratory phenotype', a shift which has shown to be accompanied by a high constitutive production of MMP-2.(314) These synthetic cells may secrete a fibrous ECM, and may therefore be related to the collagen and other ECM components present in the old aortic intima.(203;319) The intima does not contribute much mechanically to the vessel wall, but is a rich source of substances and signal transduction mechanisms that may influence the properties of the whole vessel wall, and may be viewed as

a foundation for the subsequent development of atherosclerosis.(362) Increased MMP-2 activity and intimal thickening with age in the human aorta therefore, in part explains the increased susceptibility of older arteries to disease. Increased MMP-1 expression may also be involved in age-related intimal thickening. MMP-1 is also associated with the phenotypic modulation of the VSMC to a 'synthetic state' and may therefore also contribute to intimal thickening due to the increased deposition of fibrous ECM components.(339) While vascular MMP-1 expression was not investigated in this study, circulating levels of the enzyme displayed a weak relationship with age. It is therefore possible that increased MMP-1 activity with ageing also contributes to the age-associated increase in aortic intimal thickness.

Circulating AGEs also demonstrated a positive relationship with age. The relationship between increased AGE formation with ageing has been previously described as AGEs accumulate slowly over time under physiological conditions.(292;293)

4.5.3 ECM Turnover during Ageing in Large Elastic versus Smaller Muscular Arteries

Non-invasive studies have previously demonstrated that age-related changes and the progression of atherosclerosis are non-uniform throughout the vascular tree and affect large elastic arteries such as the aorta to a greater extent than muscular arteries.(316-318) The histological evaluation and matrix-degrading enzyme quantification of the aorta and IMA, provides evidence that the age-related

changes observed in the aorta do not occur in the IMA. The relationship between MMP-2 up-regulation was present in the aorta but not the IMA and there was little variation in the size of the small intima of the IMA, which was absent in 1 IMA tissue sample.

4.5.4 Vascular Remodelling and Arterial Stiffening

While the intimal thickness of the human aorta increases with age, medial collagen concentration of the aorta is related specifically to aortic stiffness. Increasing evidence from animal studies and non-invasive human studies suggest that aldosterone may be related to aortic collagen accumulation in arterial stiffening, in addition to increasing arterial stiffness through short-term functional alterations.(326;336;363) The direct relationship observed between aortic medial collagen concentration and aortic stiffness lends further support to the possibility that aldosterone antagonism reduces arterial stiffness in humans through the prevention of collagen accumulation as demonstrated in rats, in addition to possible short-term functional alterations. While medial collagen content is increased in stiffer aortae, variation in collagen subtypes, the majority of which is collagen type I, with smaller amounts of types III and V, also contributes to vascular stiffening. Although collagen subtypes were not directly quantified in vascular tissue, the measurement of circulating metabolites of collagen type I in relation to vascular stiffness, demonstrated that vascular stiffening is associated with degradation of collagen type I, both in subjects with hypertension and in healthy control subjects. In addition, is likely that degradation of collagen type I

in arterial stiffness is mediated largely by MMP-1, as not only was MMP-1 related to measures of arterial stiffness, but also to ICTP (collagen degradation) levels. Therefore, arterial stiffness is associated with an accumulation of total collagen content in the aortic media, yet an increase in collagen type I degradation in relation to collagen type I synthesis. It is likely that only a small amount of collagen type I degradation occurring in the vasculature may affect vascular stiffness, as both ICTP (collagen degradation) and MMP-1 levels were present in very low levels (2.0-6.7ng/ml and 6.5-27.5ng/ml respectively). Several studies have previously assessed alterations of collagen subtypes in hypertension and arterial stiffening using animal aortae. Stiffer arteries in hypertensive rats were specifically related to increased collagen type III but not type I.(98) Decreased amounts of collagen type I have also been reported in hypertensive rats, accompanied by an increase in collagen type V, and no change in the proportion of collagen type III .(99) Although the authors did not find a direct relationship between collagen type V and arterial stiffness, the hypertensive rats did display increased arterial stiffness. A small increase in the degradation of collagen type I, which is the main collagen type of the aorta, accompanied by increases other collagen subtypes, particularly type III and type V is a likely explanation for the increase in total collagen concentration, but a decrease in collagen type I, in stiffer arteries.

The results of this study also demonstrate the direct relationship between elastin fragmentation of the aorta and aortic stiffness, as opposed to age. While it has been demonstrated that there is a loss of elastic tissue with ageing in the human

aortic arch and lower abdominal aorta,(155) more recently increased vessel stiffness due to ageing in rats was shown to be related to increased fenestration of the elastic lamina. Stiffer aortae in this study have demonstrated not only increased elastin fenestration but also increased aortic expression of MMP-9, an enzyme which has been shown to specifically degrade arterial elastin.(150) Although activated MMP-9 was not detected in aortic or IMA tissue samples, which may be related to decreased sensitivity of the aortic homogenate supernatant, activated MMP-9 was detected in the plasma of both normotensive and hypertensive subjects, where it was also related to increased vessel stiffness. The relationship between circulating MMP-9 levels and arterial stiffness has previously been documented(219) as has MMP-9 gene expression and arterial stiffness.(246)

While aortic collagen and elastin expression and degradation are related to vessel stiffening, increased AGE cross-links on these structural proteins are also related to stiffer arteries. AGE formation in the vascular wall may contribute to arterial stiffness by increasing the mechanical stiffness of collagen fibres(364;365) or through the modulation of vascular tone.(286;287;289;290) Specifically the CML and CEL AGE structures are related to vascular stiffening, as the anti-AGE antibody (CD12) used in this study has specificity for a common epitope on these structures. AGE formation has previously been associated with vascular stiffening, as a drug that breaks these cross-links (Alagebrium), increases aortic compliance. Although circulating levels of AGEs have been associated with impaired left ventricular function in patients with type-1 diabetes,(366) and have

also been related to the severity of coronary artery disease in non-diabetic patients,(367) this is the first study to demonstrate a direct relationship between aortic stiffness and circulating AGEs.

The relationship between AGEs and vascular stiffening was stronger when control subjects were removed from the group and only hypertensive subjects were analysed. The importance of AGE formation in hypertension-associated vascular alterations is becoming increasingly clear as discussed in section 4.4.4.

4.5.5 ECM Turnover during Arterial Stiffening in Large Elastic versus Smaller Muscular Arteries

As with the vascular structural adaptations demonstrated with ageing, the results of the studies carried out on the aortic and IMA tissue demonstrate that alterations in vascular structure with arterial stiffening affect the large central arteries compared to the muscular arteries. Increased medial collagen content, elastin fragmentation and MMP-9 expression was related to PWV only in the aorta, but not in the IMA. This result is as expected, as PWV is essentially a marker of aortic stiffness. On the other hand, increased circulating markers of collagen type I degradation were related to both PWV and AIx. Whereas PWV is a marker of aortic stiffness, AIx is determined in part by PWV but also represents much more the vasomotor tone in the small medium-sized muscular vessels downstream in the circulation. Although the metabolites of collagen type I display a stronger relationship with PWV than with AIx, both PWV and AIx are significantly related to increased plasma MMP-1 levels, also reflective of increased collagen

type I degradation. These results suggest that while collagen type I degradation may be a more important determinant of aortic stiffness, it is also related to arterial stiffening of the smaller muscular arteries.

While plasma MMP-1 levels are related to both PWV and AIx, plasma AGE levels displayed a relationship only with PWV, suggesting a relationship with aortic stiffness only. This is of particular interest, considering the conflicting data obtained from animal and human studies in relation to the contribution of AGE formation in elastic versus muscular arteries. The results of this study support the findings of the study which evaluated changes in aortic and systemic arterial compliance in humans following treatment with Alagebrium. Administration of the drug in humans reduced aortic stiffness,(293) as measured by PWV, but did not affect systemic arterial resistance, which had previously been reported in animal studies.(292)

4.5.6 The Relationship between Vascular Ageing and Arterial Stiffening

It has been demonstrated that alterations occur in the vessel that are specifically related to the ageing process, while altered ECM matrix-degradation and vascular protein turnover occur specifically in relation to vascular stiffening. However, it is well documented that vascular stiffening, occurs not only during vascular pathologies but also with age in healthy populations.(9;107;313) Therefore, while vascular stiffening may progress regardless of age, the alterations that occur in the vasculature with age may also lead to increased vascular stiffness.

The age-related increase in MMP-2 activity may have implications for vascular stiffening. MMP-2 degrades similar vascular components as MMP-9, which is specifically related to vascular stiffening. The degradation of arterial elastin by MMP-2 may also be implicated in age-related aortic stiffening.

The increased activation of MMP-2 with age in the aorta is associated with an age-associated increase in intimal thickness. Although intimal-thickening is associated specifically with ageing, and has previously been demonstrated as a separate entity of vascular damage compared to arterial stiffening,(331) it is possible that the high collagen content present in the aortic intima may contribute to some degree to the age-associated increase in vascular stiffening.

Aside from the potential for specific age-related changes to impact upon aortic stiffness, several alterations have been identified that occur in matrix-degrading enzyme activity and matrix proteins that are related to both vascular ageing and vascular stiffening.

Although more strongly related to measures of arterial stiffness, circulating MMP-1 levels also increase with age. Apart from the relationship with vascular collagen type I degradation, MMP-1 may contribute to the age-associated increase intimal thickness. The same is true of plasma AGEs, suggesting that under physiological conditions the accumulation of AGEs will contribute to increased stiffness of vascular collagen and possibly elastin, and also alteration of vascular tone.

Circulating MMP-2 was also related to aortic stiffness, despite the relationship between vascular MMP-2 and age. Although this relationship was lost following

correction for age and MAP, it further demonstrates the close relationship between age and arterial stiffening.

4.5.7 Conclusion

These studies have addressed several issues in relation to the remodelling of the vasculature. Mechanisms of arterial remodelling have been investigated in terms of both ageing and arterial stiffening, where common mechanisms have often been assumed for the two. My studies have demonstrated that while independent alterations occur during ageing and arterial stiffening, several mechanisms are also common to both. The investigation of vascular remodelling in the aorta in comparison to the IMA has also demonstrated that altered matrix-degrading activity, structural protein expression and cross-link formation occurs in the large elastic arteries in relation to both ageing and arterial stiffening, to a greater extent than the smaller muscular arteries.

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