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GENETIC POLYMORPHISMS AND ARTERIAL STIFFNESS

Sixiang Zhou

A thesis submitted for the degree of

Doctor of Philosophy

to

University of Dublin, Trinity College

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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. Except where otherwise acknowledged this thesis is my own work. The research in this thesis was carried out at the Trinity Center for Health Sciences, St James's Hospital, Dublin.

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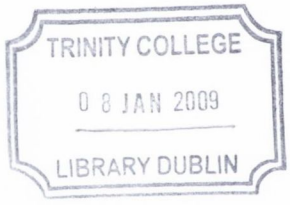

Summary

Arterial stiffness exerts a major influence on cardiac afterload and coronary perfusion. Indices of arterial stiffness have therefore been proposed as surrogate markers of cardiovascular risk and as potential therapeutic targets. Aging, genetic and environmental factors together lead to structural and functional changes of the arterial wall, which may result in decreased elasticity and increased stiffness. The process of vascular stiffening has been proven to be closely associated with ageing. Whilst the genetic contributions to arterial stiffness are far from clear, the current view is that this is a polygenetic process which may also be influenced by pathological and environmental factors.

The aim of this thesis was to determine if some specific genes contribute to arterial stiffening. Blood samples from the untreated hypertensive patients were collected and genetic DNA of these samples was extracted. Polymorphisms of candidate genes including the collagen III gene, the elastin gene, the matrix metalloproteinases (MMP-9) gene, the adiponectin gene, the receptor of advanced glycation end products (RAGE) gene and the G-protein beta 3-subunit gene were studied using restriction fragment length polymorphism (RFLP) methods. MMP-9 and plasma adiponectin levels were also measured in blood samples from untreated hypertensive subjects. Aortic stiffness was measured by pulse wave velocity (PWV), and augmentation index (AIx) was measured by applanation tonometry to estimate wave reflection.

The studies demonstrate that some specific genetic polymorphisms can affect arterial stiffness and indeed blood pressure. The collagen III 2209G>A polymorphism has proved to be an independent determinant of not only systolic blood pressure and diastolic blood pressure but also of PWV and Aix. The elastin 549G>A polymorphism can lead to higher blood pressure and arterial stiffness within the AA genotype. Moreover, both the COL3A1 2209G>A and ELN 549G>A polymorphisms show age-gene interaction with arterial stiffness. Aortic PWV and blood pressure were

modulated by -1562C>T and -836G>A polymorphisms in the MMP-9 gene—both polymorphisms were independent predictors of both systolic and diastolic blood pressure. The -276G>T polymorphism of the adiponectin gene was seen to influence aortic PWV, (but not the AIx), blood pressure, plasma adiponectin concentrations and insulin resistance. In addition, the 45T>G polymorphism of the adiponectin gene is independently, and as a haplotype together with -276G>T, strongly associated with higher blood pressure and greater arterial stiffness in untreated hypertensive patients. AA homozygotes of -374T>A and C allele carriers of the 429T >C RAGE gene may confer a significant protective effect against the development of cardiovascular events as they exhibit lower blood pressure and less aortic stiffness. On the other hand a polymorphism of the G-protein beta 3 subunit had no impact on either blood pressure, PWV or AIx.

In summary, this thesis shows that specific polymorphism sites that affect vascular structure and function may play a significant role in determining blood pressure and the extent of arterial stiffness. These findings may, in part, explain the propensity of individuals with these polymorphisms to cardiovascular disease. The observations also have the potential to identify new therapeutic targets.

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List of Abbreviations

AAA	Abdominal aortic aneurysms
AASI	Ambulatory Arterial Stiffness Index
ACAT-1	Acyl-coenzyme A:cholesterol acyltransferase-1
ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end products
AIx	Augmentation index
AMI	Acute myocardial infarction
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	One-way analysis of variance
Aortic DBP	Aortic diastolic blood pressure
Aortic SBP	Aortic systolic blood pressure
AT1	Angiotensin II type 1
BMI	Body index
BP	Blood pressure
CA	coronary aneurysms
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CHD	Coronary heart disease

CML	Carboxy (methyl) lysine
COL3A1	Collagen type III-a1 gene
CRP	C-reactive protein
DBP	Diastolic blood pressure
DN	Dominant negative
ECG	Electrocardiogram
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EEL	External elastic lamina
ELISA	Enzyme-Linked ImmunoSorbent Assay
ELN	Elastin gene
ENaC	Epithelial sodium channel
eNOS	Endothelial NO synthase
ESRD	End-stage renal disease
FA	Fatty acid
FACIT	Fibril associated collagen with interrupted helices
FAP	Fibroblast activation protein
FATP	Fatty acid transporter protein
GNB3	Heterotrimeric G protein
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment of insulin resistance
ICAM	Intracellular adhesion molecule

IEL	Internal elastic lamina
IL	Interleukin
IMA	Internal mammary artery
IRS	Insulin-receptor substrate
LDL	Low density lipoprotein
LH	Lithium heparin
LP	Lipoprotein
MAP	Mean arterial pressure
MFS	Marfan syndrome
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MP	Mean pressure
MPs	Mononuclear phagocytes
MT-MMPs	Membrane-type metalloproteinases
NF-kB	Nuclear factor-kappa B
NO	Nitric oxide
NRCMs	Neonatal rat cardiac myocytes
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PP	Pulse pressure
PWA	Pulse wave analysis
PWV	Pulse wave velocity

RAGE	Receptor for advanced glycation end products
RFLP	Restriction fragment length polymorphism
SBP	Systolic blood pressure
SHRs	Spontaneously hypertensive rats
SMCs	Smooth muscle cells
SNPs	Single nucleotide polymorphisms
SR	Scavenger receptors
sRAGE	soluble receptor for advanced glycation endproducts
SVAS	Supravalvular aortic stenosis
TGs	Triglycerides
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumour necrosis factor-alpha
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth cells
WKY rats	Wistar-Kyoto rats
Zc	Characteristic impedance

Chapter 1

Introduction

1.1 Arterial functions

Arteries are distributed between the heart and peripheral arterioles, serving a cushion as well as a conduit function. Left ventricular ejection is intermittent, but flow through the arterioles is continuous. The conduit function is to supply an adequate blood flow to peripheral tissues and organs in accordance with their metabolic needs. Conduit-function efficiency is the consequence of the width of the arteries and the very low resistance of large arteries to flow. The conduit function is highly efficient and can accommodate an increase in the flow to some tissues, like muscle, by up to 10-fold. This physiological adaptability is mediated through acute changes of arterial flow velocity and/or diameter. Diameter changes are dependent on the endothelium, which responds to alterations in shear stress [1]. The acute endothelium-dependent vasodilatation is limited in several clinical conditions including: atherosclerosis [2], and hypertension [3]. The long-term alterations of conduit function occur through narrowing or occlusion of arteries with restriction of blood flow and resulting ischemia or infarction of downstream tissues. Atherosclerosis is the most common disease that disturbs conduit function. In conduit arteries, basal blood flow remains constant until the lumen diameter is narrowed by 50%. Beyond 70–80% reduction of the lumen

diameter (critical stenosis), basal blood flow is reduced as is the ability to increase flow during activity.

The cushioning function of arteries is to dampen the oscillations in pressure and blood flow, from intermittent ventricular ejection, to the steady flow required in peripheral tissues and organs [4]. During systolic contraction, roughly 40% of stroke volume is forwarded directly to peripheral tissues, while the remainder is stored in capacitive arteries (mainly aorta and elastic-type arteries). Approximately 10% of the energy produced by the heart is directed to the distension of arteries and is ''restored'' during diastole as recoil in the aorta, squeezing the stored blood forward into the peripheral tissues, thereby ensuring continuous perfusion of organs and tissues. Since the energy produced by the heart should serve principally for tissue perfusion, the energy used for arterial distension and recoil should be as low as possible.

The dampening function of the arterial tree is altered primarily during the aging process and in conditions associated with ''sclerotic'' remodeling of arterial walls, which is associated with increased collagen content and modifications of extracellular matrix [5-7]. Arteriosclerosis is primarily manifest as medial degeneration that is generalized throughout the thoracic aorta and central arteries, causing dilatation, diffuse hypertrophy, and stiffening of the arteries [4□8]. Age-related arterial alterations leading to stiffening are heterogeneous, being more pronounced in the aorta and central, elastic-type arteries than in the peripheral muscular-type limb arteries [9].

1.2Arterial stiffness

1.2.1 Definitions of arterial stiffness

Arterial stiffness is a term used to define the incapacity of the artery to expand and contract during the cardiac cycle. More appropriately we should examine arterial compliance, or dispensability which decline as a pathological consequence of aging and metabolic disorders. [10] Although these terms are interrelated, they do not have the same meaning (Table 1.1). [11] Compliance is the absolute change in area for a

given change in pressure. In the arterial system compliance relates to the change in artery diameter caused by left ventricular ejection. Compliance mainly shows the function in the large conduit arteries, which store blood during systole. The term C1 is used to designate compliance in the larger, proximal arteries, and C2 is used to refer to distal, or oscillatory, compliance of the smaller arteries. Dispensability is used to define compliance relative to the initial volume or diameter of an artery. A large artery may have less dispensability than a smaller artery because the percentage increase in area at a given pressure is smaller in the larger artery than in smaller artery, but its compliance will be greater because of the larger absolute volume increase in large artery. A loss of arterial elasticity results in reduced arterial compliance and dispensability. When pressure increases, a point is eventually reached with less dispensability occurring at higher pressures as a consequence of the elastic properties of the arterial media. [12] At low pressures elastic fibres take up pressure, whereas at higher pressures the tension is absorbed by the more rigid collagen fibres and compliance consequently decreases. Differences in arterial compliance should therefore generally be corrected for blood pressure.

Table 1.1 Terms to describe arterial stiffness [11].

Term	Definition	Method
Compliance	Arterial segment volume /diameter change with pressure change	Ultrasonography
Distensibility	Compliance relative to initial volume/diameter	Ultrasonography
Pulse pressure	Difference between systolic and diastolic blood pressure	Blood pressure measurement
Pulse wave velocity (PWV)	The speed of the pulse wave over an arterial segment	ECG-gated tonometry, ultrasound, or doppler
Augmentation index (AIx)	Augmentation of aortic pulse wave by wave reflection expressed as a ratio of aortic pulse pressure	Carotid or radial tonometry
Capacitive (large) artery compliance (C1)	Change in volume throughout exponential diastolic pressure decay	Diastolic pulse contour analysis by radial tonometry
Oscillatory (small) artery compliance (C2)	Change in volume per oscillatory pressure change throughout exponential diastolic pressure decay	Diastolic pulse contour analysis by radial tonometry

1.2.2 Arterial stiffness and wave reflection

Left ventricular ejection produces a forward traveling pulse waveform, and backward traveling waves occurring as a consequence of wave reflection. The latter occur throughout the arterial tree at the branching points of arteries [13], at regions of increased arterial stiffness, and also the high-resistance arterioles which are considered by some to be the major sites of wave reflection in the circulation [4]. Also, the waveform is different in the aorta and in the periphery. Pulse pressure increases by 18% to 31% between the aortic arch and the brachial artery in younger individuals. [7] However, as a consequence of aging, stiffer vessels result in acceleration of both the advancing and the reflected waves. The reflected waves occur earlier and go back along the aorta at greater speed, arriving not in the diastolic period but in systole period. Consequently, summation of the advancing and reflected waves occurs earlier in the cardiac cycle and amplifies the aortic systolic pressure. Amplification of pulse pressure thus changes dramatically with age. In younger individuals, the summation of the forward and the backward wave at each point of the arterial tree results in progressive elevation of systolic blood pressure in peripheral arteries. The more rapid pulse wave transit in older persons results in less amplification of PP from central to peripheral blood vessels, as the reflected waves have occurred earlier and traveled back faster and increased the peak aortic pressure, which is closer, or similar to, that in the periphery [7].

1.2.2.1 Arterial stiffness and coronary heart disease

Several mechanisms may explain the association between aortic stiffness and coronary heart disease. Arterial stiffening may lead to early pulse wave reflection causing an increase of central systolic blood pressure, a decrease of diastolic blood pressure, and a consequent increase of pulse pressure. The elevation of systolic blood pressure increases myocardial oxygen demand, reduces ejection fraction, and increases ventricular load, thereby leading to left ventricular hypertrophy. [14] Moreover, because myocardial blood supply depends largely on the pressure level during diastole

and the duration of diastole, [15] the decrease of diastolic blood pressure can affect coronary perfusion, resulting in subendocardial ischemia. [15] A raised pulse pressure, due to increased vascular stiffness, may induce arterial remodeling, increased wall thickness, and the development of plaques. [16] Additionally, stiffer arteries may contribute to rupture and ulceration of atherosclerotic plaques, [17] which is likely to result in increased shear stress and subsequent rupture. Finally, it has been shown that pulse pressure, which is a consequence of arterial stiffness, is independently associated with arterial plaque ulceration, supporting the hypothesis that cyclical hemodynamic forces are important determinants of plaque rupture. [18]

1.2.2.2 Arterial stiffness and hypertension

Not surprisingly, there is a strong correlation between arterial stiffness and the presence of hypertension. Liao et al measured carotid arterial elasticity by high-resolution B-mode ultrasonography and expressed their results as adjusted arterial diameter change, Peterson's elastic modulus, Young's elastic modulus, and β stiffness index. These investigators suggested that the loss of arterial elasticity in large and medium arteries and the resulting adverse effects on target organs such as the kidneys may be responsible for the relationship between greater arterial stiffness and the increased risk for hypertension that was observed in the ARIC cohort. [19]

Results from a more recent study also suggest that reduced aortic elasticity, as reflected by increased PP, may play a primary role in the development of hypertension. Mitchell et al [20] used calibrated tonometry and pulsed Doppler to assess arterial stiffness and pulsatile hemodynamics in 128 patients with uncomplicated systolic hypertension and 30 age- and gender-matched normotensive patients in the control group. The PWV was assessed using tonometry and body surface measurements. Characteristic impedance (Zc) was calculated from the ratio of change in carotid pressure and aortic flow in early systole. Hypertensive patients had higher PP than patients in the control group; this was primarily attributable to higher Zc, which accounted for nearly one-half of the excess PP in men and more than two-thirds in

women. Increased Zc in hypertensive patients was attributable to decreased effective aortic diameter. These findings were considered to be consistent with the view that aortic function may play a primary active role in the pathophysiology of systolic hypertension.

1.2.2.3 Arterial stiffness and stroke

Several mechanisms may explain the association between arterial stiffness and stroke. First, arterial stiffness may favor the occurrence of cerebrovascular events through an increase in central PP. A growing body of in vitro studies shows that cyclic stretching exerts a greater influence than static load on the phenotype and growth of vascular smooth muscle cells [21-23]. Thus, the amplitude of the PP may influence arterial remodeling at the site of both the extracranial and intracranial arteries. It may increase the carotid wall thickness, the development of plaques, [16, 24, 25] the likelihood of plaque rupture, [17] and the prevalence and severity of cerebral white matter lesions. [8]. In the Rotterdam Study, atherosclerosis, indicated by increased common carotid intima-media thickness and plaques, was related to cerebral white matter lesions.[26] Second, the measurement of aortic stiffness, which integrates the alterations of the arterial wall, may also reflect parallel lesions present at the site of cerebral vasculature. Thus, fibrosis, medial smooth muscle necrosis, breaks in elastin fibers, calcification, and diffusion of macromolecules within the arterial wall, has been described at the site of the cerebral vasculature. [4, 27, 28] Third, coronary heart disease and heart failure, which are favored by high PP and arterial stiffness, [29, 30], are also risk factors for stroke.

1.2.2.4 Arterial stiffness and end-stage renal disease

Clinical and epidemiologic studies have shown a high prevalence of systolic hypertension in end-stage renal disease (ESRD). The principal factor responsible for increased systolic BP and PP in ESRD patients is increased arterial stiffness with

increased PWV and early wave reflections. [31, 32] Indeed, in comparison with age- and mean blood pressure-matched non-uremic patients, arterial stiffness is greater in ESRD, especially in younger uremic subjects. The stiffening is more pronounced in the aorta than in peripheral arteries. It has recently been shown that in ESRD patients that aortic stiffness was an independent risk factor for total mortality and cardiovascular morbidity and mortality.[33] Increased arterial stiffness was found to be associated with the presence of arterial calcification and a low HDL-cholesterol level, but not with other metabolic or hormonal disturbances commonly observed in ESRD.[32] Several studies that include ESRD patients indicate that sodium overload may induce arterial stiffening independently of BP changes. In ESRD patients, a positive relationship between interdialytic body weight gain and aortic PWV was observed, supporting the potential role of sodium excess. Besides the increase in peripheral systolic BP and aortic BP, the most obvious consequence of arterial stiffening in ESRD patients is an early return of wave reflections to the aorta and the disappearance of aortic-to-peripheral pressure amplification. [34] This phenomenon, which normally occurs after the sixth decade, appears already during the fourth decade in ESRD patients and means that for a similar brachial systolic BP, the aortic systolic BP is higher in ESRD patients than in non-numeric. The second factor associated with an early return of wave reflections in ESRD patients is a shorter effective length of the arterial tree [34-36]. This is related to a shorter body size, principally shorter body height, resulting from malnutrition and growth retardation frequently observed in azotemic children and adults with nephropathies starting in childhood. With a comparable arterial stiffness and peripheral resistance, ESRD patients with short body height have an increased effect of wave reflections on aortic pressure. Low body height was also found to be associated with cardiovascular risk in men and women in the general population. Whether arterial stiffness and altered wave reflection occur at the early phase of renal diseases still remains unknown.

1.3 Measurement of arterial stiffness

The increased demand from clinicians and researchers for the assessment of arterial stiffness has led to the development and commercial availability of several methods that are more practicable than conventional techniques. Non-invasive measurements of arterial stiffness are essentially derived using 3 main methodologies; 1) pulse pressure, 2) pulse wave velocity□3) waveform analysis. A number of alternative devices based on these methods have recently been described [37].

1.3.1 Pulse pressure

Pulse pressure is the difference between systolic and diastolic blood pressure and is the consequence of cardiac contraction and is strongly influenced by the properties of the arterial tree. [38] As the pulse pressure is mainly determined by stiffness of the large arteries and stroke volume, and pulse wave reflection, it constitutes a surrogate marker for arterial stiffness. [39]

Brachial pulse pressure, which can be assessed easily and reliably in routine clinical practice, provides a simple surrogate measure of large artery stiffness. A number of, but not all, observational and interventional studies have identified pulse pressure as an important, independent predictor of cardiovascular risk in older subjects aged over 50 years. [40-47]

Unfortunately, assessment of arterial stiffness by pulse pressure can be quite inaccurate. The brachial blood pressure is strongly determined by the phenomenon of pulse wave amplification from the aorta to the peripheral arteries. Due to pulse wave amplification, Figure 1.1[48], the peripheral systolic blood pressure and consequently also the pulse pressure can differ markedly between central end peripheral arteries. [49, 50] Thus, pulse pressure measured in the brachial artery is amplified compared to the proximal aorta. Pulse wave amplification decreases with age and is most prominent in the young. [51] Hence, measurement of arterial stiffness based on the brachial artery may lead to the misclassification of subjects at high risk, particularly those below 60 years.

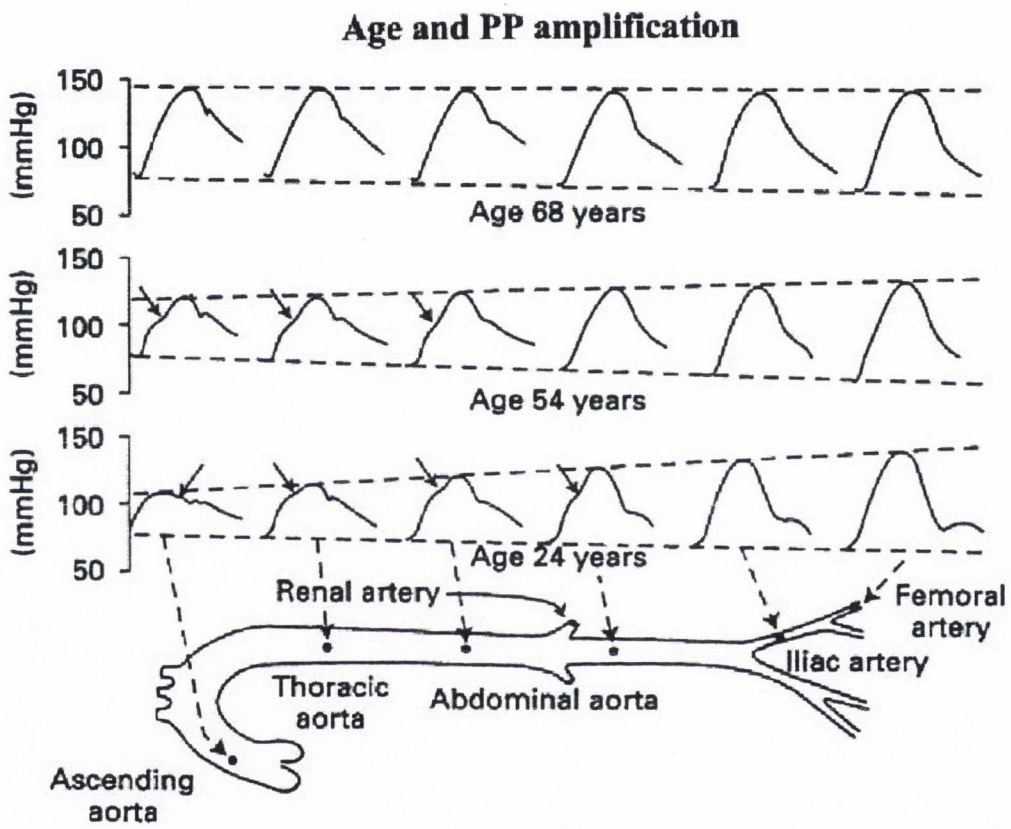


Figure 1.1 [48] Propagation of the PP wave from central to peripheral arteries in patients 24, 54, and 68 years of age. In older patients, the more rapid propagation of PW reduces PP amplification, resulting in nearly identical central and peripheral BP.

1.3.2 Pulse wave velocity

Arterial stiffness can be assessed noninvasively by measurement of pulse wave velocity (PWV), a simple and reproducible method. [4, 52, 53] Pulsations arrive progressively later at more peripheral sites with the delay depending on the distance from the heart and on PWV. (Figure 1.2) The speed of pulse wave transits from the aorta to the peripheral arteries is mainly determined by the artery wall stiffness and lumen diameter. PWV can be calculated by measuring the time for the pulse to pass between two points with known distance. The measurement usually involves taking separate recordings from two sites and relating them to the R wave of a simultaneously recorded ECG. A variety of methods can be applied to register the pulse wave such as doppler ultrasound, or applanation tonometry. Since the aorta is the major component of arterial stiffness, the carotid-femoral pulse wave velocity, which is a measure of aortic stiffness, is the most commonly used in the evaluation of regional stiffness. Carotid-femeral pulse wave velocity is mainly a measure of velocity and is also quite commonly used when conduit artery stiffness is examined.

Assessment of pulse wave velocity is relatively simple and the method has been widely applied and has been found to be both robust and reproducible. Studies show that pulse wave velocity is an independent predictor of cardiovascular disease and mortality in both hypertensive patients and in patients with end-stage renal disease. [54, 55] Furthermore, aortic pulse wave velocity is a powerful independent predictor of mortality in diabetic and elderly population samples. [56-58] PWV has been used to evaluate the vascular effects of both vasoactive substances [59] and antihypertensive drug therapy. [60, 61]

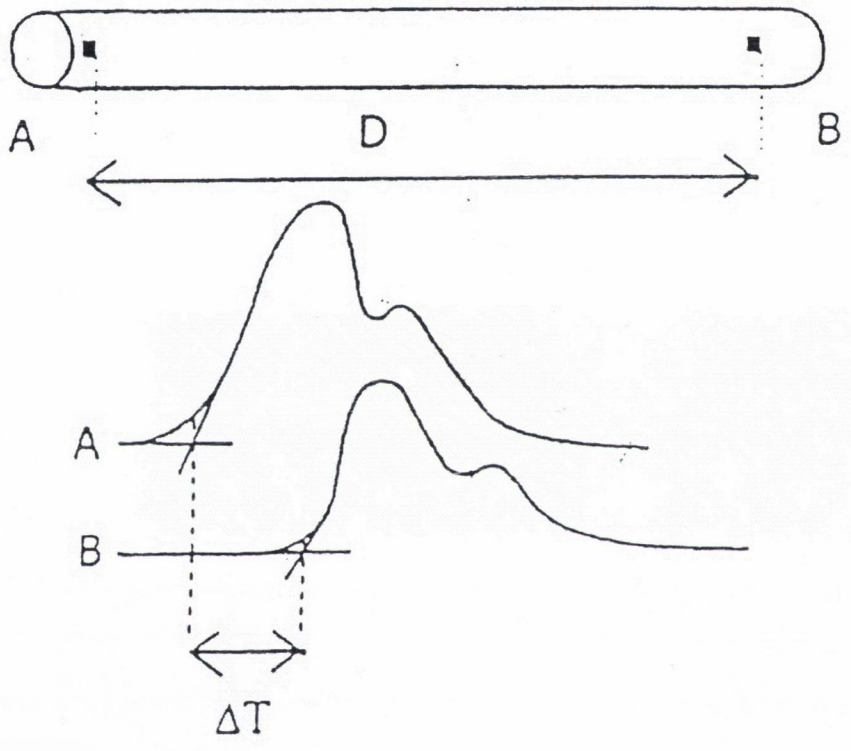


Figure 1.2 The carotid pressure waveform (A) and the femoral artery waveform (B) showing the delay in the transmission of the pulse between the two arterial points shown as ΔT

1.3.3 Pulse wave analysis

As the left cardiac ventricle contracts it creates a forward pressure wave that travels to the periphery throughout the arterial tree. When the forward wave reaches the branching points of arteries, regions of increased arterial stiffness, and high-resistance arterioles a backward wave occurs as a consequence of wave reflection. [4, 13] The reflected waves are superimposed on the wave that travels forward resulting in an arterial waveform that varies throughout the arterial tree.

Pulse wave analysis (PWA) is a non-invasive method to measure arterial stiffness. [62] Applanation tonometry uses a Millar transducer to record pressures at the radial or the carotid artery, and a validated generalized transfer function based upon a comparison with intra-arterial pressures in patients undergoing surgery is then applied to generate the corresponding central waveform. [63-64]

The augmentation index (AIx) is a hemodynamic measure related to arterial stiffness. It is defined as the increment in pressure from the first systolic shoulder to the peak pressure of the aortic pressure waveform expressed as a percentage of the peak pressure.

The aortic or central augmentation index (AI) is calculated as the ratio of the pressure difference between the 'shoulder' of the aortic wave and 'peak' central systolic pressure (□P=P1-P2) and the pulse pressure (PP) according to the formula: AI= (□P/PP) × 100 (Figure 1.3) [62].

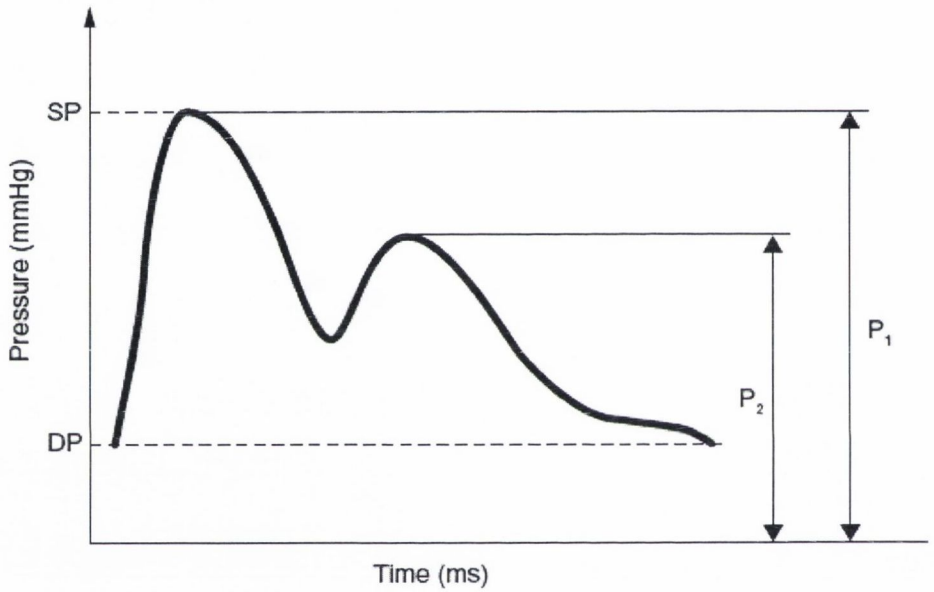


Figure 1.3 Typical waveform of central aortic pressure in young people (<40 years old). P1, central initial peak, generated by cardiac ejection; P2, central second peak, generated by reflective wave; SBP, central systolic blood pressure; DBP, central diastolic blood pressure.[62]

Arterial stiffening increases the amplitude and the velocity of the reflected waves. If the reflected wave travels down the arterial tree and back fast (high PWV – with stiffer arteries, e.g. in the elderly), it returns early to the ascending aorta and merges with the incident wave at an early point. Thus it adds to pressure and augments the waveform at systole, producing a second (late) systolic peak that is higher than the first peak. In contrast, if PWV is low (as in young healthy individuals), the reflected wave arrives at a later point of the cardiac cycle and thus, the augmentation is seen at diastole as a convexity rather than concavity decay of pressure (Figure. 1.4) [63].

Recently, it was reported that increased AIx was associated with the presence and severity of coronary artery disease (CAD), particularly in younger and middle-aged male patients [65]. However, in hypertensive patients the impact of AIx on the prognosis remains unclear.

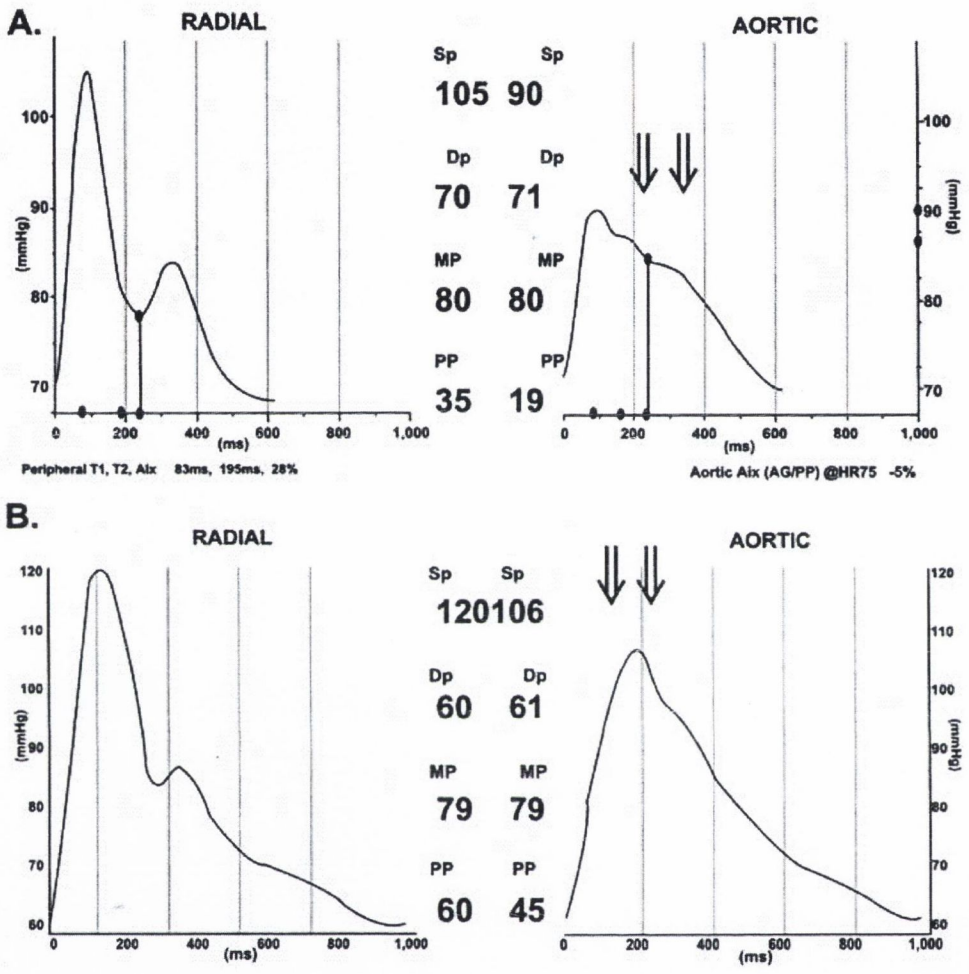


Figure 1.4 [63] (A and B) Wave reflections in young and old persons (Pulse Waveform Analysis: Radial waveform is shown on the left, derived central aortic waveform is shown on the right). The reflected wave is marked with arrows. SBP = systolic blood pressure, DBP = diastolic blood pressure, MBP = mean blood pressure, PP = pulse pressure.

1.4 A: 8 year old boy: return of reflected wave in diastole with convex diastolic contour.

1.4 B: 60-year old diabetic: return of reflected wave in early systole with marked pressure boost in late systole.

Note the huge difference in pulse pressure with nearly equal mean pressure.

1.4 Determinants of arterial stiffness

Although increased arterial stiffness (decreased arterial elasticity) has been considered intrinsic to the aging process of the arterial wall, it has since been demonstrated that factors such as smoking, hypertension, gender difference, exercise, and diabetic mellitus are important predictors of reduced arterial elasticity, independent of age.

1.4.1 Aging

Aging has a greater effect on arterial stiffening than any other factors [4]. Using various assessment techniques, an age-dependent increase in arterial stiffness in both healthy and diseased populations has been described. It has been shown that in vivo aortic stiffness decreases sharply with age in the first decade of life, reaching a minimum at 10 years of age, and thereafter increasing with age in both genders. [66] Using multivariate models, age is the major clinical determinant of aortic stiffness, a finding confirmed in several populations in different countries. [66-68]

The underlying basis for these age-dependent changes is still incompletely understood. Increasing fatigue, fracture and fragmentation of the elastin fibers or lamellae within the media of the elastic arteries seems to be the cause of the increase of the arterial stiffness with age. In the other way, age causes arterial elastin to decrease while collagen increases [8].

The effects of aging are different on proximal, predominantly elastic arteries, compared to distal, predominantly muscular arteries. [4, 69, 70] Central arteries stiffen progressively with age, whereas stiffness of muscular arteries changes little with age. [4] A number of clinical studies have analyzed the effects of age on aortic stiffness, mainly with the method of PWV. The increase of central artery stiffness with age is responsible for earlier wave reflections and changes in pressure wave contours. Kelly et al [71] observed that wave reflections were responsible for an increase of about 25% in pulse pressure (PP) between age 30 and 60 years. The effects of age on peripheral

artery stiffness are less pronounced; it has been shown that the peripheral PWV (carotid–radial or femoral–tibial) changes with age are approximately two to three times less marked than those of the central aorta [68]. Similar results have been observed when local cross-sectional distensibility coefficients were evaluated at the sites of the carotid and femoral arteries of the same subjects. Carotid artery distensibility was strongly correlated with age, whereas no such correlation was observed at the site of the femoral artery. [4, 72]

The increase in aortic stiffness with age occurs gradually and continuously and is similar for men and women [4]. Crosssectional studies have shown that aortic PWV increases with age by approximately 0.1 m/sec per year (about 1%) [67]. Similar changes were observed at the site of the carotid artery [4]. However, some studies have suggested that the increase in large artery stiffness follows a nonlinear quadratic evolution, with a more pronounced increase after the age of 55 years [73, 74]. This result corroborates the well-known epidemiologic observation of an increasing prevalence of systolic hypertension, the main clinical manifestation of large artery stiffness, after the age of 55 years. [75]

1.4.2 Smoking

Although smoking is known to alter the arterial wall, particularly the endothelial function, and to accelerate atheromatosis in several arterial territories, little is known about the influence of tobacco consumption on arterial stiffness. Failla et al [76] showed that in smokers without any overt cardiovascular disease, acute cigarette smoking reduced distensibility in both medium and large arteries. It has also been demonstrated that cigarette smoking increased PWV in both normotensive and hypertensive subjects [77]. The acute effects of smoking were also investigated in chronic smokers by several investigators [78-81]. All of these studies showed that acute smoking significantly decreased carotid and brachial distensibility, and increased BP. One recent study on the duration of smoking cessation showed that reversal of the deleterious effects of smoking on arterial stiffness is likely to take >10 years to achieve

levels of stiffness similar to that of never smokers [82]. In middle-aged women, no association between aortic PWV and history of smoking was observed.

1.4.3 Hypertension

Stiffness and characteristic impedance of the aorta and large elastic arteries increase with an elevation in arterial pressure [4]. In the early stage of hypertension, compliance of peripheral muscular arteries may be paradoxically increased due to passive arterial dilatation [70]. Changes are largely due to the physical effect of increased arterial pressure per se (increased stiffness at higher pressure) and are largely reversed by antihypertensive therapy [4]. They are the same changes seen with aging - early return of wave reflection and greater augmentation of the central aortic and carotid pulse. Arterial stiffening in hypertension may be increased by the structural degeneration caused by prolonged high arterial pressure, creating a vicious cycle. Arterial degeneration, medial necrosis, and aneurysm formation are accelerated by hypertension.

1.4.4 Gender differences

As estrogens have vasorelaxant effects, women have a higher aortic compliance than men throughout the middle-aged period of their life. However, after the menopause the aortic compliance level of women approaches that of men. This was found by various investigators, using different noninvasive techniques, including pulse wave velocity measurements by Doppler flowmeters [66], ultrasound-determined pulsatile diameter changes of the distal aorta [83] and echocardiographic measurements of the aortic root [84]. In a recent analysis from the Framingham Heart Study, women aged 37-86 years had modestly lower carotid-femoral PWV and moderately lower carotid-radial PWV than men of comparable age [85].

1.4.5 Exercise

Chronic exercise has beneficial effects on arterial function [86]. In healthy volunteers, AIx and PWV were significantly lower in endurance trained athletes than in sedentary controls. In contrast, in acute exercise PWV, wave reflections and arterial stiffness increase, probably related to the increase in blood pressure [50]. Aortic characteristic impedance is unchanged, however, because the increase in aortic cross-sectional area offsets these changes in compliance and PWV. Moreover, the disparity between central and peripheral arterial pressures increases [87]. This may lead to an overestimation of myocardial work because the aortic systolic pressure increases far less than the brachial systolic pressure.

1.4.6 Diabetes mellitus

Type 1 diabetes is associated with enhanced wave reflections and an increased AIx [88]. Patients with type 2 diabetes have stiffer aortas and arteries than nondiabetic subjects, as assessed by various techniques, including ultrasound measurements of the carotid arteries and Doppler ultrasound measurements of PWV [89, 90]. The effects of chronic hyperglycemia on nonenzymatic glycosylation of matrix proteins may, in part, explain this difference [91]. Glycosylation of collagen and elastin and accumulation of advanced glycosylation end-products have been shown to cause vessel stiffening [92]. Moreover, there appears to be a multiplicative effect of atherosclerosis and vessel stiffening in diabetes [93]. Diabetic patients undergoing coronary artery bypass grafting had stiffer aortas than matched control-patients [91]. Children with a positive parental history of diabetes had stiffer arteries than children without such a parental history [94]. These findings have been confirmed in healthy young adults with a positive family history of type 2 diabetes [95].

1.4.7 Genetic factors

Because of the numerous components of the arterial system, it does not seem likely that the influence of gene polymorphisms on arterial stiffness can be limited to a single

gene. In cohorts of subjects with mild-to-moderate hypertension, several candidate genes have been found to be significantly associated with aortic stiffness for the same age and BP as in control populations. Thus, polymorphisms of aldosterone synthase [96] and angiotensin II type 1 (AT1) receptor gene [97], angiotensinogen gene, or nitric oxide synthase gene, [98-100] have been shown to be significantly associated with increased aortic pulse-wave velocity. The AT1 receptor gene polymorphism A1166C is the most important candidate to consider. First, the stiffness alterations associated with this polymorphism are more pronounced in older than in younger hypertensive subjects. In particular, the increase in pulse-wave velocity with age is steeper in hypertensive individuals with the C allele than in the 1166A homozygous [74]. Second, recent human studies showed that the AT1 1166C allele was associated with increased vascular reactivity to several vasoconstrictors in vivo [101-103], which is also seen with the T mutant of the constitutive nitric oxide synthase gene (G894T polymorphism). In hypertensive populations, subjects with the C allele of the AT1 receptor gene polymorphism exhibit a greater pressure independent effect on aortic pulse-wave velocity than do the other allele groups for the same degree of chronic ACE inhibition. [104]

Matrix metalloproteinases (MMPs) are potential candidate proteins, because they are involved in matrix homeostasis and arterial wall remodeling. This is particularly true of MMP-3 (stromelysin-1), which acts on various substrates, including fibronectin, elastin, and collagens. Aortic stiffness has been shown to be greater in subjects older than 60 years and homozygous for the 5A promoter polymorphism of MMP-3 than in age-matched subjects homozygous of the 6A polymorphism. [105]

Zhang et al described a functional -1562 C/T polymorphism in the promoter region of MMP-9 [106] characterized by a single nucleotide change from cytosine to thymidine 1562-bp upstream from the start of transcription (C/T) which has been shown to be related to the presence and severity of arteriosclerosis [107] and associated with large vessel stiffening in patients with CAD [108] and healthy subjects. [109]

1.5 Arterial morphology

1.5.1 Structure of the normal arterial wall

The basic morphological structure of larger arteries consists of cells and matrix arranged in three transmural zones, which are the tunica intima, the tunica media and the tunica adventitia (Figure 1.5). The intima is the region bound on the luminal side by a one cell thick layer of endothelial cells on top of basement membrane. The intima is bound peripherally by the internal elastic lamina (IEL), a fenestrated sheet of elastic fibres, which provides support for the endothelial cells. Between the endothelium and elastic tissue, vascular smooth cells (VSMCs) and various components of the exracellular 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. 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, which is thinner than the IEL and an inter-lamellar zone, which consists of circular layers of smooth muscle cells (SMCs), with a slight helical orientation. VSMCs are the exclusive cellular component of the medial layer, but they do not represent a homogenous cell population. They have different mixtures of phenotypes, including contractile, proliferative, synthetic, or apoptotic behaviour [110]. The relative occurrence of each of these phenotypes depends on age, location in the vascular tree and pathological conditions. Despite these variations, it has been shown that the medial SMCs do not contribute significantly to the passive properties of the vessel wall. [111] In between the SMCs are oblique collagen and interconnecting bands or fibrils of elastic tissue between the elastic laminae. These form small compartments in which SMCs lie in a proteoglycan matrix. A connecting molecular unit distributes stress and maintains the viscoelastic properties

of the wall [112]. The external 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). It is 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 adventitia are the primary sauce of the tensile strength of the vessel wall.

Figure 1.5 The basic morphological structure of arteries

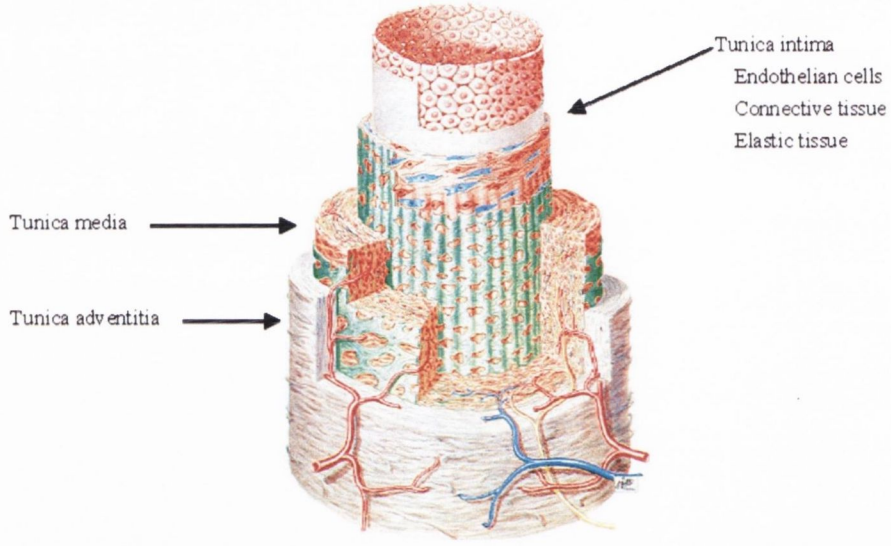


Figure 1.5 The three main layers of the artery. The innermost of the initma is bound on the lumen side by the endothelium and peripherally by the internal elastic lamina. The middle layer, the media is separated from the outer layer, the adventitia by the external elastic lamina.

Cite from: http://herkules.oulu.fi/isbn951426973X/html/equation2222.png

1.5.3 Endothelial function

The 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 [113, 114]. The endothelium can release hormonal factors that control relaxation and contraction and are therefore important in BP control, blood flow [115]. The endothelial cells produce a wide range of substances such as NO, prostacyclin, endothelin, vascular endothelial growth factor, interleukins, tissue plasminogen activator, angiotensin converting enzyme (ACE) and von Willebrand factor. Synthesis of NO occurs through enzymatic oxidation of L-arginine.

The function of each vessel and the role of its respective endothelium vary according to its location in the body. In the larger arteries, a healthy endothelium provides a surface that limits the activation of clotting and inflammation, blocks the transfer of atherogenic lipid particles into the arterial wall, and prevents adhesion of platelets and monocytes. In the resistance arteries, endothelial cells contribute to the regulation of blood flow and blood pressure. In the precapillary arterioles, the endothelium plays a role in the transport of nutrients and hormones, including glucose, fat, and insulin. [116]

Endothelial dysfunction is characterized by loss of endothelium-dependent vasodilatation and can be considered an early phase in the pathogenesis of cardiovascular disease. Decreased production, increased degradation or decreased sensitivity to NO are involved in endothelial dysfunction. Therefore, the term 'decreased NO bioavailability' is often used to describe the pathophysiological processes that involve NO in endothelial dysfunction. [117]

Reduced bioavailability of NO impairs vascular smooth muscle relaxation and thus causes functional stiffening of the arteries. Several studies have established an effect of

endothelial dysfunction on arterial stiffening [118-122]. Interestingly, a recent study has shown evidence that arterial stiffness itself may disturb endothelial function and NO release and thereby accelerate the stiffening process. [123]

1.6 The extracellular matrix

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 basement that supports the cellular elements of the vessel wall. Collagen and elastin, the connective tissue proteins are the two major extracellular constituents of the vascular wall (Figure1.6).

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 [126, 127]. For these reasons, the walls of such arteries are 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.

Figure 1.6 Human aorta stained with hematoxylin and eosin

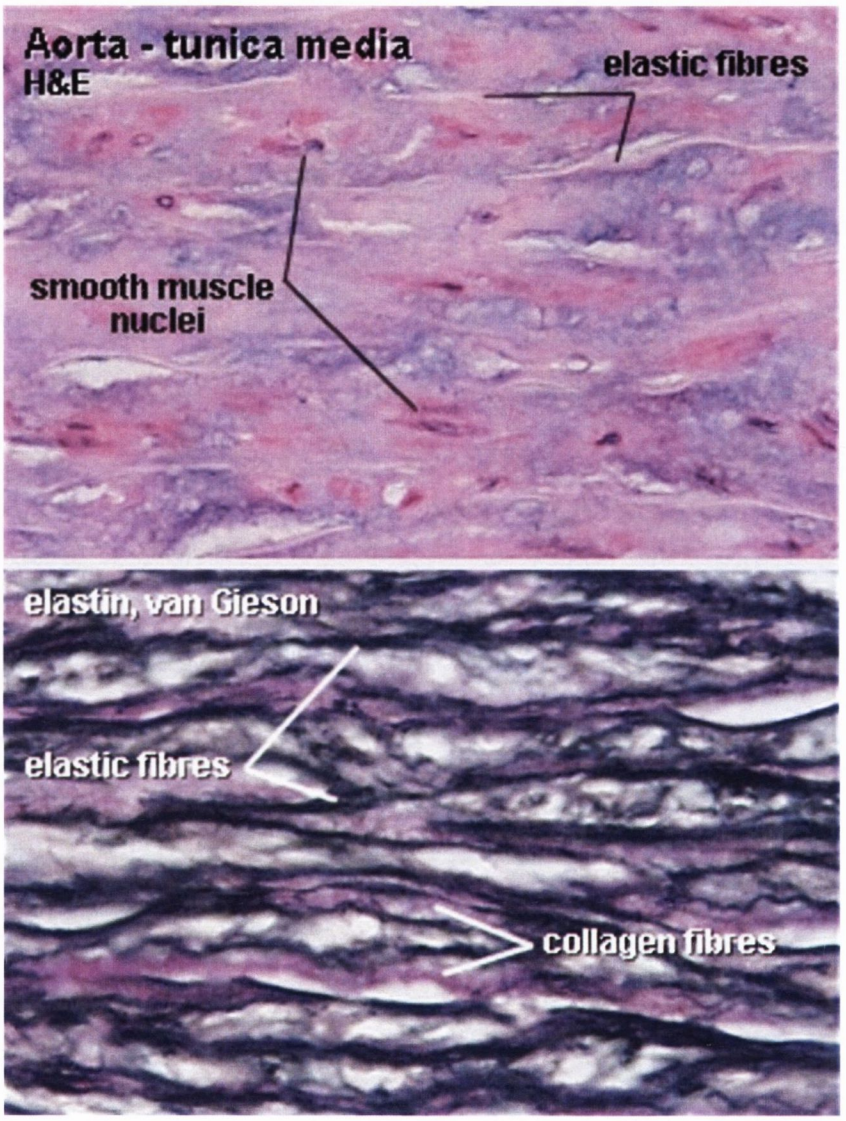


Figure 1.6: Section of human aorta (elastic artery) stained with hematoxylin and eosin (top) demonstrates smooth muscle cell nuclei and elastin fibres. The elastin van Gieson stain (bottom) demonstrates the large proportion of collagen and elastin fibres in the vessel wall.

From: http://www.ucc.ie/bluehist/CorePages/Vascular/Vascular.htm

1.6.1 Collagen

The term 'collagen' is used as a 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 [128] and so far 26 genetically distinct collagen types have been described [129-135]. 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. (Table 1.2)

Table 1.2 Classification and tissue distribution of collagen

Type	Subgroup	Molecular forms	Distribution
I	Fibrillar	[α1(I)]2α2(I) [α1(I)]3	Most tissue
II	Fibrillar	[α1(II)]3	Cartilage, cornea, Vitreous humor, intervertebral disc.
III	Fibrillar	[α1(III)]3	Soft tissue, with type I collagen
IV	Network forming	[α1(IV)]2α2(IV) [α1(I)]3 [α1(IV)]2α4(IV) other types	Basement membranes
V	Fibrillar	α(V)] 3α1(V) α2(V)α3(V) other forms	Minor amounts in most tissues with collagen type I
VI	Beaded filamentforming	α1(VI) α2(VI) α3(VI)	Minor amounts in most tissues
VII	Anchoring fibrilforming	[α1(VII)]3	Skin, cervix, oral mucosa
VIII	Network forming	[α1(VIII)]2α2(VIII)	Many tissues
IX	FACIT	α1(IX) α2(IX) α3(IX)	With collagen type II
X	Network forming	[α1(X)]3	Hypertrophic cartilage
XI	Fibrillar	α(XI) α2(XI) α1(II)	With collagen type II
XII	FACIT	[α1(XII)]3	Many tissues with type I collagen
XIII	Transmembrane domain	Unknown	Minor amounts in many tissue
XIV	FACIT	[α1(XIV)]3	Many tissues with type I collagen
XV	MULTIPLEXINS	Unknown	Many tissues
XVI	FACIT	[α1(XVI)]3	Many tissues
XVII	Transmembrane	[α1(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 muscles.
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

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. 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 collagen and anchoring collagens) and network forming collagens. [136]

Fibril forming collagens, which are the most abundant collagen family, account for about 90% of all collagens. Collagens type I, Π, ΙΙΙ, V and XI make up the fibril forming family and, of this family, type I, ΙΙΙ and V have been identified in the aorta and are mainly responsible for tensile strength and elastic resilience [137-139]. They lend stability and strength to the tissue in which they are present [140, 141]. Type ΙΙΙ collagen is co-localized with type I collagen in tissue such as the blood vessels and skin [142] and type III collagen is essential for normal collagen I fibrillogenesis in the cardiovascular system and other organs. [143] Collagen type V has also been localized 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 [144]. The elastic lamellae of human thoracic aorta are associated with collagen type V fibres, along with collagen type I and ΙΙΙ. [145]

1.6.1.1 Collagen regulation

Theoretically, the characteristics and amounts of collagen are determined at a very young developmental stage and thereafter remain quite stable because of very low turnover. The bulk of vascular collagen is produced by VSMCs, but may also be produced by endothelial cells, adventitial fibroblasts and macrophages. [146, 147]

Ageing is associated with adverse haemodynamics and metabolic alterations, which can accelerate vascular degenerative changes in both men and women. [148] Increased blood pressure, or hypertension, also stimulates excessive collagen production [149]. At the histological level arterial ageing manifests as a two- to three-fold increase of intima-media thickness during the normal life span [150, 151]. Collagen degradation is

regulated by catabolic matrix metalloproteinases. Histological examination of stiffened arteries shows damaged endothelium, increased collagen content, broken elastin molecules, hypertrophied vascular smooth muscle layers, inflammatory activity, and increased matrix metalloproteinases [152, 153]. Due to its slow turnover rate, collagen is particularly susceptible to nonenzymatic glycation and cross-linking. This leads to a more unorganized and dysfunctional collagen fibre structure with inferior elasticity.

1.6.1.2 Collagen changes and vascular disease

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 increase again in the established hypertensive state [154]. 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 Π inhibition through AT1 receptors [155]. Another study found no difference in aortic collagen content between SHRs and WKYs [156]. Changes in the total and proportions of various collagen subtypes have been investigated in hypertensive rats [157]. 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 latter 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 increased aortic stiffness. Induced hypertension and hyperlipidemia in rabbits displayed up-regulation of collagen type 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. [149]

Collagen changes in the hypertensive heart also occur. Myocardial fibrosis is known to occur in humans with left ventricular hypertrophy associated with hypertension [158]. The myocardial fibrosis results in an increase in the interstitial and perivascular content of fibrillar collagen type I and III [159]. Collagen changes occur in atherosclerosis [160]. Increased staining intensity for collagens type IV, V and IX was found in vessels that had more severe atherosclerotic lesions [136]. (Table 1.3)

Increased accumulation of collagen type I and III has also been reported in atherosclerotic lesions [161]. Experimentally induced atherosclerosis in sheep displayed reduced staining for collagen type IV as the lesion progressed, and was localized in the basement membrane in control tissue. [162] Collagen type IV exhibited a diffuse immunostaining throughout the vessel wall in the atherosclerotic sheep, with more marked concentration around the SMCs. [163] Type VIII collagen is present in the atherosclerotic media and intima [136] and in hypercholesterolamicm rabbits, the in situ distribution patterns of collagen type VIII in the carotid artery, implies a role for this collagen type in neointimal growth during the early phases of atherogenesis. [164]

The collagen content of atherosclerotic arteries of smokers is reported to be higher than that of the atherosclerotic arteries of non-smokers. [146]

Table 1.3 Collagens of the normal vessel wall and its expression in atherosclerotic lesions [136]

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

↑ Accumulated in atherosclerotic lesions.

1.6.2 Elastin

The elastic properties of the aorta depend largely on the presence of elastin in the wall which enables 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 and keeps the blood flow in the vascular system relatively constant. [165]

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 amount of elastin 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 tissues, and is a major component in the abdominal aorta and carotid arteries.

1.6.2.1 Elastin regulation

Elastin molecules are stabilized by cross-linking to form desmosine and isodesmosine. Disruption of these crosslinks contributes to weakening of the elastin array with predisposition to mineralization by calcium and phosphorous, together increasing arterial stiffness. [166-168]

Mature crossline 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 physiologically [169]. The main elastases belong to the MMP family, together with cysteinyl and serine proteinases [170]. Around 80-90% of serum elastase activity is due to matrix metalloproteinases [171]. Of the MMP family, MMP-9/92kDa gelatinase has the greatest affinity for aortic elastic fibres [172] although other MMPs can also degrade elastin, with varying affinity.

1.6.2.2 Elastin changes and vascular disease

Rupture and fragmentation of the IEL in the arteries are common features of patients with heritable connective tissue diseases [173]. Supravalvular aortic stenosis (SVAS) is a congenital vascular disease causing the narrowing of the aorta [174]. The condition can lead to hypertension, cerebrovascular accident and heart failure. Vascular lesions in affected arteries show disorganized, thickened elastic fibres, excessive hypertrophied SMCs and extensive deposition of collagen. A deficiency in the elastin gene and therefore a quantitive deficiency in elastin can lead to SVAS [174, 175]. It may be that the elastin deficiency makes the elastic fibres more susceptible to haemodynamic stress and damage or 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's syndrome (MFS), [176] a connective tissue disorder of which the main manifestation is dilation of the aortic root. Vascular tissues of MFS patients display disorganized and fragmented elastic fibres [177]. Studies carried out using mice with mutations in the fibrillin-1 gene [178] 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 a role for elastin in arterial development. [179] Late arterial development was affected as the arterial diameter declined and the lumen eventually becomes obliterated. These changes were a result of sub-endothelial accumulation of SMCs, a process involving cell proliferation, migration and reorganization—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 [180].

Angiotensin Π, which induces hypertension, has been shown to be related to elastin gene expression in the affected arteries. [181]

Sustained hypertension, which is associated with increased vessel stiffness, is associated with the destruction and the reconstruction of elastin fibres [182]. A deficiency in the elastin gene has been suggested as a cause for essential hypertension [183]. 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 the carotid artery balloon injury rat model, expression of potent elastolytic cysteine proteases is increased [184]. Expression of these two elastolytic enzymes- cathepsin S and K is increased and preferentially located to the developing neointima, suggesting an association with pathological intimal formation.

1.7 The Matrix metalloproteinases

The MMPs are a family of calcium and zinc dependent enzymes that can collectively degrade the entire ECM [185]. Although many proteases can breakdown the ECM molecules, the MMPs are believed to be the physiologically relevant mediators of matrix degradation.

1.7.1 Classification of MMPs

At present 26 human MMPs have been identified all of which have a high degree of similarity in their domain structures. MMPs are named based on their substrate specificity, their molecular weight or by Arabic numerals. MMPs have been divided into four groups based on substrate specificity—the collagenases, the gelatinases, the stromelysins and membrane-type metalloproteinases (MT-MMPs). (Table 1.4)

Table 1.4 MMP family

MMP	Name	Latent/active MW	Substrates
MMP-1	Interstitial Collagenase-1	55/45000	Collagens type I, II, III, gelatins, proteoglycans
MMP-2	72kDa gelatinse	72/66000	Gelatin, elastin
MMP-3	Stromelysin-1	57/45000	Collagens IV, V, IX, X, elastin, gelatin casein etc.
MMP-7	Matrilysin	28/19000	Proteoglycations, gelatin, elastin etc.
MMP-8	Neutrophil collagenase-3	75/58000	Collagens type I, II, III, gelatins, proteoglycans
MMP-9	92kDa gelatinase	92/86000	Gelatin, elastin
MMP-10	Stromelysin-2	57/45000	Collagens IV, V, IX, X, elastin, gelatin casein etc.
MMP-12	Macrophage metalloelastase	54/45000	Collagen, gelatin, elastin
MMP-13	Collagenase-3	60/48000	Collagens, gelatin
MMP-14	MT-MMP-1	66/56000	Collagens, casein, fibronectin
MMP-15	MT-MMP-2	72/60000	Gelatin, fibronectin

Latent/active MW=latent or active molecular weight. MT-MMP=membrane type MMP.

Table cited from matrix metalloproteinases-www.biotrak.apbiotech.com

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 3/4 and 1/4 the size of the original molecule [185, 186]. MMP-13 has been identified more recently and appears to be critical in bone metabolism and homeostasis. [187]

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 [185] the fragments produced due to collagen degradation by MMP-1. MMP-2 and –9 can also degrade elastin [188]. It is also generally thought that the gelatinases have substrate specificity for intact collagen type III basement membrane while some reports suggest that type III collagen is a poor substrate for these enzymes [189, 190]. 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 [191]. 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, X, elastin, gelatin, laminins, fibronectin and proteoglycan proteins [192]. In addition, MMP-3 activates other MMPs including MMP-1 and MMP-9 [193]. 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.

1.7.2 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-alpha (TNF-α) [194]. Others such as heparin, corticosteroids and transforming growth factor (TGF-α) 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 state and require activation before exerting their proteolytic activity. Plasmin is a potent activator of most MMPs (although plasmin-independent pathways do exist) [195,196]. 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 [197]. 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 [198]. 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. [199]

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 characterized [200]. TIMPs are secreted (but may also be found at the cell surface) as 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 [201, 202]. Although TIMP-1 acts

against all members of the collagense, stromelysin and gelatinase subgroups of the enzyme it is a poor inhibitor of MMP-9 and a number of MT-MMPs. It forms high affinity, irreversible (in vito) 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 [203] while on the other hand, TIMP-2 present at low concentrations, bound to MT-MMP is required for MMP-2 activation [207]. Activated MMP-9 is also preferentially inhibited by TIMP-2, although it exists in a complex with TIMP-1, which shows activation of the latent pro-form. [201, 205]

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 [206] while TIMP-2 stimulates the growth of lymphoma cells [207]. It has also been demonstrated that TIMP-2 stimulates the proliferation of fibrosarcoma cells [208] and dermal fibrolasts [209] and may therefore be related to vascular cell proliferation and vascular matrix production.

1.7.3 MMPs and the vessel wall

MMPs play a fundamental role in physiological and pathological vascular matrix degradation and collectively are capable of degradation of the entire ECM. Through their collagenolytic and elastinolytic effects, MMPs degrade the ECM by creating uncoiled, less effective collagen and broken and frayed elastin molecules, respectively.

Vascular collagenases include MMP-1, MMP-8, and MMP-13. MMP-1 is responsible for the degradation of native collagens type I, Π, ΙΙΙ, which is expressed by endothelial cells, while MMP-8 is stored in polymorphonuclear neutrophils (PMN) [210,211]. The gelatinase, MMP-2 and –9 are expressed in macrophages, PMN and vascular endothelial and SMCs [210,212]. 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.

1.7.4 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 remodeling is thought to be the cause of most prevalent vascular pathologies. MMPs therefore play a crucial role in vascular diseases including hypertension, [213-215] aortic aneurysms [216-218] and atherosclerotic remodeling. [219-221]

Human abdominal aortic aneurysms (AAA) are characterized by progressive dilatation of the aortic wall [222, 223]. The ECM remodeling in AAA is characterized by structural disorganization 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-9 is involved in aneurysm formation, while it is suggested that MMP-9 is involved in aneurysmal expansion at a later stage [224]. It has also been shown that cultured VSMCs derived from aneurysmal aorta produce three-fold higher levels of MMP-2 than from age-standardized atherosclerotic tissue [225]. 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 [226]. Using a rat model of aneurysm formation associated with arterial dilation, TIMP-1 levels were over-expressed by sending syngeneic rat SMCs transfected with TIMP-1 onto the luminal surface of the vessels [227]. 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 analyzed for possible associations with coronary aneurysms (CA) [228]. 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, [229] 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-, IL-1 and PDGF [230]. These cytokines and growth factors are known to stimulate MMP synthesis in human aortic VSMCs and vascular endothelial cells [231-233]. 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 were observed 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 functional role in plaque destabilization in atherosclerosis. Advanced atherosclerosis is characterized 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 [234]. 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 destabilization of plaques in regions prone to rupture [235, 236]. This is due to the breakdown of collagen and thinning of the fibrous cap rendering these enzymes an important determinant of the vulnerability of atherosclerotic plaques.

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 remodeling and neointimal formation, processes characterized by altered ECM turnover.

Intimal hyperplasia caused stenosis 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, plays a key role in the development of hyperplasia, along with excessive degradation and accumulation of ECM. Neointimal formation in these veins is accompanied by increased MMP-9 production [233]. In addition, when sections of intact saphenous vein were cultured with a therapeutic concentration of doxycyline (a potent MMP inhibitor) neointimal thickness and MMP-9 production were 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 [237]. 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. [238]

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 [239]. This increased expression may, in part, contribute to pathological remodeling 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. [240]

1.7.5 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 [241-243]. 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 remodeling after angioplasty in pigs, although no effect was seen on neointima formation [244, 245]. It has also been reported that batimatat reduced neointima formation 7 days after balloon injury in rat arteries but not beyond 14 days [246] a subsequent study did however report a reduction in neoinitmal formation due to batimastat administration in balloon injured rat carotid arteries up to 75 days after arterial injury [247]. Hydroxamate compound 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. [248]

Recently, ONO-4817 which belongs to a different class of drugs tetracycline has been shown to suppress neointimal formation in hypercholesterolemic hamsters. ONO-4817 prevented vascular stenosis in hamsters by inhibiting both SMC proliferation and migration [249]. Despite intense research, the only currently approved MMP inhibitor, also a tetracycline derived compound is the third generation periostat. This drug is only

licensed for the treatment of prriodontitis. Present studies aimed at developing MMP inhibitors are therefore aimed at producing drugs with improved pharmacokinetic properties and improved selectivity for specific MMPs. [250]

1.8 Adiponectin

1.8.1 Adipokines

For many years adipose tissue was viewed playing a passive role in total body lipid and energy homeostasis. Adipose tissue was considered to be the site where excess energy was stored, in the form of triglycerides (TGs), and was released in the form of fatty acid (FA) when the energy form was needed in the body [251].

It is currently proven that adipose tissue is not only a source of energy but also an active hormonal system involved in metabolic control. Many adipokines have been identified (Table 1.5) [252]. They all integrate in a communications network with other tissues and organs such as the skeletal muscle, adrenal cortex, brain and sympathetic nervous system and participate in appetite and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and haemostasis (Table 1.6) [252].

Table 1.5 Adipokines and their main effects [252]

Adipocytokines	Effects on
LPL	Lipid metabolism
HSL	Lipid metabolism
Perilipin	Lipid metabolism
aP2	Lipid metabolism
CETP	Lipid metabolism
RBP	Lipid metabolism
IL-6	Inflammation, atherosclerosis, insulin resistance
TNF-α	Inflammation, atherosclerosis, insulin resistance
Adipsin/ASP	Immune–stress response
Metallothionein	Immune–stress response
Angiotensinogen	Vascular homeostasis
PAI-1	Vascular homeostasis
Adiponectin	Inflammation, atherosclerosis, insulin resistance
PPAR-γ	Lipid metabolism, inflammation, vascular homeostasis
CRP	Inflammation, atherosclerosis, insulin resistance
IGF-1	Lipid metabolism, insulin resistance
TGF-b	Cell adhesion and migration, growth and differentiation
Monobutyrin	Vasodilation of the microvessel
Uncoupling proteins	Energy balance and theromoregulation
Steroid hormones	Lipid metabolism, insulin resistance
Leptin	Food intake, reproduction, angiogenesis, immunity
Resistin	Inflammation, insulin resistance
P450 arom	Lipid metabolism
Apelin	Insulin resistance
Visfatin	Insulin resistance
ZAG	Lipid metabolism, cancer cachexia

Table 1.6 Adipokines and their metabolic effects in humans [252]

Adipocytokines	Metabolic effects	Future Investigations into the
Adiponectin	Inhibition of monocyte adhesion to endothelial cells, macrophage transformation to foam cells, endothelial cell activation.	Detrimental effects of hypoadiponectaemia in obesity, type II diabetes mellitus, cardiovascular disease
Leptin	Satiety signal, inhibits lipogenesis, stimulates lipolysis, improves insulin sensitivity, angiogenic activity.	Effect on vascular structure
IL-6	Impairs appetite, lost fat tissue with no effect on lean mass, inhibits gluconeogensis, increases hepatic *de novo* synthesis of fatty acid and cholesterol.	Molecular mechanisms trough IL-6 can elicit proinflammatory or anti-inflammatory effects.
PAI 1	Inhibits activity of tissue-type plasminigen activator, an anticlotting factor.	Effects of tissue-type plasminogen activator, its inhibitor in type 1 and 2 diabetes mellitus
Adipsin	Stimulates triglyceride storage in adipose cells through stimulation of glucose transport, enhances fatty-acid re-esterification and inhibits lipolysis	Role on coronary artery disease
TNF	Stimulates release of FFA by adipocytes, reduces adiponectin synthesis and impaired insulin signalling.	Antifibrosis treatment for NASH
Resistin	Controversial effects on glucose metabolism Endothelial dysfunction?	Insulin resistance in muscle and liver
Angiotensinogen	Acts through vasoactive peptide angiotensin II, Correlates significantly with blood pressure.	Role on pharmacogenetic for hypertension
Aromatase	Converts androstenedione to estrone driving fat to subcutaneous and breast tissues.	Role in inflammation
11-Hydroxysteroid dehydrogenase	Regenerates metabolically active cortisol from cortisone in humans	Role in inflammation

1.8.2 Adiponectin and its biological function

Adiponectin, the most abundant known hormonal factor produced by adipocytes, was originally identified by four independent groups in the mid-1990s using different experimental approaches, in both mice and humans [253-256]. As a result, adiponectin is also called Acrp30 [256], AdipoQ [256], apM1 [254] and GBP28 [255].

The circulating plasma range of adiponectin in human subjects is 3–30 ug/ml, accounting for 0.01% of total plasma protein, being considerably more abundant than other adipokines, such as leptin (2–8 ug/l) or tumor necrosis factor (TNF)-α (8 ng/l) [257]. Adiponectin exerts its biological function through two receptors, AdipoR1 and AdipoR2, which are primarily expressed in skeletal muscle and liver respectively [258]. Studies have shown that AdipoR1 is also present in endothelial cells [259], cardiomyocytes [260], and pancreatic-β cells [261], whereas AdipoR2 is also expressed in endothelial cells [262]. These observations provide a potential molecular pathway through which adiponectin may exert its functions on these tissues. Both receptors have seven transmembrane domains but with different structures [263]. The expression of both AdipoR1 and AdipoR2 is significantly decreased in muscle and adipose tissue in hyperinsulinemic and hyperglycemic states [263]. Increasing evidence from experimental models indicates that adiponectin plays a vital role in lipid metabolism, [264] glucose metabolism, [265] insulin resistance [264, 266] and vascular diseases.

Several clinical studies have shown that adiponectin levels are negatively correlated with serum triglycerides and small dense low density lipoprotein (LDL), and positively correlated with high-density lipoprotein (HDL) [267-269]. Cross-sectional and intervention studies show that the relationship between adiponectin and plasma lipids is independent of age, gender, BMI and insulin sensitivity [270-272]. Adiponectin stimulates glucose metabolism by promoting the phosphorylation and activation of adenosine monophosphate-activated protein kinase (AMPK), a stress-responsive kinase, in skeletal muscle [273], liver [273] and adipocytes [274]. AMPK activation is

believed to be mediated, at least in part, by adiponectin binding to the cell surface receptors AdipoR1 and AdipoR2 [258]. Recently, T-cadherin was also identified as an adiponectin receptor [275], but its role in intracellular signaling remains unclear. Adiponectin increases insulin receptor tyrosine kinase activity in skeletal muscle, and increases the expression of proteins involved in fatty acid metabolism. Recent studies in humans have shown that plasma adiponectin regulates insulin receptor tyrosine phosphorylation [276]. Moreover, adiponectin gene deficient mice display reduced plasma FFA clearance and elevated TNF-α levels, increased TNF-a expression in adipocytes and reduced levels of fatty acid transporter protein mRNA (FATP-1) in muscle. Knock-out mice fed on a fat and carbohydrate rich diet develop insulin resistance and display impaired insulin-receptor substrate (IRS-1)-associated phosphatidilinositol-3 activity in muscle [277]. Similarly, supression of adiponectin expression reverses the reduction of FATP-1 expression, the increase of TNF-a, and insulin resistance. [277]

Recent studies support adiponectin as an anti-inflammatory vasoprotective adipokine. First, the pathogenesis of atherosclerosis is summarized and the influence of adiponectin on cardiovascular disease is described. Early in atherosclerosis, various lipoproteins such as low density lipoproteins (LDL) and lipoprotein (LP) are deposited in the intima of the vascular wall (Fig. 1.7). LPs are oxidized and easily adhere to some endothelial cells such as vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1, and E-selectin (Fig. 1.7). Mononuclear cells can attach to endothelial cells and move into the subendothelial space (Fig. 1.7). Once they arrive in the vessel wall, monocytes may transform into macrophages and take up oxidized LDL through scavenger receptors (SR) whilst turning into foam cells. This process is supported by acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) in macrophages, which catalyzes the formation of cholesteryl esters. As a secondary event, the synthesis of vasodilating NO by endothelial NO synthase (eNOS) is impaired (Figure 1.7) [278].

Figure 1.7 Influence of adiponectin on endothelial and vascular function [278]

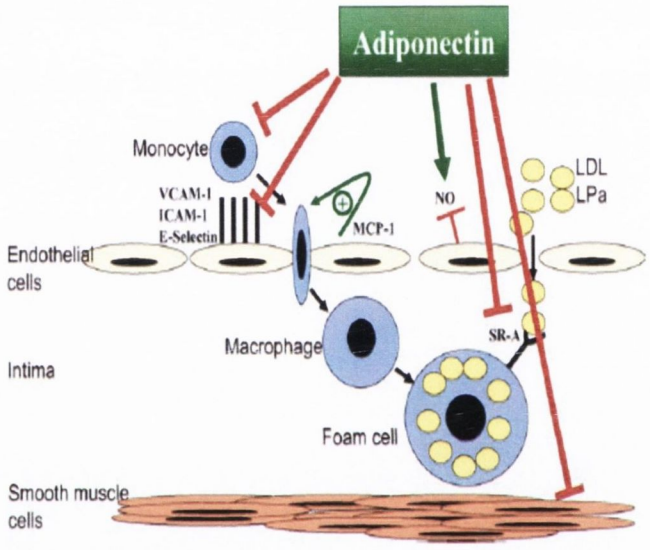


Figure 1.7 Major steps in the pathogenesis of atherosclerosis. Adiponectin inhibits upregulation of adhesion molecules, attachment of monocytes to endothelial cells, transformation from macrophages to foam cells, as well as proliferation and migration of vascular smooth muscle cells. Moreover, NO production of endothelial cells is stimulated by this adipokine.

1.8.3 Adiponectin and coronary artery disease

Plasma levels of adiponectin are significantly decreased in obese patients, and the levels of adiponectin are strongly and negatively correlated with BMI [279]. Moreover, type 2 diabetic patients were found to have lower levels of plasma adiponectin concentrations than non-diabetic subjects, independent of BMI [277]. Plasma levels of adiponectin were significantly decreased to a greater level in patients with coronary artery disease (CAD) than in age- and BMI-adjusted control subjects [280]. And in patients with type 2 diabetes, plasma adiponectin levels were shown to be obviously lower in patients with CAD than in patients without CAD. The presence of microangiopathy, such as retinopathy and microalbuminuria, did not seem to affect the plasma adiponectin levels. Therefore, adiponectin levels may be particularly associated with macroangiopathy in patients with type 2 diabetes. A recent study reported that hypoadiponectinemia was significantly and independently correlated with CAD even after adjustment for several coronary risk factors [281]. In this study, male subjects with hypoadiponectinemia (< 4.0 mg/l) had a 2-fold increase in CAD prevalence, independent of other coronary risk factors. The definition of hypoadiponectinemia (< 4.0 mg/l) was defined by the 25^{th} percentile in this study.

It is well established that atherosclerosis is an inflammatory disease [282]. Among inflammatory markers, as C-reactive protein (CRP) is one of the most beneficial and reliable biomarkers in cardiovascular risk assessment [283-285]. It was reported recently that plasma adiponectin levels correlate negatively with the CRP levels in patients with CAD [286]. This study also showed that not only was CRP mRNA expressed in the human adipocyte, but also that the levels of CRP mRNA in human adipose tissue correlated negatively with the levels of adiponectin mRNA in that tissue [286]. Another study in patients with acute myocardial infarction (AMI) reported that the reduction in plasma adiponectin during the early time course of AMI was negatively correlated with the plasma CRP levels [287]. CRP is generally produced in the liver, under regulation by cytokines, such as IL-1h, IL-6, and TNF-a [285].

However, a recent study showed the presence of CRP mRNA in atherosclerotic plaques [288]. Therefore, the expression of CRP may be negatively regulated by adiponectin in adipose tissue.

It is clear that most patients with the metabolic syndrome are closely related to insulin resistance [289]. Hyperinsulinemia associated with an insulin-resistant state may lead to decreased adiponectin levels. The evidence that adiponectin accumulates in the arterial walls of the injured vessels but not in non-injured walls, might suggest the possibility of circulating adiponectin consumption in patients with CAD [290]. Hypoadiponectinemia was also found to be an independent predictor of cardiovascular events in patients with end-stage renal disease [291]. The plasma levels of adiponectin in end-stage renal disease patients are relatively higher than in the general population.

1.9 RAGE

1.9.1 Glycation of macromolecules: A cytotoxic process

Nonenzymatically glycated proteins have been shown to play a role in the development of a variety of pathogenic processes involving inflammation, renal failure, and, diabetic complications.

At the molecular level, glucose and proteins react non-enzymatically under physiological temperature and PH to form a Schiff base [292]. This undergoes rearrangements to form a complex structure known as an advanced glycation end product, or AGE [293].

Initial studies on AGEs focused on their slow formation involving long-lived proteins such as vessel wall type I collagen [294]. This occurs naturally with aging and could be accelerated under conditions of hyperglycemia as happens in diabetes and in inflammatory/prooxidative environments such as atherosclerosis [295]. Recent studies indicate that AGEs not only form slowly on extracellular proteins, but also form rapidly intracellularly with intermediates of glucose metabolism [296]. The

predominant AGE structure occurring in vivo is a carboxy (methyl) lysine (CML) compound, which is found to be elevated in diabetic subjects with renal failure and vascular disease [297]. AGEs elicit their effect by a number of mechanisms. First, AGEs were involved in the occurance of vascular disease by their ability to cross-link proteins of the vascular vessel wall, altering their structure and function which leads to vessel thickening and vascular leakage [298]. The use of an AGE inhibitor in animal models blocked these effects, suggesting AGE may be a mechanism for vascular disease [298]. Second, rapid intracellular AGE formation can alter cellular function by changing protein structure and function. AGEs have been shown to have the ability to interact with specific cellular receptors that can degrade AGEs or can elicit signal transduction pathways resulting in the activation of proinflammatory and procoagulant pathways. A number of receptors have been identified, which include the AGE-receptor complex (galectin-3, OST-48, and 80K-H) [299], macrophage scavenger receptors (type I and II) [300], LOX-1 [301], CD-36 [302], and the receptor for advanced glycation end products, or namely RAGE [303]. Despite the binding ability, the main biological function of these receptors and the related pathologies is unclear except for RAGE.

1.9.2 RAGE: A multiligand receptor

RAGE was first identified from bovine lung as a 35-kDa protein that bound AGEs in a dose-dependent manner [303]. RAGE was first thought to act as a scavenger receptor for AGEs; however, further studies demonstrate RAGE to act as a signal transducer after binding AGEs to up-regulate gene expression [304]. RAGE was found in low levels in most tissues except lung [305]. RAGE was also found to be at high levels in the developing embryonic rat brain; however, it is not thought to be a key factor for neuronal development, as RAGE knockout mice develop normally, live a natural life span, and are fertile [306]. At the cellular level, RAGE is expressed by a variety of cells, including endothelial cells, vascular smooth muscle cells, podocytes, and neuronal cells [305]. Studies revealed under proinflammatory conditions such as

diabetic vascular disease, that RAGE expression is highly increased [305].

RAGE can bind amphoterin, also known as HMGB-1 (high mobility group-1), a protein involved in neurite outgrowth and linked to tumor invasion [307]. Most recently, RAGE was found to bind to a variety of the S100/calgranulin family of proteins, including S100A12 (extracellular newly identified RAGE-binding protein, or EN-RAGE) and S100B [308]. This protein family consists of at least 15 proteins; their concentration level will be up-regulated under inflammatory disease conditions [309]. The fact that RAGE can bind multiple ligands is consistent with both the heterogeneous nature of AGE structures and the multiligand nature of other immunoglobulin receptors such as CD36 [310]. Bronstein et al have demonstrated the successful crystalization of S100A12 and its functionality [311]. It was shown that S100A12 forms a hexameric assembly, and it was proposed this interacts with three RAGE extracellular domains in a trimeric assembly to activate signaling [312].

1.9.3 RAGE and vascular disease

The measurable method for testing the progressive pathogenesis of vascular disease in diabetes is the hyperpermeability of the vascular endothelium [313]. To investigate this, some in vivo studies of RAGE were focused on a rodent model [314]. Administration of anti-RAGE IgG or sRAGE (soluble extracellular domain minus the transmembrane domain and tail) (2.25 or 5.15 mg/kg) strongly blocked the increase in vascular permeability in diabetic animals [314]. In addition, blockade of RAGE could also reverse vascular hyperpermeability in diabetic rats treated 11 weeks after induction of diabetes. This therefore suggested that vascular hyperpermeability was not only reversible, but also indicated a role for RAGE and its blockade in established diabetic vascular disease. And a rodent model was set for accelerating diabetic atherosclerosis [315]. Macrovascular disease is by far the most common complication to affect human diabetic subjects, accounting for around 70% of their mortality [316, 317].

The effects of sRAGE blockade on the proliferation and migration of SMC in the arterial wall led to the investigation of other macrovascular disease processes where neointima expansion is a central mechanism; also called restenosis induced by angioplasty [305]. Evidence suggests that especially in diabetic subjects, angioplasty is associated with restenosis in 40-50% of subjects [318]. One of the major underlying mechanisms of restenosis is neointimal hyperplasia resulting from proliferation of SMC [318]. In a rat model of accelerated neointimal expansion, demonstrated by enhanced SMC proliferation, RAGE was seen to be up-regulated, with sRAGE reducing SMC proliferation in animal's models [319]. RAGE knockout mice were markedly resistant to neointimal expansion after arterial denudation. SMC was shown to be the principal RAGE-expressing cell type involved in neointimal expansion, in studies employing mice transgenetically expressing the DN-RAGE form using a SMC specific promoter, which also demonstrated a resistance to vascular injury [305].

Apart from macrovascular disease, microvasculature disease can affect the eyes, kidneys, and peripheral nerves because of diabetic vascular disease. Renal disease may be the most important disease, which occurs less frequently than retinopathy but accounts for the main mortality of all diabetes complications [320].

An insulin-resistant db/db mouse model of diabetes was used to test the role for RAGE in the activation of podocytes and the subsequent increased excretion of serum albumin and loss of renal function [321]. In long-term studies of renal disease, animals were assessed with sRAGE treatment after 27 weeks. In the sRAGE-treated group, decreased glomerular and mesangial expansion was seen in conjunction with decreased rates of albumin excretion [321]. These data suggest a role for RAGE in the renal disease of diabetes. Diabetes was induced by streptozotocin in both wild-type and RAGE knockout mice and cell and morphological changes assessed. The increase in VEGF expression seen in the diabetic wild-type mice was blocked in the diabetic RAGE null mice, along with a lack of mesangial matrix expansion and thickening of the glomerular basement membrane [276]. Studies by other groups designed to

genetically alter RAGE expression have shown a role for RAGE in renal disease [322]. Yamamoto et al. developed a double transgenic mouse model that developed diabetes (islet cell insulin knockout). It expresses high levels of RAGE in the vascular endothelium [322]. The double transgenic animal demonstrated enhanced albuminuria, mesangial expansion, and glomerulosclerosis compared to the single transgenic diabetic animal. However, in this study, significant transgenic expression of RAGE was also seen in monocytic cells, the infiltration of which accelerates glomerular disease [322].

1.10 Aims of the present study

Arterial stiffness is a major predictor of cardiovascular risk independent of classical risk factors. Identification of molecular, cellular and genetic causes of arterial stiffness is essential to explore and understand mechanisms involved in increasing arterial stiffness. In my research I chose some candidate genes and studied some polymorphism sites of these genes. My candidate genes include COL3A1 gene, ELN gene, MMP-9 gene, adiponectin gene and RAGE gene.

The physical properties of the arterial walls largely depend on the two extracellular proteins elastin and collagen [323]. The proportion of elastin and collagen in the arterial wall is regulated by a slow dynamic process of formation and degradation. Elastin and collagen degradation is regulated by catabolic matrix metalloproteases. Disturbances of this balance typically lead to higher collagen content and a diminished proportion of elastin, which reduces arterial elasticity [324]. Collagen production is also stimulated by elevated blood pressure [149]. At the histological level arterial ageing manifests as a two- to three-fold increase of intima-media thickness during the normal life span [150, 151]. Histological examination of stiffened arteries shows damaged endothelium, increased collagen content, broken elastin molecules, hypertrophied vascular smooth muscle layer, inflammatory activity, and increased matrix metalloproteinases [152, 153]. Both the role of collagen gene and elastin gene

in tissues and the mechanisms by which mutations in the two genes produce disease are still largely unknown. Since both the COL3A1 2209 G>A polymorphism and the 549 G>A polymorphism of elastin gene lead to an amino acid substitution, they may change the structure of collagen and elastin in the arterial wall, which could modulate BP and arterial stiffness.

Matrix metalloproteinases (MMPs) are potential candidate proteins, because they are involved in matrix homeostasis and arterial wall remodeling. MMP-9 is an inducible protein, and tissue and plasma levels therefore reflect stimulatory and genetic factors. The MMP-9 gene is located on chromosome 20, and a number of polymorphisms in the promoter, coding, and untranslated regions that exhibit tight linkage disequilibrium have been reported [106]. Of all the variants, the most extensively studied is the 1562C>T polymorphism, upstream from the transcription start in the promoter region. Previous data indicate that this variant is functional with increased transcriptional activity in macrophages [106]. It has also been associated with presence and severity of CVD, increased MMP-9 levels, [325] and systemic arterial stiffness in patients with coronary artery disease (CAD) [108]. A second nonconservative single nucleotide polymorphism 836G>A locate at codon 279, resulting in an amino acid substitution, has also been reported (R279Q). This is associated with increased MMP-9 levels and future cardiovascular events in patients with angina. [325] I hypothesized that the two variants in the MMP-9 gene would be related to arterial stiffness and MMP-9 levels.

Both type 1 and type 2 diabetes are associated with an increased risk of cardiovascular disease [326, 327]. It has also been established that both type 1 and type 2 diabetes increase arterial stiffness [87, 328]. However, the effects of diabetes on arterial stiffness and on the role of arterial stiffening in the pathogenesis of cardiovascular disease in patients with diabetes are still are still unclear. In diabetic patients where adiponectin levels are reduced PWV is increased [267]. Reduced adiponectin levels are associated with increased future coronary heart disease events in men with type 2 diabetes [329]. Two polymorphisms were found in the adiponectin gene, one is

SNP45T>G in exon 2 and the other is SNP276G>T in intron 2, both two polymorphisms were closely associated with type 2 diabetes in the different populations. I hypothesized that the two polymorphisms in the adiponectin gene would be related to arterial stiffness and affect adiponectin expression levels.

RAGE contributes to arterial stiffening through AGEs by forming irreversible cross-links between slow-turnover proteins such as collagen and elastin [330-333]. The non-enzymatic protein glycation process forms cross-linked molecules that are structurally more rigid and less susceptible to degradation [334]. RAGE could impair endothelial function through AGEs by quenching nitric oxide (NO) and by increasing the generation of oxidants [335]. Furthermore, by binding to RAGE, AGEs initiate inflammatory responses that can increase vascular stiffness via activation of metalloproteinases, a phenomenon that contributes to endothelial dysfunction and promotes atherosclerosis [336-338]. Two functional -374T>A and -429T>C polymorphisms were found in the promoter region of the RAGE gene and these two polymorphism sites could potentially exert significant effects on transcriptional activity of RAGE gene which could lead to the changing of RAGE expression level and in turn affect arterial stiffness.

The present studies that focused on arterial stiffness were performed in order to answer the following questions:

I. Is collagen gene exon 31 polymorphism associated with higher blood pressure or arterial stiffness?

II. Is Elastin gene 549 G>A polymorphism associated with the development of arterial stiffness?

III. Could the MMP-9 haplotype affect blood pressure and arterial stiffness in patients with essential hypertension?

IV. What is the relationship between the Adiponectin gene haplotype and higher blood pressure and greater arterial stiffness in patients with essential hypertension?

V. Is RAGE haplotype in relationship with higher blood pressure and greater arterial stiffness?

Chapter 2

General Methods

2.1 Research subjects

2.1.1 Patient consent:

This studies described in this thesis were approved by the St James's Hospital/Federated Dublin Voluntary Hospital Research Ethics Committee. Informed consent was obtained from all subjects taking part in the study. The nature of the study was first explained to potential volunteers and an information leaflet was given to subjects. Subjects were referred to the Hypertension Clinic at St. James's Hospital for assessment.

2.1.2 Research subjects with essential hypertension:

1. Healthy untreated hypertension patients whose BP are greater than 140/90 mmHg.
2. Patients with a history or clinical evidence of CAD (including a electrocardiogram) peripheral vascular disease, cerebrovascular disease, valvular disorders, rhythm disturbances and signs and symptoms of congestive heart failures were excluded.
3. The patients were studied supine, having fasted and abstained from smoking, alcohol or caffeinated containing beverages in the 12 hours prior to the study.

2.2 Measurements

Haemodynamic measurements were carried out at the Hypertension Clinic, St James's Hospital, Dublin. Overall some 261 patients participated. Baseline demographic data, including age, height, weight, waist circumference and laboratory measurements, renal

and hepatic function, fasting glucose and lipids were entered into a database.

2.2.1 Blood pressure measurement

Blood pressure was recorded in the dominant arm using a validated oscillometric technique (Omron 705, Omron Matsusaka Co., Ltd., Kyoto, Japan). Three readings separated by 1-minute intervals were taken, and the mean was used for analysis. Peripherral pulse pressure was calculated as the difference between brachial systolic and diastolic BP.

2.2.2 Measurement of PWV

Carotid-femoral PWV was determined with the foot-to-foot method (Complior, Colson, Dupont Medical). Briefly, 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 between the transducers allows calculation of PWV in meters/second (m/sec). The distance traveled by the pulse wave was measured over the body surface with a tape measure as the distance between the recording sites and fed into the software and 10 consecutive waves were sampled. (Figure 2.1)

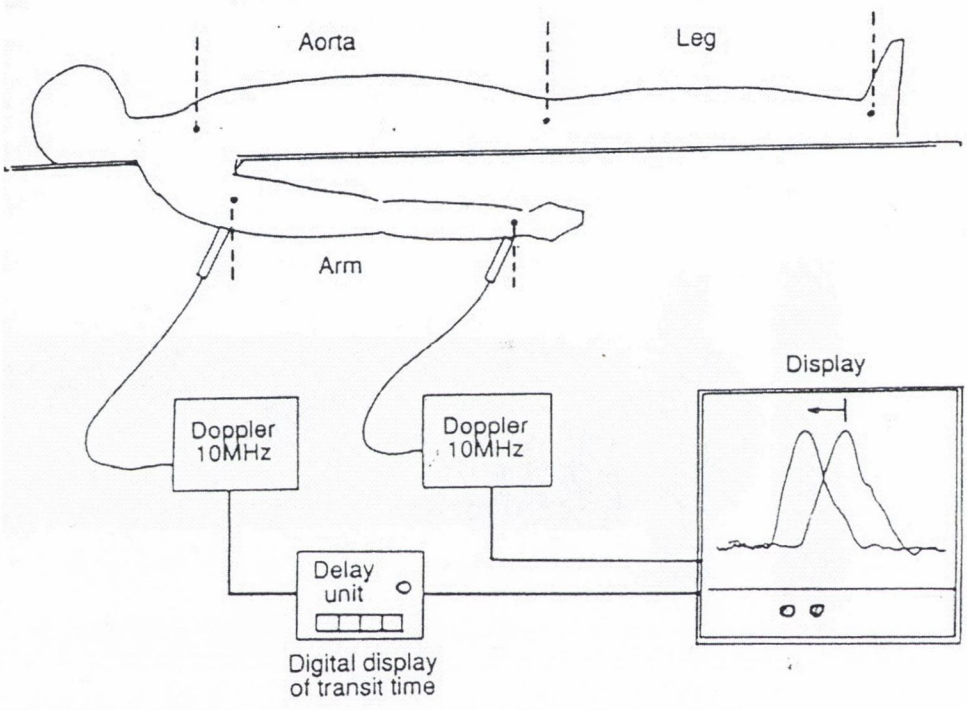


Figure 2.1 Method of measuring pulse wave velocity. Pressure waves are recorded simultaneously along the path of wave travel from the heart. The delay from the foot of the proximally-recorded to the distally-recorded waves is measured, and wave velocity is calculated as the distance between sites divided by the delay. Wave velocity is expressed in meters per second. It is always considerably higher than peak flow velocity in the aorta or other arteries.

2.2.3 Pulse Waveform Analysis

Assessment of arterial stiffness was performed noninvasively with the commercially available SphygmoCor system (AtCor Medical). In brief, peripheral pressure waveforms were recorded from the radial artery at the wrist, using a previously validated transfer function, relating radial to aortic pressure waveform within the system software (SphygmoCor, Aretor Medical, version 7.0). After 20 sequential waveforms had been acquired, a validated [339, 340] generalized transfer function was used to generate the corresponding central aortic pressure waveform. AIx and AP (Figure 2.2) were derived from this with the technique of PWA [62].

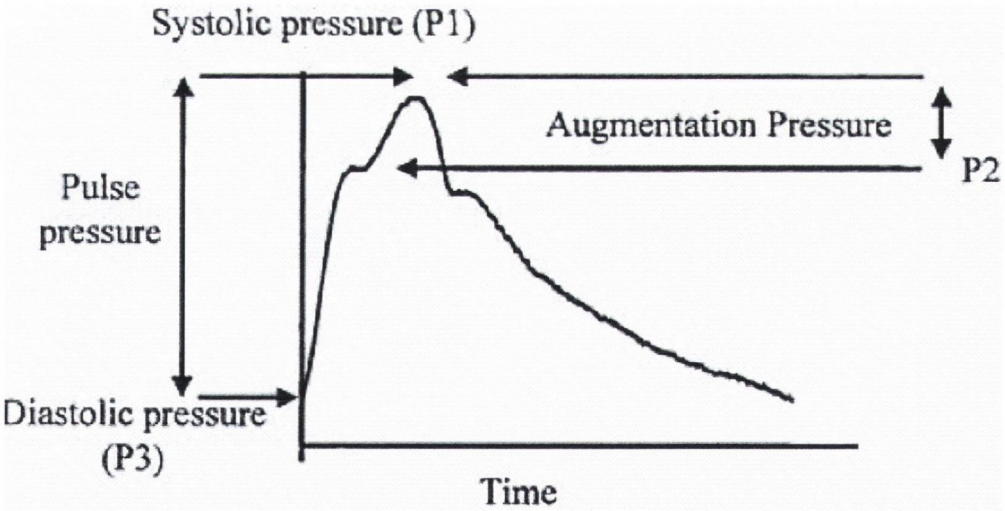


Figure 2.2 Augmentation pressure

Cited from http://www.medscape.com

The merging point of the incident and the reflected wave (the inflection point) was identified on the generated aortic pressure waveform. AP was the maximum systolic pressure minus pressure at the inflection point. The AIx was defined as the AP divided by pulse pressure and expressed as a percentage. Larger values of AIx indicate increased wave reflection from the periphery or earlier return of the reflected wave as a result of increased pulse wave velocity (attributable to increased arterial stiffness) and vice versa. In addition, because AIx is influenced by heart rate, an index normalized for heart rate of 75 bpm (AIx@75) was used in accordance with Wilkinson et al [50]. Time to return of the reflected wave (Tr) was the time from the beginning of the derived aortic systolic pressure waveform to the inflection point and can be used as a substitute for pulse wave velocity (a higher pulse wave velocity will lead to a shorter Tr) [341]. Only high-quality recordings, defined as an in-device quality index ≥80% (derived from an algorithm including average pulse height, pulse height variation, diastolic variation, and the maximum rate of rise of the peripheral waveform) and acceptable curves on visual inspection by 1 investigator were included in the analysis. All PWA measurements were taken in the sitting position in a quiet, temperature controlled room (22±1°C) after a brief period (at least 5 minutes) of rest.

2.3 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 5ml lithium heparin (LH) coated tubes and transported to the laboratory immediately for centrifugation. In addition, standard heamatological (full blood count) and biochemical (renal function test, glucose, cholesterol 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). LH plasma was then separated into 150 µl aliquots. The left blood samples and LH plasma samples were stored at -80°C until use.

2.4 Genomic DNA extraction (after QIAamp DNA Blood Maxi Kit – instrutions)

1. Pipet 500 µl QIAGEN Protease into the bottom of a 50 ml centrifuge tube.
2. Add 5–10 ml blood and mix briefly.
3. Add 12 ml Buffer AL, and mix thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for at least 1 min. Invert multiple tubes simultaneously by clamping them into a rack using another empty rack, grasping both racks, and inverting them together.
4. Incubate at 70°**C** for 10 min.
5. Add 10 ml ethanol (100%) to the sample, and mix by inverting the tube 10 times, followed by additional vigorous shaking.
6. Carefully transfer half of the solution from step 5 onto the QIAamp Maxi column placed in a 50 ml centrifuge tube, taking care not to moisten the rim. Close the cap and centrifuge at 1850 x *g* (3000 rpm) for 3 min.
7. Remove the QIAamp Maxi column, discard the filtrate, and place the QIAamp Maxi column back into the 50 ml centrifuge tube. Load the remainder of the solution from step 5 onto the QIAamp Maxi column. Close the cap and centrifuge again at 1850 x *g* (3000 rpm) for 3 min.
8. Remove the QIAamp Maxi column, discard the filtrate, and place the QIAamp Maxi column back into the 50 ml centrifuge tube.
9. Carefully, without moistening the rim, add 5 ml Buffer AW1 to the QIAamp Maxi column. Close the cap and centrifuge at 4500 x *g* (5000 rpm) for 1 min.
10. Carefully, without moistening the rim, add 5 ml Buffer AW2 to the QIAamp Maxi column. Close the cap and centrifuge at 4500 x g (5000 rpm) for 15 min.
11. Place the QIAamp Maxi column in a clean 50 ml centrifuge tube, and discard the collection tube containing the filtrate.
12. Pipet 1 ml Buffer AE, equilibrated to room temperature, directly onto the membrane of the QIAamp Maxi column and closes the cap. Incubate at room temperature for 5 min, and centrifuge at 4500 x g (5000 rpm) for 2 min.

13. For maximum yield: Pipet 1 ml fresh Buffer AE, equilibrated to room temperature, onto the membrane of the QIAamp Maxi column. Close the cap and incubate at room temperature for 5 min. Centrifuge at 4500 x g (5000 rpm) for 5 min.

2.5 Genomic DNA concentration and purity test using NanoDrop ND-1000 spectrophotometer (v3.0.1)

1. Clean the surfaces of and surrounding the arm and pedestal with water. Wipe dry.
2. Pipet 1–2 µl of distilled water onto the pedestal. Gently close the sampling arm so that a column of liquid is formed between the arm and the pedestal.
3. Open the software using the desktop icon. After initialization, select the Microarray Module. Select a sample type DNA.
4. Lift the sampling arm and wipe both surfaces clean with a soft tissue. Pipet 1–2 µl of blank onto the pedestal and gently close the sampling arm. Click "Blank" to make a baseline measurement.
5. Click "Start Report" to begin recording. For each sample:
   A. Wipe the surfaces clean;
   B. Pipet 1–2 µl of sample onto the pedestal;
   C. Gently close the arm (press gently to ensure formation of liquid column, if desired);
   D. Type in the sample ID;
   E. Click "Measure";
   F. If desired, you can "Re-blank" between samples.

2.6 Genotyping

2.6.1 Collagen 2209 G>A polymorphism

For genotyping of 2209 G>A, DNA fragments containing 2209 G>A were amplified

by genomic PCR using primers Forward 5'-TGCTGGTGCCCCTGGTGAA-3' Backward 5'-ACCCTGAAAATAAGTGAGA-3'; PCR conditions included predenaturation at 95°C for 15 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The amplified PCR product (435 bp) was then digested with 10U of the restriction enzyme, BbU at 37°C for 3 hours (Promega, USA) and the product run on a 2% agarose gel stained with ethidium bromide. A non-digested fragment was a single band of 183 bases pairs (bp) (AA), the homozygous G allele of the exon 31 polymorphism was digested as 119 and 64 bp in length (GG), and heterozygote (GA) showed three fragments of 183, 119 and 64 bp. [342]

2.6.2 Elastin 549G>A polymorphism

polymerase chain reaction restriction fragment length polymorphism (RFLP) method was used for genotyping Elastin 549G>A polymorphism, DNA fragments containing 549 G>A were amplified by genomic PCR using primers Forward 5'-TACTTACGGGGTTGGAGCTG-3' Backward 5'- AGAGCCGAGCAGACAAGAA G -3'; PCR conditions included predenaturation at 95°C for 15 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The amplified PCR product (280 bp) was then digested with 10U of the restriction enzyme BfaI at 37°C for 3 hours (Biolabs, UK) and the product run on a 2% agarose gel stained with ethidium bromide. A non-digested fragment was a single band of 280 bases pairs (bp) (AA), the homozygous G allele of the elastin polymorphism was digested as 201 and 79 bp in length (GG), and heterozygote (GA) showed three fragments of 280, 201, and 79 bp.

2.6.3 MMP-9 –1562 C>T and 836G>A polymorphism

The MMP-9 -1562 C>T polymorphism was studied used polymerase chain reaction

restriction fragment length polymorphism (RFLP). The MMP-9 promotor polymorphism -1562C>T was amplified from 100 ng of genomic DNA using the forward (5'-GCCTGGCACATAGTAGGCCC-3') and reverse (5'-CTTCCTAGCCAG CCGGCATC-3') oligonuclleotide primers. PCR conditions included predenaturation at 95°C for 15 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 1 minute 30 seconds, extension at 72°C for 1.5 minutes and a final extension at 72°C for 10 minutes. The amplified PCR product (435 bp) was then digested with 10U of the restriction enzyme, BbU overnight (Promega, USA) and the product run on a 2% agarose gel stained with ethidium bromide. CC homozygotes showed a single band at 435 bp, CT heterozygotes showed bands of 435, 247 and 188 bp, while TT homozygotes showed band sizes of 247 and 188 bp. Successful genotyping of the MMP-9 C/T polymorphism was achieved for all 261 cases. [343]

The MMP-9 836G>A polymorphism was screened using restriction fragment length polymorphism (RFLP). An area surrounding the polymorphism was amplified by PCR using the oligonucleotide primers: forward (5'-CTCGCCCCAGGACTCTACA C-3') and reverse (5'-GTGGAGGTACCTCGGGTCGGG-3'). PCR is produced using thermo-start Taq (ABgene) and PCR conditions included enzyme activation at 95°C for 15 minutes followed by 30 cycles of 95°C for 30 seconds, 69°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR product was digested with the restriction enzyme BsoBI (Biolabs, UK) and run on a 2% agarose gel stained with ethidium bromide. As one of the digest bands is only 15bp, it can not be shown on the agarose gel. GG homozygote showed a single band of 194 bp, GA heterozygote showed bands of 194, 179, while AA homozygote showed band sizes of 179 only [343]. Successful genotyping of the MMP-9 G>A polymorphism was achieved for 261 cases.

2.6.4 Adiponectin 45T>G and -276G>T polymorphism

Blood samples were obtained from each subject and genomic DNA was isolated by QIAamp Blood Kit (Qiagen, Hilden, Germany). For genotyping of SNP45 and

SNP276, DNA fragments containing both SNP45 and SNP-276 were amplified by genomic PCR using primers 5'-TCCTTTGTAGGTCCCAACT-3' and 5'-GCAGCA AAGCCAAAGTCTTG-3'. The PCR profile was as follows: 15 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 30 seconds at 56°C, 1 minute at 72°C; and finally 7 minutes at 72°C. Then PCR products were treated with BspH1 (New England Biolabs, Beverly, MA), which showed 375 and 128 bp fragments (T allele of SNP45) or 503 bp fragment (G allele of SNP45). Since SNP276 did not alter any restriction sites, the second PCR was performed using these PCR products as a template and mutagenic primers; forward primer (5'-ACACTGATATAAACGCCATGAA-3') and reverse primer (5'-GCAGCAAAGCCAAAGTCTTG-3'). The PCR condition was as follows: 10 minutes at 95°C; 40 cycles of 30 seconds at 95°C, 30 seconds at 56°C, 60 seconds at 72°C; and finally 7 minutes at 72°C. The PCR products were treated with BglI (New England Biolabs), which showed 147 and 21 bp fragments (G allele of SNP276), or 168 bp fragment (T allele of SNP276). [344]

2.6.5 RAGE -374T>A and -429T>C polymorphisms

Blood samples were obtained from each subject and genomic DNA was isolated by QIAamp Blood Kit (Qiagen, Hilden, Germany). For genotyping of -374T>A, DNA fragments containing -374T>A were amplified by genomic PCR using primers 5'-CCTGGGTTTAGTTGAGATTTTTT-3' for upstream and 5'-GAAGGCACTTC CTCGGGTTCT-3' for downstream primer. The conditions of amplification were: 95°C for 15 minutes; 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute; and finally 10 minute at 72°C. The PCR product (671bp) was then subjected to Tsp509 I (New England Biolabs, Beverly, MA, USA) digestion for 16 hours at 65°C and resolved by electrophoresis on 3% agarose gels. Genotypes were scored according to the patterns of DNA bands. Treatment of the T allele with Tsp I gave rise to five fragments of 284 bp, 217 bp, 110 bp, 44 bp, and 16 bp. In the case of the A allele, digestion resulted in four fragments of 327 bp, 284 bp, 44 bp, and 16 bp. [345]

The -429T>C polymorphism of the RAGE promoter was analyzed by PCR-RFLP:

Forward and reverse primers (forward: 5'-GGGGGCAGTTCTCTCCTC-3' and reverse: 5'-TCAGAGCCCCCGATCCTATTT-3'). The conditions of amplification were: 35 cycles of 94°C for 1 minute, a primer-annealing step of 56°C for 1 minute, and primer extension at 72°C for 2 minutes, followed by a final extension for 10 minutes at 72°C. For polymorphism –429 the PCR products were digested with AluI and incubation lasted for 3 hours at 37°C. Digestion products were run on 3% agarose gel. [346]

2.6.6 G-protein beta-3 gene 825C>T polymorphism

Blood samples were obtained from each subject and genomic DNA was isolated by QIAamp Blood Kit (Qiagen, Hilden, Germany). For genotyping of 825C>T, DNA fragments containing 825C>T was amplified by genomic PCR using forward and reverse primers (forward: 5'-TGACCCACTTGCCACCCGTGC-3' and reverse: 5'-GCAGCAGCCAGGGCTGG-C-3'). The conditions of amplification were 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. PCR products were digested by BseDI (New England Biolabs) for 3 hours. The TT genotype shows an unrestricted band size at 268bp, CC homozygote shows two bands of 116bp and 152bp, the CT heterozygote shows three bands of 268bp, 116bp and 152bp. [347]

2.7 Enzyme-Linked immunoSorbent assay (ELISA) (after the R&D instrutions)

2.7.1 MMP-9

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 100 µl of Assay Diluent RD1-34 to each well.
4. Add 100 µl of standard, control, or sample per well. Cover with the adhesive strip

provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µl) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µl of MMP-9 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µl of Substrate Solution to each well. Incubate for 30 minutes at room temperature, protect from light.
9. Add 50 µl of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

2.7.2 Adiponectin

1. Prepare all reagents, working standards and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack and reseal.
3. Add 100 µl of Assay Diluent RD1W to each well.

4. Add 50 µl of standard, control or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µl) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µl of Adiponectin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 µl of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 50 µl of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

2.8 Statistical Analysis

Data were analysed using JMP version 6.0(SAS for Windows, Cary, NC, USA). All Results are expressed as mean±SEM for continuous variables and percentages for categorical data, $p<0.05$ considered significant. Non-parametric tests (Wilcoxon Rank

Sums) were used to compare data stratified by genotype. Chi-square analysis was used to compare categorical variables. Analysis of covariance was used to determine whether the effects of genotype were independent of the major determinants of arterial stiffness including age, gender, blood pressure and other factors.

Because there were differences between individual studies statistical methods specific to each are described in the separate chapters.

CHAPTER 3

COL3A1 gene exon 31 polymorphism is associated with higher blood pressure and greater arterial stiffness in patients with essential hypertension

3.1 Introduction:

Type I and type III collagen totally account for 80-90% of aortic collagen content and occur in the intimal, medial and adventitial layers. Both type I and type III collagens are characterized as a long, continuous triple helical structure with each chain containing about 330 repeats of the amino acid sequence-glycine, praline and hydroxyproline. It is important for collagen I fibrillogenesis, as demonstrated during experiments on mice lacking the COL3A1 gene, which code for type III procollagen [143]. 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.

As described in the introduction, collagen changes appear to have a role in hypertension. The genetic basis for this is poorly understood. In hypertensive and hyperlipidemic rabbits, there is up-regulation of collagen type III. Collagen type III was spread more uniformly across the wall, including the adventitia [149]. The treatment of neonatal rat cardiac myocytes (NRCMs) with aldosterone showed significantly increased expression levels of COL3A1 mRNAs at 24 and 48 hours. [348]

The A1 chains of type III collagen are encoded at the collagen type III-a1 gene (COL3A1) which is located on chromosome 2q24.3-q31 [349]. The gene is 44 kb long

and it comprises 52 exons. Type III collagen is a homotrimer of A1 (III) collagen chains and is expressed in many tissues but is primarily a component of extensible connective tissue such as skin, lungs, gut, vascular system and uterus. It is often coexpressed with type I collagen. The polymorphisms of exon 31 2209G>A, encoding threonine instead of alanine at position 570 of the amino acid sequence has been found in relation to floppy mitral valve/mitral valve prolapse [350]. Mutations in the COL3A1 gene result in the vascular form of Ehlers-Danlos syndrome (EDS); EDS type IV, if they alter the sequence in the triple-helical domain [351.

Since the COL3A1 polymorphisms were hypothesized to be associated with the severity of MVP and EDS type IV, and as the COL3A1 2209 G>A polymorphism leads to an amino acid substitution it may cause the structure of collagen to change. Whether this change could modulate BP and arterial stiffness in a hypertensive population is unknown. I tested the hypothesis that COL3A1 2209G>A coding regions play an independent role in modulating BP and arterial stiffness in a population of never-treated patients with essential hypertension. Also, I examined the role of this polymorphism in the relationship with the change of blood pressure. Moreover, I examined the role of the COL3A1 2209G>A polymorphism in relation with age and arterial stiffness.

3.2 Methods

261 never treated Caucasian apparently healthy hypertensive subjects with a diagnosis of essential hypertension based on three outpatient measures of BP were studied. All the subjects were tested for genotypes of COL3A1 2209 G>A polymorphism by using of a polymerase chain reaction-restriction fragment length polymorphism (RFLP), (see Chapter 2). The haemodynamic measurements are described in Chapter 2.

The 261 research subjects are divided into 3 groups according to their genotypes of COL3A1 2209 G>A polymorphism. Result analysis is described in Chapter 2.

To study the polymorphism in the relationship with arterial stiffness in relation to age, I divided each genotype group into three subgroups according to the age; subgroup age is under 40 years of age, 40 to 50 years of age and over 50 years of age.

3.3 Results:

3.3.1 Distribution of COL3A1 2209G>A genotypes

The frequencies of the COL3A1 2209G>A genotypes were AA: 7.3%, GA: 48.7% and GG: 44%. Allele frequencies were A: 31.6% and G: 68.4%. Genotype distributions fit the Hardy–Weinberg equilibrium.

3.3.2 Clinical characteristics according to genotype

Demographic and clinical characteristics of the patient population according to the COL3A1 2209G>A genotypes are given in Table 3.1. There were no significant genotype differences in relation to age, gender, BMI, smoking, or creatinine.

Table 3.1 Clinical and biochemical database comparison according to the genotype of COL3A1

	COL3A1			
	AA (n=19)	GA (n=127)	GG (n=115)	p
Age (years)	46±1	46±1	47±2.5	0.90
Gender, male (%)	52%	54%	43%	0.53
BMI(kg/m^2)	30±0.5	29±0.4	29±1	0.28
Waist (cm)	96±1	95±1	94±2	0.68
Hip (cm)	108±1	106±1	104±1	0.41
Smokers (%)	31%	32%	27%	0.84
Total Cholesterol (mmol/l)	5.2±0.1	5.1±0.1	5.5±0.2	0.03
HDL (mmol/l)	1.3±0.02	1.3±0.03	1.4±0.06	0.70
Trigly (mmol/l)	1.7±0.1	1.6±0.1	1.7±0.2	0.78
Glucose (mmol/l)	5.6±0.1	5.3±0.1	5.3±0.1	0.57
Creatinine (umol/l)	87±1.5	86.5±1	86±3	0.81

3.3.3 Association between COL3A1 2209G>A polymorphism and blood pressure

For the COL3A1 2209G>A polymorphism, the systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP) were significantly higher in AA homozygote compared with GA heterozygote and GG homozygote. This polymorphism exhibited a gene dose-dependent effect on systolic ($p<0.05$) (Figure 3.1), diastolic ($p<0.05$) (Figure 3.2), pulse pressure ($p<0.05$). To assess the relationship between BP and the polymorphisms, a stepwise regression model was constructed with known or likely confounders of BP (Table 3.2). After age, the COL3A1 2209G<A genotype was the major determinant of systolic BP. Heart rate also emerged as a significant predictor. This polymorphism was also an independent determinant of both brachial systolic and diastolic BP. There was no significant difference of aortic SBP, DBP and PP between the genotypes.

Table 3.2 Comparison of the blood pressure and arterial stiffness according to the genotype of COL3A1

	COL3A1			
	AA (n=19)	GA (n=127)	GG (n=115)	p
Brachial Systolic BP (mmHg)	155±2	151±1	146±4	0.04
Brachial Diastolic BP (mmHg)	91.5±1	90±1	86±2	0.04
Aortic Systolic BP (mmHg)	144±2	138±2	139±4	0.13
Aortic Diastolic BP (mmHg)	93±1	90±1	91±3	0.08
Brachial Pulse Pressure (mmHg)	63.5±1	59±1	57±2.5	0.05
Aortic Pulse Pressure (mmHg)	52±4	48±1.5	47±1	0.10
PWV (m/sec)	11.1±0.5	10.6±0.2	10±0.2	0.01
AIx (%)	30.3±1	27±1	23±4	0.04
Heart rate (min^{-1})	74±5	71±1	68±1	0.02

3.3.4 COL3A1 2209G>A polymorphism and arterial stiffness

Aortic PWV and AIx were significantly higher in COL3A1 2209AA homozygotes ($p<0.05$) (Table 3.2). There was a significant gene dose-dependent effect of the COL3A1 2209G>A polymorphism on both PWV ($p=0.002$) (Figure 3.3) and AIx ($p=0.04$) (Figure 3.4). In a stepwise multiple regression model, with PWV and AIx as the dependent variable and the known or likely confounders of arterial stiffness as independent variables age emerged as a major determinant of aortic PWV and AIx in the study cohort with a significant independent contribution from the COL3A1 2209G>A polymorphism.

Figure 3.1 SBP in different genotypes

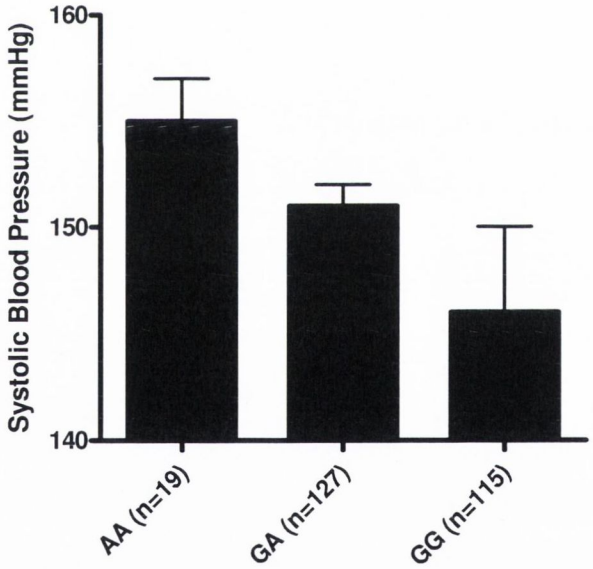


Figure 3.2 DBP in different genotypes

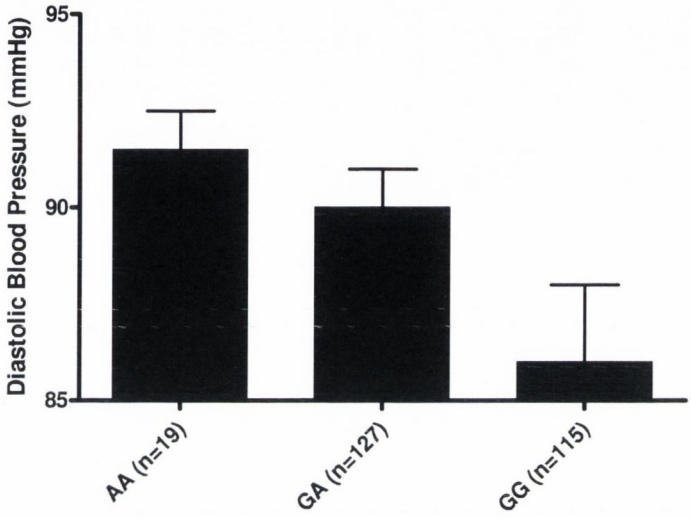


Figure 3.3 PWV in different genotypes

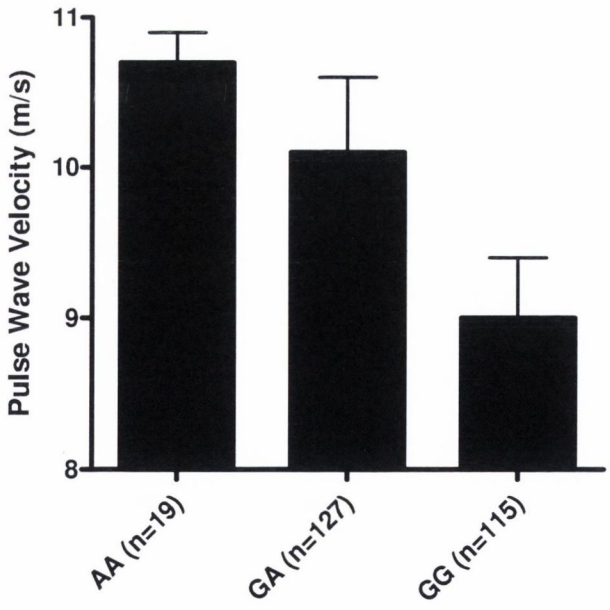
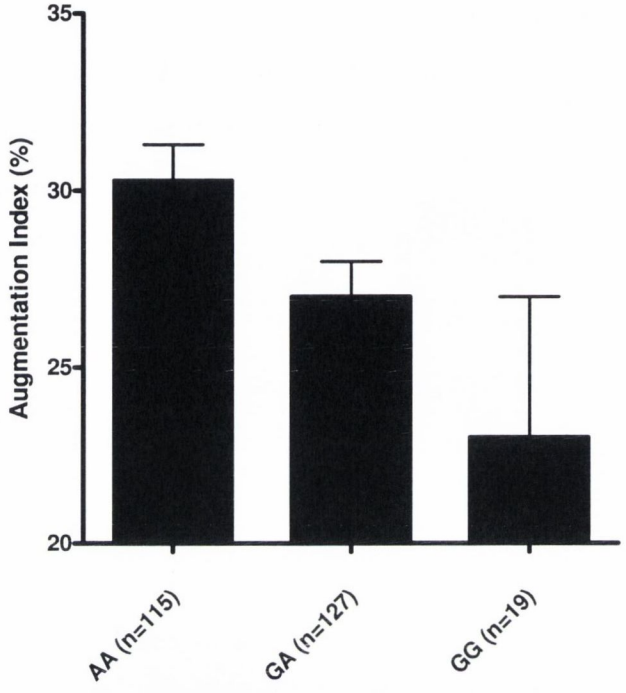


Figure 3.4 AIx in different genotypes



3.3.5 Influence of age on the relationship between COL3A1 genotypes and arterial stiffness

After each genotype group was divided into three subgroups according to age, there was a significant age-gene interaction for PWV. For the AA homozygote group, PWV increased 36% from 34 to 59 years of age; GA heterozygote increased 23% from 31 to 59 years of age, while only 14% for GG homozygote group increased from 31 to 57 years of age. In contrast, no age-gene interaction was found with AIx and BPs. (Table 3.3)

Table 3.3 Age-gene interaction for arterial stiffness and BPs in COL3A1 polymorphism

	Age	Age subgroup	Brachial Systolic BP (mmHg)	Brachial Diastolic BP (mmHg)	Aortic Systolic BP (mmHg)	Aortic Diastolic BP (mmHg)	Pulse Pressure (mmHg)	PWV (m/sec)	AIx (%)
AA	34±2	Under 40 (5)	161±8	98±4	153±3	93±5	63±5	9.4±0.6	25±3
	46±1	40-50 (6)	161±5	99±3	151±4	102±4	62±4	10.6±0.6	28±4
	59±2	Over 50 (8)	165±7	94±4	155±9	100±2	70±4	12.8±0.8	34±4
GA	31±1	Under 40 (37)	144±2	89±2	129±2	89±2	56±2	9.2±0.2	24±2
	45±1	40-50 (34)	155±3	95±2	144±3	96±2	60±2	10.4±0.2	28±2
	59±1	Over 50 (56)	158±2	91±1	148±2	93±1	67±2	11.4±0.3	29±1
GG	31±1	Under 40 (33)	143±3	85±2	130±2	88±2	57±2	9.2±0.3	20±2
	44±1	40-50 (34)	147±3	91±2	137±3	92±2	57±2	10.3±0.3	26±2
	57±1	Over 50 (48)	156±3	91±1	149±3	93±1	65±2	10.5±0.3	27±2

3.4 Discussion

The present study shows that the COL3A1 2209G>A polymorphism is associated with arterial stiffness in patients with essential hypertension. The greater stiffness of AA homozygote carriers is strongly supported by the results of stepwise regression analysis which shows the COL3A1 2209G<A polymorphism to be an independent determinant of not only SBP and DBP but also PWV and AIx. But this polymorphism does not seem to be associated with aortic SBP and DBP.

Collagen is a vital connective tissue protein and is one of the major extracellular constituents of the vascular wall. The absolute and relative quantities of collagen largely determine the biomechanical properties of major arteries and veins [124]. Collagen forms the fibrous scaffolding of the vessel while the proteoglycans and glycoproteins (along with solutes and water) form the inter-fibrillary matrix. Collagen plays a pivotal role in maintaining the structural integrity of the arterial wall. In the aorta and other large vessels, the collagen not only contributes to wall mass, but also determines the passive physical properties of the vessel wall. Collagen fibres are load bearing in the circumferential direction, whereas elastic fibres provide longitudinal as well as circumferential support. The relative proportions of collagen in the ECM determine the mechanical properties of the arterial wall, largely in the tunica media. In the pulmonary artery, the medial component is composed of about 27% collagen. In the adventitia of the same vessel the collagen increases to around 63%. In the thoracic aorta the proportion of collagen in the media is greater than that of the pulmonary artery. Collagen makes up around 37% of the media and 77% of the adventitia. The large proportion of collagen in the aortic media lends strength, allowing the vessel to withstand the pulsatitle load imposed upon it from the heart. [125]

Interstitial collagen type III is highly resistant to proteolytic attack, due to its triple helical structure, but can be cleaved by matrix metalloproteinase (MMP) collagenases at a specific site, approximately three-quarters of the length from the N-terminus of

each chain. MMP-2 and -9 are closely related at the structural level. Purified MMP-9 was able to cleave the soluble, monomeric forms of native collagen types III at 37°C and 25°C, respectively. Activity against collagen III was abolished by metalloproteinase inhibitors and was not present in the concentrated crude medium of mock-transfected cells, demonstrating that it was MMP-9-derived. Digestion of type III collagen generated a three-quarter fragment, as shown by comparison with MMP-1-mediated cleavage [352]. Fibroblast activation protein (FAP), a membrane bound proteinase works in synchrony with other proteinases to cleave partially to degrade or denature collagen III. [353]

Collagen can form irreversible cross-links with advanced glycation end products (AGEs) under nonenzymatic protein glycation [330, 331]. AGE-linked collagen is stiffer and less susceptible to hydrolytic turnover. This results in an accumulation of structurally inadequate collagen molecules [334]. In addition, several neurohumoral factors, particularly those related to the angiotensin II and aldosterone systems may modulate collagen accumulation [354].

The G-2209A mutation leads to the substitution of a threonine by an alanine at position 570. The amino acid substitution site may lead the collagen to be more difficult to degrade by the MMPs and FAP or other proteolytic attack, which causes the increase amount of collagen relatively. McNulty et al. have shown previously a strong association between aortic stiffness and collagen type I turnover in hypertensive patients [355]. As type III collagen is co-localized with type I collagen in tissue such as the blood vessels and skin [143] and is essential for collagen I fibrillogenesis, it can be imagined that the change of type III collagen can affect the amount of type I collagen and then lead to the change in the arterial stiffness.

Increased collagen deposition in the vascular wall has been demonstrated in conduit [356] and resistance arteries from essential hypertensive patients [357. 358] and from animal models of hypertension [359, 360]. The distribution of elastin and collagen varies markedly along the longitudinal aortic axis [4]. In the proximal aorta, elastin is

the dominant component, whereas in the distal aorta, the collagen-to-elastin ratio is reversed, and in peripheral arteries, collagen predominates [361, 362]. As the content of collagen in the center artery is lower than in the peripheral arteries, the effect of the COL3A1 polymorphism on blood pressure is different. In my study, I found that the systolic, diastolic blood pressure and pulse pressure measured in brachial artery is significant different within the three genotypes, while there is no significant difference of the aortic systolic, diastolic blood pressure and aortic pulse pressure happened in the three genotypes.

The AA homozygote carriers have significantly higher PWV than the GA heterozygote and GG homozygote carriers, suggesting in GG homozygote collagen is more easily degraded while in AA homozygote less collagen is degraded so that less arterial stiffness occurs despite the going ageing process. The AA homozygote carriers have significantly higher AIx than the GA heterozygote and GG homozygote carriers, suggesting that the GG homozygote collagen is more easily degraded while in AA homozygote collagen is harder to degrade so that less arterial stiffness occurred despite the going ageing process.

In my research, the PWV was increased by 36% from 34 to 59 years of age in AA homozygote group, while the increase in GA and GG genotype group is only about 23% and only 14% respectively even with a greater age interval. The reason why the AA homozygote has the age-gene interaction may be attributed to an amino acid change making collagen fibres increasingly insoluble and more refractory to the actions of collagenases and other proteinases. Also the amino acid change may accelerate the AGE-linked collagen formation and in turn make the arteries stiffer with older age. Studies regarding age-related collagen changes in the vasculature have examined the changes in the rat aorta with the aging process and have found that the collagen concentration increases until a certain age and then reaches a plateau. [363]

An association between increased heart rate and the COL3A1 2209G>A genotype was observed in the study (Table 3.2). The mechanism is not clear from this study. Whether

there are changes in cardiac collagen content associated with such a polymorphism has not been studied although GG is associated with mitral valve prolapse. A high resting heart rate has been shown to be related to the development of coronary atherosclerosis and of cardiovascular events and death in several studies [364-366]. In fact, the findings of both increased aortic stiffness with concomitant increase in pulse pressure and a significant increase in heart rate might indicate that individuals with the COL3A1 AA genotype are at a higher risk for cardiovascular events.

My study did not detect any relationship between COL3A1 2209G>A and aortic SBP or DBP. The reason maybe explained by location of elastin and collagen in the proximal aorta elastin is the dominant component, whereas in the distal aorta, the collagen-to-elastin ratio is reversed, and in peripheral arteries, collagen predominates. Consequently the change of collagen content could cause more structural and mechanical properties changes in distal or peripheral arteries than in the proximal aorta. This may explain why only brachial BPs but not aortic BPs have strong association with COL3A1 2209G>A genotypes.

Chapter 4

Elastin gene SNP 549 G>A play a role in the development of arterial stiffness in essential hypertensive patients

4.1 Introduction

As described before arterial stiffness is an independent risk factor of cardiovascular mortality in subjects with end-stage renal failure, [54] hypertension, [30] diabetes mellitus [367] and in those aged over 70 years [57, 368]. Morphological studies indicate that arterial distensibility depends on different factors such as age and blood pressure [369]. Aging is thought to be the main variable responsible for functional changes in the arterial wall leading to an increase in arterial stiffness [370]. Aging changes in the human arterial wall are especially apparent in the load-bearing media in which there is progressive disorganization, with loss of the orderly arrangement of elastin fibers. Furthermore, morphological studies have indicated that the effects of aging on elastin are different in proximal elastic arteries such as aorta than in distal muscular arteries. [371, 372]

Some experimental [373] studies have also suggested that genetic factors may be implicated in the mechanism of arterial stiffness. Elastin is one of the major determinants of arterial distensibility of large blood vessels [5] that forms the principal component of elastic fibers from the media layer of arteries [374]. Elastin gene has been located on chromosome 7 in humans [375]. Several polymorphisms of the elastin gene have been described, [376, 377] but no intermediate phenotype has been demonstrated for these genetic variants [378]. However, a deletion involving 7q11.23

and resulting in hemizygosity of the elastin gene has been identified as the mechanism responsible for the Williams-Buren syndrome, [379] which is characterized by supravalvular aortic stenosis, hypertension, or peripheral stenoses. Furthermore, Tromp et al [380] identified a G-to-A polymorphism of the elastin gene in exon 16 at position 549 resulting from a variant that converted the codon -GGT-(glycine) at amino acid position 422 to -AGT-(serine).

As the 549 G>A polymorphism leads to an amino acid substitution it may change the structure of elastin. The main objective of this study was to evaluate the relationships between the 549 G>A polymorphism of the gene-encoding elastin and arterial stiffness. I also examined the role of this polymorphism in the relationship with the change of blood pressure. Moreover, I examined the role of this polymorphism in the relation with age and arterial stiffness.

4.2 Methods

I collected the clinical database of the research subjects as described in chapter 2 and 3. All the subjects were tested for their genotypes of ELN 549 G>A polymorphism by using of a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), (see Chapter 2). The haemodynamic measurements are described in Chapter 2.

The 261 research subjects are divided into 3 groups according to their genotypes of ELN 549 G>A polymorphism. Result analysis is described in Chapter 2.

To study the polymorphism in the relationship between arterial stiffness and age, I again divided each genotype group into three subgroups according to the age; subgroup age is under 40 years of age, 40 to 50 years of age and over 50 years of age.

4.3 Results:

4.3.1 Distribution of COL3A1 2209G>A genotypes

The frequencies of the ELN 549G>A genotypes were AA: 35(13.4%), GA: 110(42.2%) and GG: 116(44.4%). Allele frequencies were A: 34.5% and G: 65.5%. Genotype distributions fit the Hardy–Weinberg equilibrium.

Table 4.1 Clinical and biochemical database comparison according to the genotype of ELN 549G>A polymorphism

	COL3A1			
	AA (n=35)	GA (n=110)	GG (n=116)	p
Age (years)	49±2	47±1	46±1	0.43
Gender male (%)	54%	54%	47%	0.50
BMI(kg/m^2)	29.5±1	29±0.5	30±0.5	0.58
Waist (cm)	94±3	96±1	95±1	0.66
Hip (cm)	105±2	106±1	107±1	0.37
Smokers (%)	20%	33%	33%	0.26
Total Cholesterol (mmol/l)	5.1±0.1	5.3±0.1	5.1±0.1	0.54
HDL (mmol/l)	1.4±0.06	1.3±0.03	1.4±0.03	0.69
Trigly (mmol/l)	1.5±0.1	1.6±0.1	1.7±0.1	0.76
Glucose (mmol/l)	5.7±0.2	5.3±0.1	5.4±0.1	0.10
Creatinine (umol/l)	88±2	88±2	86±1	0.26

4.3.2 Clinical characteristics according to genotype

Demographic and clinical characteristics of the patient population according to the ELN 549G>A genotypes are given in Table 4.1. There were no significant genotype differences in relation to age, gender, BMI, smoking, cholesterol, glucose or creatinine.

4.3.3 Association between ELN 549G>A polymorphism and blood pressure

For the ELN 549G>A polymorphism, the systolic blood pressure (SBP), diastolic

blood pressure (DBP), pulse pressure (PP), aortic systolic blood pressure (aortic SBP) and aortic diastolic blood pressure (aortic DBP) were significantly higher in the AA homozygote compared with the GA heterozygote and GG homozygote. (Figure 4.1-4.4)

There was no gene dose effect between the three groups. The blood pressure levels were nearly the same between the GA heterozygote and GG homozygote group. To assess the relationship between BP and the polymorphisms, we constructed stepwise regression models with known or likely confounders of BP. After adjusting for age, gender, smoking, cholesterol and glucose, the ELN 549G>A genotype was the major determinant of brachial, aortic SBP, DBP and PP.

4.3.4 ELN 549G>A polymorphism and arterial stiffness

PWV and AIx were significantly higher in the ELN 549AA homozygote ($p<0.05$) (Table 3.2). There was a gene dose-dependent effect of the ELN 549G>A polymorphism on both PWV and AIx. Figure 4.5 and 4.6 show that the PWV and AIx in the GA and GG group similarly. PWV and AIx in the AA homozygote group are much higher than in the other two groups. In a stepwise multiple regression model, with PWV as the dependent variable and the known or likely confounders of arterial stiffness as independent variables, age, SBP, ELN genotype and heart rate emerged as major determinants of aortic PWV. While in a stepwise multiple regression models, with AIx as the dependent variable, age, PP, aortic SBP, aortic DBP, heart rate and the ELN polymorphism were shown to be the determining factors of the AIx. Female gender is also a strong determinant of AIx.

Table 4.2 Comparison of the BPs and arterial stiffness according to the genotype of ELN 549G>A

	ELN			
	AA (n=35)	GA (n=110)	GG (n=116)	p
Brachial Systolic BP (mmHg)	165±2	151±2	149±2	0.0001
Brachial Diastolic BP (mmHg)	96±2	90±1	89±1	0.01
Aortic Systolic BP (mmHg)	150±2	140±2	139±2	0.003
Aortic Diastolic BP (mmHg)	97±2	92±1	90±1	0.03
Brachial Pulse Pressure (mmHg)	67±3	59.5±1	59±1	0.05
Aortic Pulse Pressure (mmHg)	58±3	51±1	48±1	0.001
PWV (m/sec)	11.9±0.4	10.1±0.2	10±0.2	0.0001
AIx (%)	34±2	27.5±1	27±1	0.05
Heart rate (min^{-1})	69±2	68±1	70±1	0.71

Figure 4.1 SBP in different genotypes

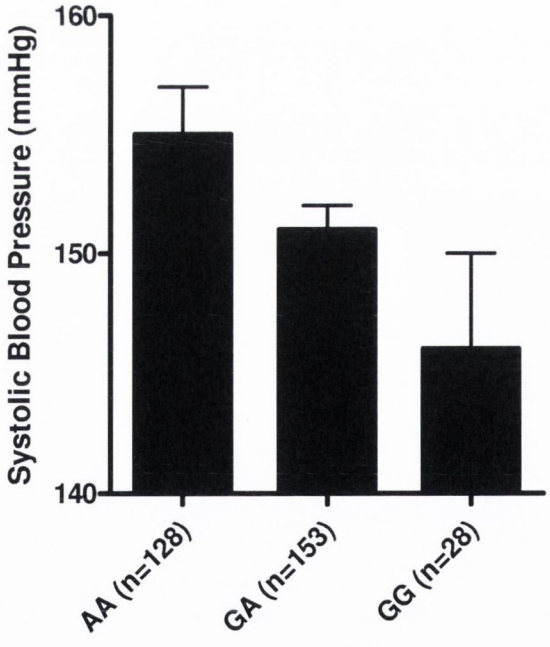


Figure 4.2 DBP in different genotypes

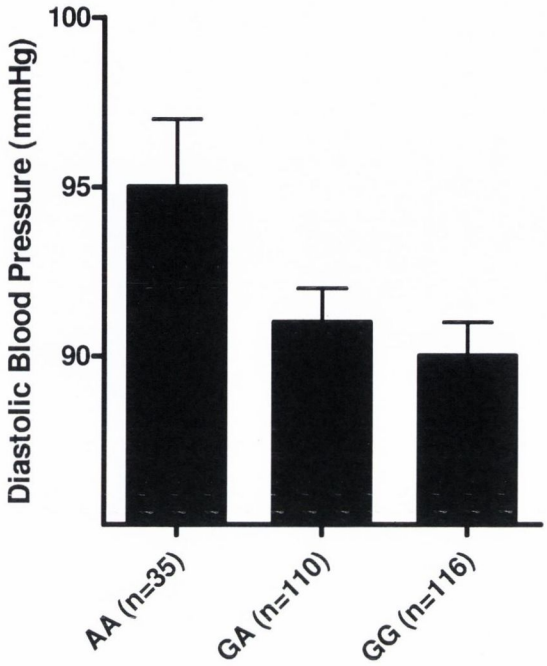


Figure 4.3 Brachial PP in different genotypes

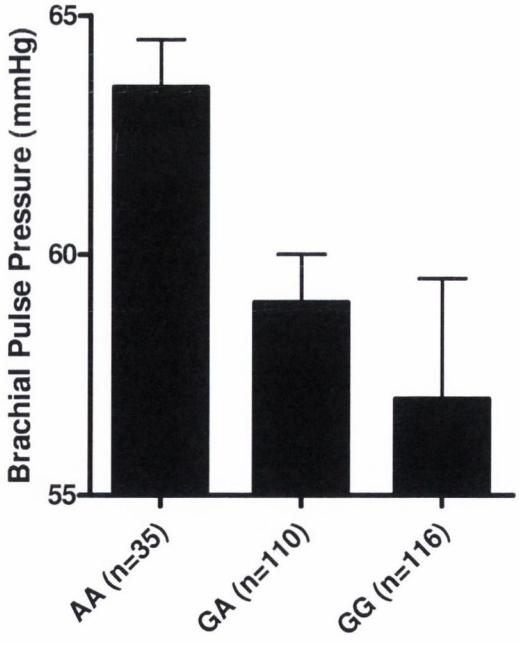


Figure 4.4 Aortic PP in different genotypes

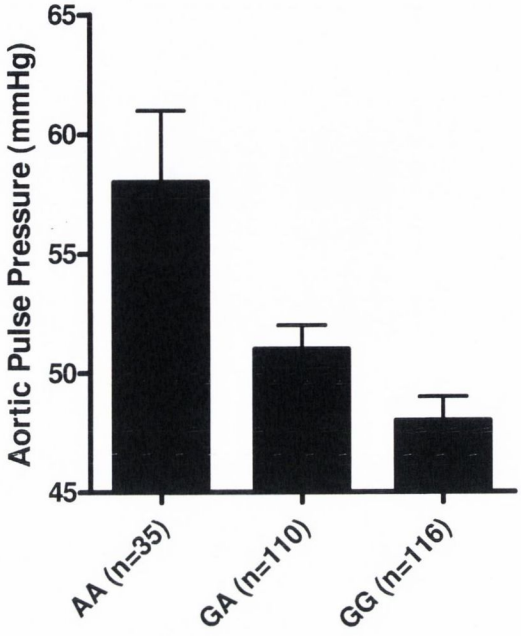


Figure 4.5 PWV in different genotypes

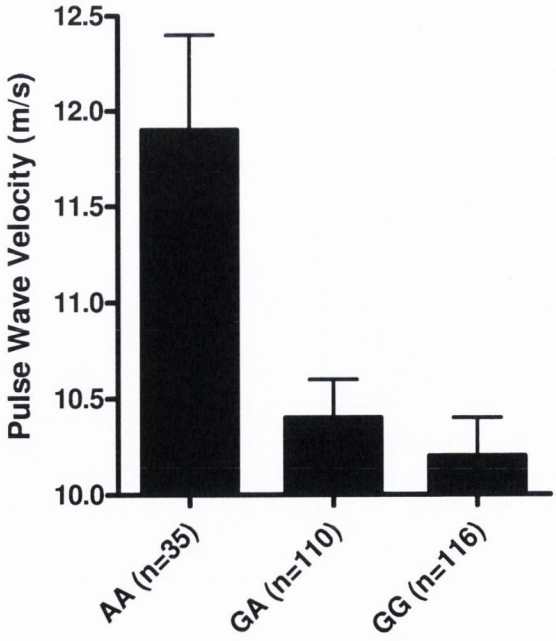
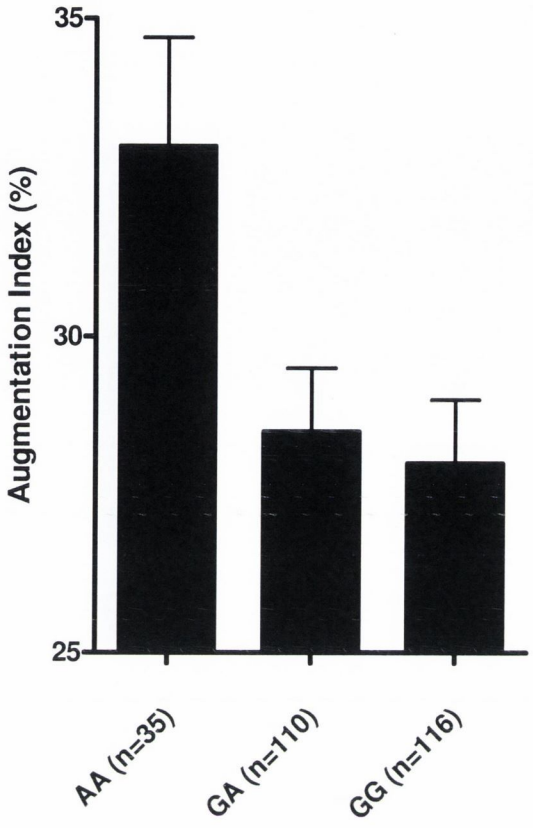


Figure 4.6 AIx in different genotypes



4.3.3 Influence of age on the relationship between ELN genotypes and arterial stiffness

After each genotype group was divided into three subgroups according to age, there was a significant age-gene interaction with PWV. For the AA homozygote group, the PWV increased 36% from 36 to 59 years of age; the GA heterozygote increased 20% from 31 to 58 years of age, while the GG homozygote group increased 24% from 30 to 58 years of age. In contrast, no age-gene interaction was found with AIx and BPs. (Table 4.3)

Table 4.3 Age-gene interaction for arterial stiffness and BPs in the ELN polymorphism

	Age	Age subgroup	Brachial Systolic BP (mmHg)	Brachial Diastolic BP (mmHg)	Aortic Systolic BP (mmHg)	Aortic Diastolic BP (mmHg)	Pulse Pressure (mmHg)	PWV (m/sec)	AIx (%)
AA	32±2	Under 40 (6)	157±8	96±6	139±8	93±6	61±5	10±0.6	34±3
	44±1	40-50 (10)	164±6	98±4	153±5	101±5	66±4	11.2±0.4	34±2
	59±2	Over 50 (19)	168±3	95±2	156±5	96±3	73±3	13.6±0.8	32±2
GA	31±1	Under 40 (29)	148±3	89±2	132±4	89±2	58±2	9.3±0.2	26±2
	45±1	40-50 (40)	150±2	93±2	140±3	94±2	58±2	10.2±0.2	27±2
	58±1	Over 50 (51)	157±2	90±1	147±3	92±2	66±2	11.2±0.3	30±2
GG	30±1	Under 40 (34)	142±2	86±2	129±2	89±2	56±2	8.9±0.2	26±2
	44±1	40-50 (27)	149±3	91±2	138±2	91±2	58±2	9.9±0.3	27±2
	58±1	Over 50 (55)	156±2	90±1	146±3	92±1	66±2	11±0.2	31±1.5

4.4 Discussion

The main finding of this study is the presence of an association between arterial stiffness (PWV and AIx) and the ELN 549G>A polymorphism of the elastin gene in essential hypertensive patients independent of the usual determinants such as age and blood pressure. This association is apparent only in patients with the AA homozygote, suggesting that only the AA homozygote, not the A allele, can affect the arterial stiffness. Moreover, an age-gene interaction was found showing that the AA homozygote of the ELN gene has more effect in accelerating the arterial stiffness process with increasing age than the GA and GG genotype.

The main components of the vessel wall are collagen, elastin, and smooth muscle cell. Collagen and elastin provide structural integrity and elasticity, and are potently regulated by catabolic matrix metalloproteases (MMPs). Through their collagenolytic and elastinolytic effects, MMPs degrade the ECM by creating uncoiled, less effective collagen and broken and frayed elastin molecules, respectively. Vascular cells, as well as inflammatory cells such as macrophages and polymorphonuclear neutrophils, produce collagenases (MMP-1, MMP-8, and MMP-13) and elastases (MMP-7 and serine proteases). [381]

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 [382]. This process can be visualized microscopically as fragmentation and loss of regularity in elastic layers of the media layer. The gradual loss of elastin is accompanied by a reduction in vascular compliance.

Stiffening of elastic arteries decreases their buffering ability and increases the velocity of the propagating pressure wave. Arterial mechanical properties, in particular functional aortic compliance and intrinsic stiffness, are now established as important in the pathophysiology of arterial disease. Indeed, disruption and fatigue fracture of

elastin which is the elastic loadbearing element of the arterial wall, resulting in a transfer of stress to the more collagenous fibres, is likely to be responsible for age related arterial stiffening [383]. Hypertension aggravates this degenerative process, but, together with ageing, also alters the absolute and relative amounts of elastin and collagen in the arterial wall, [384] which is likely to result in arterial stiffening, thus setting up a vicious cycle.

Vascular wall structural abnormalities resulting from polymorphisms of some components of the arterial wall have previously been found. Several studies report a relationship between the Williams and Buren syndrome and the deletion of the elastin gene [385]. Mutations in the fibrillin-1 gene could lead to Marfan syndrome, [176] and a morphological study has shown an increase in large artery stiffness in these patients, [386] suggesting the role of the deficit of fibrillin in arterial wall properties.

Tromp et al [380] described a polymorphism site at the site 549 of extron 16 in the ELN gene. This polymorphism was confirmed to be due to a change of the amino acid which is Ser422Gly in the elastin protein. In my study, I evaluated the relationships between the 549G>A polymorphism and the elastic properties of arteries. My results suggest that mutations could be involved in structural changes in the arterial wall. It maybe because the polymorphism of ELN gene could change the amino acid from glycine to serine of the elastin in the arterial wall, and this change in the AA homozygote could make elastin in the arterial wall much more easily degraded by elastases than the GA and GG genotype carriers. Moreover, the elastin in the arterial wall with the AA homozygote may be more prone to fracture under stress than the GA and GG genotypes.

In my research, the AA homozygote has higher blood pressure than the GA and GG genotypes. It maybe that because the loss of elastin in the arterial wall (caused by the elastin expressed by the ELN gene with AA homozygote), not only caused the elastin be easily broken down by the elastases but also could be easily broken down under the mechanical strain.

In my research, the PWV was increased of 36% from 32 to 59 years of age in the AA homozygote group, while the increase in the GA and GG genotype group is only about 20% and 24% respectively with nearly the same age interval. The reason why the AA homozygote displays the age-gene interaction could be that the amino acid change caused by the AA homozygote accelerates both the elastase degradation of elastin and the fracture effect of stress on elastin. This age-gene interaction has also been found by Hanon et al [387].

The aging process is the main determinant responsible for arterial wall changes leading to arterial stiffening. With increasing age, arteries progressively stiffen and dilate. These changes are due to a degeneration of elastin fibers associated with an increase in collagenous material. Furthermore, it has been observed during aging that elastic fibers undergo thinning, splitting, fraying, and fragmentation. [388] Moreover, an increase in stiffness with age is observed for the carotid artery, whereas there is little or no consistent change in distensibility of the radial artery with age. [372]

Experiments in animals have shown that the synthesis of elastin in the medial layer of arteries is influenced by local haemodynamic conditions. At birth in rabbits, the pulmonary artery and the aorta are the same. At the age of two months, during which time the pulmonary pressure decreases from 40 mmHg to 15 mmHg and the systemic pressure increases to 80 mmHg, the ratio of elastin to collagen in the aorta is nearly twice that of the pulmonary artery [389]. 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%. [168]

The finding of increased AIx in women is consistent with the published literature [390]. The multiple regression in my study shows that the effect is only partly explained by lower height in women. The inverse correlation found between AIx and body height in the whole study group has also been noted previously, [391-393] and is probably due to earlier reflection of the aortic wave in short people.

CHAPTER 5

MMP-9 haplotype is associated with higher blood pressure and greater arterial stiffness in patients with essential hypertension

5.1 Introduction

As described in Chapter I, increased arterial stiffness and augmented wave reflection have been shown to play an important role in hypertension, stroke and end stage renal disease (ESRD) [394-396]. Arterial stiffness is the result of a complex interplay between haemodynamic forces modulated by hormones and neural stimuli, vascular inflammation and structural alteration in the vessel wall.

Matrix metalloproteinases (MMP) are a family of zinc-containing enzymes with proteolytic activity against extracellular matrix components such as elastin, proteoglycans, and collagen in both physiologic and pathologic processes [397]. MMPs play an essential role in vascular remodeling to allow blood vessels to change in size and structure for adaptation and repair. However, with excessive MMP expression and activity, inappropriate cardiovascular remodelling may occur resulting in arteriosclerosis, aneurysm formation and restenosis. [398-400]

MMP-9 (gelatinase B) is capable of degrading gelatine, fragments of collagen degraded by collagenase and type IV collagen, which forms part of the basement membrane. Circulating MMP-9 levels are increased in patients with isolated systolic hypertension, [401] in type 2 diabetic patients with coronary artery disease [402] and elevated MMP-9 levels are associated with premature coronary arteriosclerosis [403]. Recently plasma MMP-9 levels were identified as a predictor of cardiovascular mortality in patients with coronary artery disease [325] and were related to arterial

stiffness [401].

A number of SNPs have been identified in regulatory and coding regions of the MMP-9 gene. Some of them have been reported to affect *in vitro* MMP-9 expression levels, enzymatic activity and susceptibility to various inflammatory and fibrotic conditions [106]. A functional -1562C>T polymorphism in the promoter region of MMP-9 has been shown to relate to the presence and severity of coronary arteriosclerosis [107] and is associated with large vessel stiffness in patients with coronary artery disease (CAD) [108] and healthy subjects [109]. In an earlier study we have shown this polymorphism influences arterial stiffness and blood pressure [404]. Another functional polymorphism 836G>A has been found in the exon 6 and leads to the substitution of a positively charged amino-acid (arginine) by an uncharged amino acid (glutamine) that is located in the gelatinase specific fibronectin type II domains, which presumably enhances substrate binding. [405, 406]

Whether haplotypes of these two particular genetic variants in the MMP-9 gene modulate BP and arterial stiffness in a hypertensive population is unknown. In the current study the hypothesis was tested that haplotypes involving genetic variation of –1562C>T and 836G>A in promoter and coding regions of MMP-9 may play an independent role in modulating BP, arterial stiffness and plasma MMP-9 levels in a population of never-treated patients with essential hypertension.

5.2 Methods

Subjects have already been described in Chapter 2. All the subjects were tested for their genotypes of –1562C>T and 836G>A polymorphisms by using of a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), (see Chapter 2). The haemodynamic measurements are described in Chapter 2. MMP-9 levels were tested using ELISA in 80 subjects (Chapter 2).

The 261 research subjects are divided into groups according to their genotypes of

–1562C>T and 836G>A polymorphisms. Result analysis is described in Chapter 2.

5.3 Results:

5.3.1 Distribution of –1562C>T and 836G>A genotypes of the MMP-9 gene

The frequencies of the -1562C>T genotypes were *CC*: 67%, CT: 30% and TT: 3%. Allele frequencies were C: 82% and T: 18%. The frequencies of the 836G>A genotypes were GG: 23%, GA: 44% and AA: 33%. Allele frequencies were G: 45% and A: 55%. Neither genotype distributions differed significantly from that predicted by the Hardy–Weinberg equilibrium.

5.3.2 Clinical characteristics according to genotype

Demographic and clinical characteristics of the patient population according to the MMP-9 -1562C>T and 836G>A genotype are given in Table 5.1. There were no significant genotype differences in age, gender, BMI, smoking, lipids. The creatinine levels were significantly higher in the GG homozygote group than *in* the GA and AA groups of the 836G>A genotype.

Table 5.1 Clinical characteristics of the hypertensive patients according to the MMP-9 genotype at positions 1562 and 836 (n=261, mean±SEM)

		Age (years)	Gender male (%)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	Smokers (%)	Total Cholesterol (mmol/l)	HDL (mmol/l)	Trigly (mmol/l)	Glucose (mmol/l)	Creatinine (umol/l)
SNP 836	GG (n=60)	45±2	59%	29±1	95±2	105±1	20%	5±0.1	1.3±0.03	1.5±0.1	5.4±0.2	91±2
	GA (n=115)	46±1	48.5%	30±0.4	96±1	109±1	29%	5.2±0.1	1.4±0.03	1.6±0.1	5.4±0.1	85±1
	AA (n=86)	47.5±1	52%	29±0.5	94.5±1.5	104±1	27%	5.2±0.1	1.3±0.04	1.8±0.1	5.3±0.1	86±2
	p	0.52	0.35	0.17	0.65	0.29	0.59	0.84	0.21	0.16	0.57	0.001
SNP -1562	CC (n=175)	45±1	51.2%	29±0.4	95±1	106±1	28%	5.2±0.1	1.3±0.02	1.6±0.1	5.3±0.1	87±1
	CT (n=78)	48±1	52.1%	30±0.5	95±2	108±2	32%	5.3±0.1	1.4±0.04	1.8±0.1	5.5±0.1	87±1
	TT (n=8)	51±4	75%	28±1	96±4	103±3	33%	4.7±0.3	1.2±0.1	1.3±0.2	6.5±1	91±7
	p	0.14	0.41	0.58	0.98	0.70	0.77	0.11	0.61	0.25	0.08	0.87

5.3.3 Association between MMP-9 -1562C>T and 836G>A polymorphisms and blood pressure

For the -1562C>T polymorphism, systolic and diastolic blood pressures were significantly higher in TT homozygote compared with CT heterozygote and CC homozygote. Similarly for the 836G>A polymorphism, the systolic, diastolic blood pressure were significantly higher in AA homozygote compared with GA heterozygote and GG homozygote. The effects of the A allele of the MMP-9 836 and the T allele of the MMP-9-1562 polymorphisms on blood pressure and arterial stiffness were of a similar magnitude (13 and 20 mmHg differences between homozygote in systolic BP). Both polymorphisms exhibited a gene dose-dependent effect on systolic blood pressure ($p<0.01$), diastolic blood pressure ($p<0.01$). To assess the relationship between BP and the polymorphisms, we constructed stepwise regression models with known or likely confounders of BP (Table 5.1). After age, the MMP-9 genotype was the major determinant of systolic BP. Triglycerides, body mass index and heart rate also emerged as significant predictors. Both polymorphisms were also independent determinants of diastolic BP but to a quantitatively lesser degree than for systolic BP. The effects of the two genotypes were additive with a direct linear relationship observed between the -1562T and 836A alleles polymorphism and brachial SBP, DBP, PP and aortic SBP. The linear relationship was also seen in the 836A polymorphism and the aortic DBP and PP.

5.3.4 MMP-9 -1562C>T and 836G>A polymorphisms and arterial stiffness

PWV was significantly higher in the -1562TT homozygote and the 836AA homozygote ($p<0.01$) (Table 5.2). The effects of the A allele of the MMP-9 836 and the T allele of the MMP-9-1562 polymorphisms on arterial stiffness were of a similar magnitude (1.0 and 1.6 m/sec differences in PWV). There was a significant gene dose-dependent effect of both the -1562C>T ($p=0.002$) and 836G>A ($p=0.008$) on PWV. In a stepwise multiple regression model, with PWV as the dependent variable and the known or likely confounders of arterial stiffness as independent variables age

emerged as major determinants of aortic PWV in the cohort with a significant independent contribution from both the -1562C>T and 836G>A polymorphism.

In contrast to PWV, there was no significant difference in AIx between the genotypes of the two polymorphisms (Table 5.2) and the results were unchanged when AIx was adjusted for major confounders such as age, gender, heart rate, height and smoking status.

5.3.5 MMP-9 -1562C>T polymorphism and plasma MMP-9 levels

Mean plasma MMP-9 levels were higher in subjects carrying the -1562T allele (TT & CT) compared with the CC homozygote ($p=0.003$) (Table 5.2). There was a significant dose-dependent effect of the -1562C>T polymorphism on plasma levels of MMP-9 (Table 5.2). The MMP-9 genotype also significantly influenced serum MMP-9 levels after correcting for likely confounders including age, gender, BP and smoking status ($R2= 0.36$, $p<0.01$). There seems to be no relationship between the 836G>A polymorphism and the MMP-9 levels.

Table 5.2 BPs, arterial stiffness level and MMP-9 concentrations according to the MMP-9 genotypes

		Brachial SBP (mmHg)	Brachial DBP (mmHg)	Aortic SBP (mmHg)	Aortic DBP (mmHg)	Brachial PP (mmHg)	Aortic PP (mmHg)	PWV (m/sec)	AIx (%)	Heart rate (min^{-1})	MMP-9 level (ng/mL)
SNP 836	GG (n=60)	145±2	87.5±1	132±2	89±1	54±1	43±1	9.7±0.3	24±2	70±2	75±10
	GA (n=115)	152±2	89±1	140±2	91±1	62±1	50±1	10.3±0.2	28±1	69±1	90±10
	AA (n=86)	158±2	93±1	146±2	93±4	64±1	52±1.5	10.7±0.2	30±1	70.5±1	125±19
	p	<0.0001	0.003	0.0002	0.006	<0.0001	0.01	0.008	0.08	0.38	0.13
SNP-1562	CC (n=175)	150±1	89±1	138±1	91±1	59±1	49±1	10±0.1	28±1	71±1	81±9
	CT (n=78)	157±2	92±1	145±2	92±2	64±2	51±2	11±0.3	29±1	70±2	97±11
	TT (n=8)	169±5	98±3	150±5	97±4	70±4	56±4	11.6±1	27±2	69.5±3	213±53
	P	0.0002	0.004	0.01	0.22	0.008	0.26	0.002	0.63	0.95	0.006

5.3.4 MMP-9 Haplotypes

The haplotype distribution was ATAT: 1.5%, GCGT: 3.1%, ATAC: 6.9%, ACGT: 20.7%, GTGT: 0.8%, ACAC: 26.4%, ACGC: 24.5%, ATGT: 0.8%, GCGC: 15.3%. 4 subjects (1.5%) were homozygous for both the -1562T and the 836A alleles. 40 (11%) of the study population had neither at-risk allele (Homozygous GCGC haplotype). The two SNPs were in modest linkage disequilibrium (p=0.04). The MMP-9 levels, PWV and systolic blood pressure□brachial PP and aortic PP according to MMP-9 haplotypes were significantly different and as shown in figures 5.1 to 5.5. Subjects with this protective haplotype had significantly lower PWV and systolic blood pressure than any of the other haplotypes containing one or more of the at-risk alleles (Figures 5.1-5.5).

Figure 5.1 MMP-9 levels according to MMP-9 haplotypes

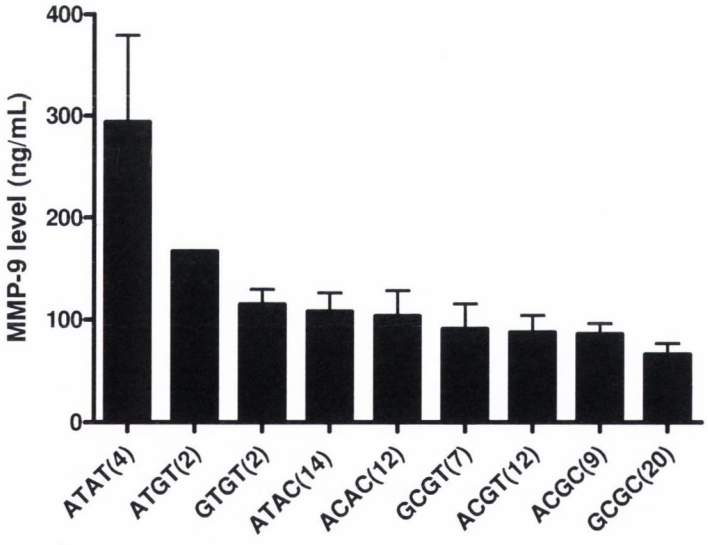


Figure 5.2 PWV according to MMP-9 haplotypes

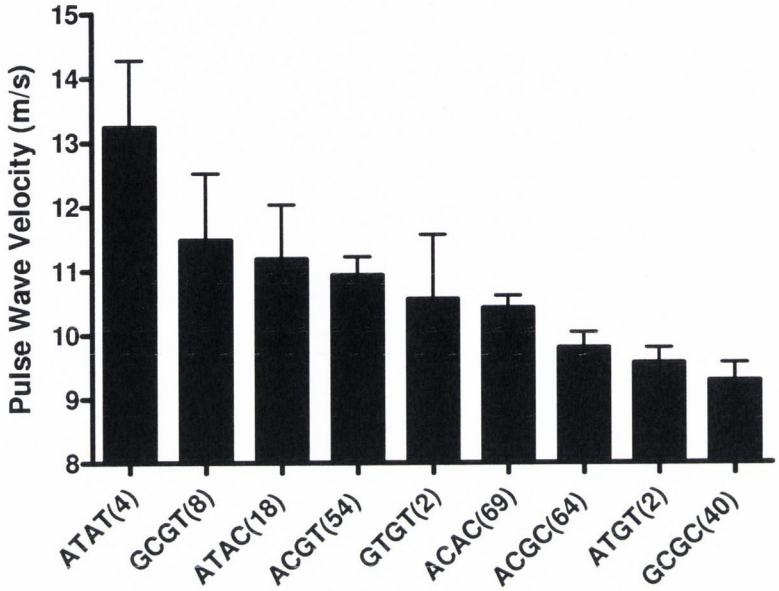


Figure 5.3 SBP according to MMP-9 haplotypes

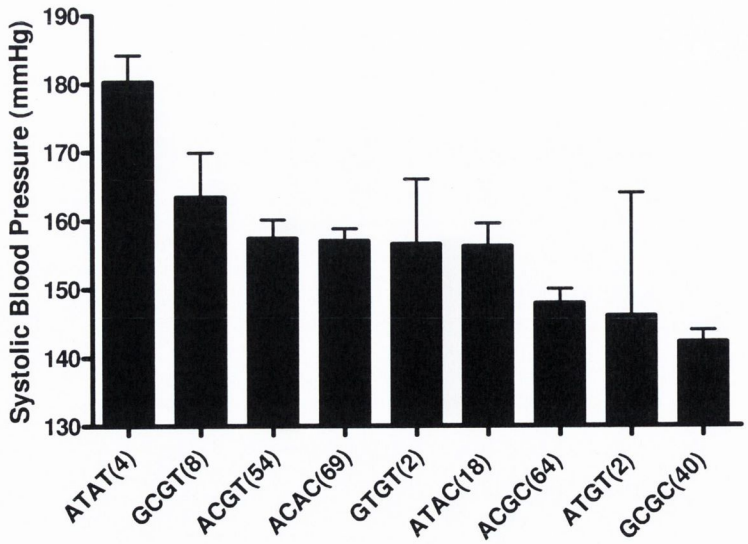


Figure 5.4 Brachial PP according to MMP-9 haplotypes

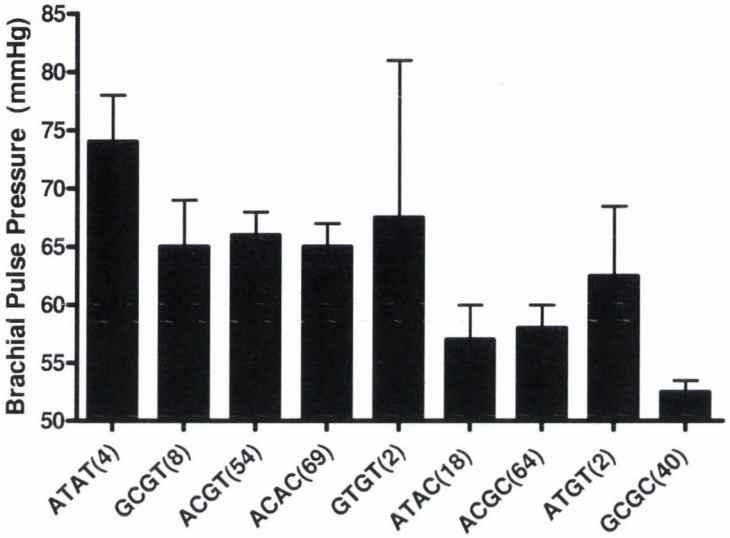
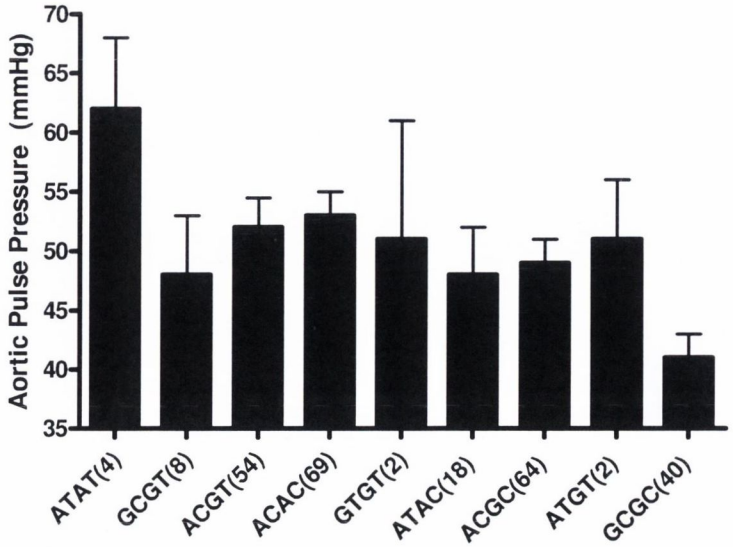


Figure 5.5 Aortic PP according to MMP-9 haplotypes



5.4 Discussion

The present study is the first to report the relationship between polymorphisms in the MMP-9 gene (-1562C>T promoter region and 836G>A coding region), blood pressure, arterial stiffness, and MMP-9 levels in never-treated hypertensive patients. I found that PWV and blood pressure were influenced by both polymorphisms in the MMP-9 gene with a significant gene-dose effect. Furthermore, both polymorphisms were independent predictors of both systolic and diastolic BP in these patients with additive effects seen for carriers of both at-risk alleles. In addition, the -1562C>T polymorphism was an independent determinant of serum MMP-9 levels in the study population, with higher MMP-9 levels seen in -1562-T allele carriers with a significant gene-dose effect.

Arterial stiffness, measured as PWV, is an independent prognosticator of cardiovascular morbidity and mortality in both healthy [361] and hypertensive populations [30, 55, 362]. Circulating MMP-9 levels are increased in hypertension [407] and predict cardiovascular risk [325]. Genetic variation in the MMP-9 gene is associated with the severity of vascular disease [107, 325] and large artery stiffness [108, 109]. Medley et al showed increased arterial stiffness, measured as aortic output and characteristic impedance, pulse pressure and MMP-9 expression in T allele carriers of the -1562C>T polymorphism in patients with coronary artery disease [108]. However, the population of this study was small (n=82). Yasmin et al also showed increased PWV and MMP-9 levels in T allele carriers; however they did not observe any significant difference in BP between the three genotypes in a normotensive population [109]. In contrast, we have shown a significant gene-dose effect of both the MMP-9 polymorphisms not only on PWV but also on BP. Both the -1562C>T and 836G>A polymorphisms were independent predictors of arterial stiffness after adjustment for factors known to influence PWV.

The higher arterial stiffness and BP in the T-allele carriers of –1562C>T and G alleles carriers of 836G>A may be secondary to excessive degradation of the arterial elastin matrix. The stiffness of the vascular wall is determined by the relative concentrations of collagen and elastin which are kept tightly regulated by a balance between production and degradation in the extracellular matrix (ECM). MMP-9 is thought to be involved in destruction of the arterial media and plaque growth [408]. Moreover, targeted deletion of the MMP-9 gene in mice attenuates collagen accumulation and enhanced expression of other MMPs after myocardial infarction, suggesting that MMP-9 plays a prominent role in extracellular matrix remodelling. [409]

MMP-9 genotypes may influence BP and arterial stiffness either through enhancing plasma MMP-9 levels or by altering the qualitative function of the MMP-9 gene product. Because of its location in the promoter, i.e. regulatory region, of the MMP-9 gene, the -1562 polymorphism might be expected to influence levels of gene expression. This was born out in the present study, which found that the T allele was associated with significantly higher plasma levels of MMP-9. This finding is consistent with previous studies, in which the presence of the T-allele was associated with increased plasma MMP-9 levels in CAD and healthy subjects [108, 109]. In these studies, higher MMP-9 expression was also associated with higher arterial stiffness [108, 109] and increased pulse pressure [108]. We have shown in a hypertensive population that the presence of the T-allele conferred increased BP and arterial stiffness associated with higher plasma MMP-9 levels with a significant gene-dose effect, with heterozygotes exhibiting levels intermediate between those of CC and TT homozygote.

In contrast, the 836 exonic variant is more likely to have a qualitative effect on MMP-9 function, which might explain the lack of association with plasma MMP-9 levels in this study, despite a clear influence on blood pressure and arterial stiffness. The 836G>A mutation leads to the substitution of a positively charged amino-acid (arginine) by an uncharged amino acid (glutamine) at position 279 within the MMP9

active site. The substitution site is located in the catalytic domain of the MMP-9 gene, particularly in the fibronectin type II domains which confers MMP-9 with high affinity binding to type IV collagen, type I gelatin and elastin [55, 362]. The digestion of type IV collagen in the epithelial basement membrane has been suggested to be a key regulatory event in the initiation of fibrosis. A plausible explanation of these results is that 836G>A represents a partial higher activity mutation within the proteinase whose presence accelerates the development of fibrosis. These findings implicate MMP-9 as a key molecule in the pathogenesis of arterial stiffness. The higher expression and/or activity of MMP-9 associated with these gene variants may be directly involved in promoting stiffness of arteries through vascular remodeling. Animal studies using MMP-9–deficient carotid artery cells have demonstrated that MMP-9 may influence arterial remodeling not only through matrix degradation but also through reorganization [410]. These theories provide a potential explanation for the observed increased PWV and BP in the T and A allele carriers.

My study shows that serum creatinine levels in the GG homozygote group of 863G>A polymorphism is higher than the other two genotype groups. Previous studies have clearly shown that subjects with end-stage renal failure present a marked increase in arterial stiffness independently of mean blood pressure values and other cardiovascular disease risk factors. In a cross-sectional analysis in a large population, a study showed that increased stiffness was significantly associated with creatinine clearance. [411]

The AIx is a hemodynamic measure related to arterial stiffness. It is defined as the increment in pressure from the first systolic shoulder to the peak pressure of the aortic pressure waveform expressed as a percentage of the peak pressure. The AIx is thought to be primarily determined by the intensity and timing of reflected waves. The central arterial pulse wave is composed of a forward traveling wave generated by left ventricular ejection and the later-arriving reflected wave from the periphery. As the stiffness of the arterial walls increases, transmission velocities of both forward and reflected waves increase. This causes the reflected wave to arrive earlier in the central

aorta and augments pressure in systole, as mentioned earlier here. The increase in pressure related to the arrival of the reflected wave determines the AIx.

However, sole use of pulse wave analysis to assess arterial stiffness is controversial. Since the pulse wave reflection returns to the aorta at an earlier phase of the cardiac cycle when the heart rate is high, there is an inverse association between heart rate and AIx that needs to be adjusted for [50]. The AIx is only, in part, determined by arterial stiffness as increases in peripheral wave reflectance may also be caused by increased peripheral vascular resistance and by the distending effect of an elevated blood pressure [62].

Haplotypes are a combination of alleles at different markers along the same chromosome that are inherited as a unit. The fundamental difference between haplotypes and individual genotypes at SNPs is that the alleles are assigned to a chromosome [412, 413]. Recently, haplotype association analysis has been suggested as a more powerful approach for identifying predisposing genes/alleles for complex conditions than individual SNPs [414, 415], because haplotypes capture almost all the variation in the gene. Haplotype analysis can improve the ability of detecting an association with variants in the gene. In our study, the two polymorphisms were in linkage and both the genotypes influenced the BP and PWV with evidence of a gene-dose effect. The combined effect of the two genotypes was additive with homozygotes for both the T and A alleles having the highest blood pressure and arterial stiffness. Although both genotypes were associated with similar absolute differences in blood pressure and PWV, the higher frequency of the 836-A allele (55.3%) versus the -1562-T allele (17.6%) means that at a population level, the 836-A allele is likely to be of greatest significance. Looked at another way, the haplotype analysis revealed a protective GC haplotype (for which one in ten of our population were homozygous) that was associated with significantly lower blood pressure and PWV. My findings support the notion that genetic variations in the MMP-9 gene can

influence the MMP-9 activity, which can lead to higher blood pressure and arterial stiffness.

This study showed a direct relationship between MMP-9 gene polymorphisms, whole genotypes, predicted haploypes, MMP-9 levels, and arterial stiffness in untreated hypertensive patients. While Yasmin et al [54] has found that MMP-9 haplotype is involved with large artery stiffening in healthy individuals my results shows that the presence of both the T and A alleles conferred increased BP and arterial stiffness associated with higher plasma MMP-9 levels with a significant gene-dose effect in untreated hypertensive patients.

Chapter 6

Adiponectin gene haplotype is associated with higher blood pressure and greater arterial stiffness in patients with essential hypertension

6.1 Introduction

Our view of adipose tissue has dramatically changed over the last decade. Once considered an inert energy depot, adipose tissue has emerged as an important endocrine organ regulating whole-body metabolism and other vital functions related to inflammation and immune responses. [416, 417]

Adiponectin is one of the molecules that is expressed and excreted only in the adipose tissue and plasma levels are relatively high, representing approximately 0.01% of total plasma proteins and the concentrations exceed those of many other common hormones. [257].

Recent studies support a role for adiponectin as an anti-inflammatory vasoprotective adipokine [418]. Adiponectin inhibits tumor necrosis factor alpha–induced expression of endothelial adhesion molecules in endothelial cells, reduces the atherogenic transformation of macrophages into foam cells by suppressing scavenger receptor expression [419, 420], and inhibits vascular smooth muscle proliferation [421]. Furthermore, globular adiponectin inhibits cell proliferation and superoxide release induced by oxidized LDL in bovine endothelial cells [259]. Importantly, adiponectin directly stimulates NO production in human and bovine aortic endothelial cells [262,422]. A number of recent studies have described the associations between circulating adiponectin levels and cardiovascular disease [267, 281, 287, 423]. Cross-sectional studies report that hypoadiponectinaemia and cardiovascular disease

have a stong relationship even after adjustment for cardiovascular risk factors such as diabetes, dyslipidaemia, hypertension, smoking and BMI [281, 287]. Hypoadiponectinaemia may explain in part why certain ethnic groups such as African-Americans and people of South Asian descent where lower levels of adiponectin have been reported [424, 425], have an increased risk of type 2 diabetes mellitus and coronary artery disease compared with other ethnic groups [426]. The study conducted by Pischon et al. demonstrated that high circulating levels of adiponectin are associated with decreased risk of myocardial infarction in men [423]. This association was independent of inflammation or glycaemic status. Taken together these findings suggest that cardiovascular risk factors such as dyslipidaemia and vascular inflammation may contribute to the relationship between hypoadiponectinaemia and cardiovascular disease. However the mechanisms that could explain the relationship between adiponectin and cardiovascular disease have yet to be elucidated.

Several genetic variants in the adiponectin gene have been identified and the associations of gene variations with type 2 diabetes have been studied [426-430]. Among them, two single nucleotide polymorphisms (SNPs) in the adiponectin gene, a silent T to G substitution at the position of nucleotide 45 in exon 2 (SNP45T>G) and a G to T substitution at the position of nucleotide 276 in intron 2 (SNP276G>T) were closely associated with type 2 diabetes in the different populations. [431-433]

Increased aortic stiffness measured as carotid-femoral pulse wave velocity (PWV) is associated with increased risk of cardiovascular events in the hypertensive [434] and diabetic [56] population. Augmentation index (AIx), a composite of wave reflection from medium-sized muscular arteries, left ventricular ejection and PWV is related to the development of coronary artery disease (CAD) [435]. PWV is significantly correlated with plasma glucose levels in subjects with impaired glucose tolerance [436] and there is a significant relationship between plasma insulin levels and aortic stiffness in the general population [437] and in subjects with the metabolic syndrome. [438]

There is also a significant association between the changes in brachial-ankle PWV and adiponectin in type 2 diabetes patients following treatment with pioglitazone which significantly reduced both brachial-ankle PWV and increased adiponectin concentrations. [439]

The primary goal of this study was to examine the association between the two polymorphisms of adiponectin gene and concentrations of circulating adiponectin and IR in a group of hypertensive population. In addition, I examined associations between the two polymorphisms and BP and arterial stiffness.

6.2 Methods

Details of the research subjects are described in Chapter 2. All the subjects were tested for their genotypes of 45T>G and 276G>T polymorphisms by using of a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), (see Chapter 2). The haemodynamic measurements are described in Chapter 2. Adiponectin levels were tested using ELISA in 80 subjects (Chapter 2).

The 261 research subjects were divided into groups according to their genotypes of 45T>G and 276G>T polymorphisms. Result analysis is described in Chapter 2.

6.3 Results

6.3.1 Distribution of 45T>G, 276 G>T genotypes of the adiponectin gene

The frequencies of the 45T>G genotypes were GG: 1% (3), GT: 23.2% (60) and TT: 75.8% (198). Allele frequencies were G: 12.6% and T: 87.4%. The frequencies of the 276G>T genotypes were GG: 53.6% (140), GT: 34.5% (90) and TT: 11.9% (31). Allele frequencies were G: 71.9% and T: 29.1%. The genotype distribution conformed to Hardy-Weinberg equilibrium for all SNPs.

In the 45T>G, there were only 3 homozygotes of GG. I combined the GG with GT heterozygotes together to represent the G allele carriers group. And with the 276G>T polymorphism, I combined the TT and GT together in the T allele carriers group following the method of Menzaghi et al [429].

6.3.2 Clinical characteristics according to genotype

Demographic and clinical characteristics of the patient population according to the adiponectin 45T>G and -276G>T genotype are given in Table 6.1. There were no genotype related differences in age, gender, BMI, waist, hip, smoking, plasma HDL, triglycerides and creatinine. The glucose, insulin and HOMA level in the G allele carrier group of the SNP 45 was significantly higher than in the TT homozygote group. Also the LDL, glucose, insulin and HOMA level in the GG group of the SNP 276 was significantly higher than in the T allele carriers.

Table 6.1 Clinical characteristics of the hypertensive patients according to adiponectin genotype at position 45 and 276 (n=261, mean±SEM)

		Age (years)	Gender male (%)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	Smokers (%)	Total Cholesterol (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	Trigly (mmol/l)	Glucose (mmol/l)	Insulin (mU/ml)	HOMA	Creatinine (umol/l)
SNP 45	G allele carriers (n=63)	47±1	51%	30±0.7	94±2	106±1	29%	5±0.1	1.3±0.02	2.2±0.2	1.5±0.1	5.7±0.2	10.5±1	0.51±0.1	86±2
	TT (n=198)	46±1	53%	29±0.3	96±1	107±1	28%	5±0.1	1.3±0.04	1.6±0.2	1.6±0.1	5.2±0.1	7±1.5	0.31±0.1	87±1
	P	0.65	0.76	0.61	0.32	0.69	0.90	0.12	0.62	0.13	0.62	0.05	0.02	0.02	0.38
SNP -276	GG (n=140)	46±1	52%	29±0.4	95±1	106±1	31%	5.2±0.1	1.32±0.03	2±0.2	1.6±0.07	5.6±0.1	6.7±1	0.31±0.08	87.6±1
	T allele carriers (n=121)	47±1	52%	29±0.5	96±1	107±1	28%	5.2±0.1	1.38±0.03	1.6±0. 3	1.5±0.08	5.2±0.1	10.7±2	0.47±0.08	86±1
	P	0.41	0.96	0.95	0.63	0.94	0.62	0.94	0.11	0.01	0.24	0.04	0.01	0.01	0.71

6.3.3 Association between adiponectin 45T>G and 276G>T polymorphisms and blood pressure

For the 45T>G polymorphism, the aortic systolic blood pressures was significantly higher in G allele carriers compared with the TT homozygote as seen in Table 6.2. Brachial systolic blood pressure and aortic diastolic blood pressure were higher in the G allele carriers group but did not achieve statistical significance. However, for the 276G>T polymorphism, the brachial systolic and diastolic blood pressure, and the pulse pressure were significantly higher in GG homozygote compared with the T allele carriers. The effects of the two genotypes were additive, with a direct linear relationship observed between the number of 45G and 276G alleles carried and the brachial SBP ($p<0.001$), DBP ($p<0.05$) and PP ($p<0.05$).

6.3.4 Adiponectin 45T>G and 276G>T polymorphisms and arterial stiffness

The PWV was significantly higher in the SNP 45 G allele carriers group ($p=0.04$) and SNP276 GG homozygote group ($p=0.02$) (Table 6.2). In contrast to PWV, there was no significant difference in AIx between the genotypes of the two polymorphisms (Table 6.2) and the results were unchanged when AIx was adjusted for major confounders such as age, gender, heart rate, height and smoking status. The effects of the two genotypes were additive, with a direct linear relationship observed between the number of 45G and 276G alleles and PWV ($p=0.001$).

Table 6.2 Comparison of the BPs and arterial stiffness according to the genotypes of adiponectin

		Brachial SBP (mmHg)	Brachial DBP (mmHg)	Aortic SBP (mmHg)	Aortic DBP (mmHg)	Brachial PP (mmHg)	Aortic PP (mmHg)	PWV (m/sec)	AIx (%)	Heart rate (min^{-1})	Adipo level (ng/mL)
SNP 45	G allele carriers (n=63)	154±1	91±1	132.5±2	80.5±2	61.5±1	51±1	10.5±0.2	29±2	68±1.5	76±6
	TT (n=198)	148±2	88±1	124.5±3	76±3	58±2	46±2	9.8±0.2	29±1	70±1	85±5
	P	0.06	0.10	0.04	0.07	0.11	0.06	0.04	0.69	0.23	0.63
SNP -276	GG (n=140)	155±1.5	91±1	132±2	80±2	63±1	51±1	10.6±0.2	29±1	70±1	74±7
	T allele carriers	149±1.5	89±1	128±3	78±2.5	59±1	48±1	10±0.2	29±1	70±1	84±6
	P	0.01	0.03	0.18	0.32	0.03	0.20	0.02	0.84	0.65	0.04

6.3.5 Adiponectin 276G>T polymorphism and plasma adipinectin levels

Mean plasma adiponectin levels were significantly higher in subjects carrying the 276T allele (TT & GT) compared with the GG homozygote (p=0.04) (Table 6.2). The adiponectin SNP276 genotype significantly influenced serum adiponectin levels after correcting for likely confounders including age, gender, BP and smoking status (R2= 0.30, p<0.0001). There was no relationship between the 45T>G polymorphism and the adiponectin level.

6.3.6 Adiponectin haplotypes

The haplotype distribution was TG: 39%, TT: 31.8%, GG: 12.2%, and GT: 17%. The two SNPs were in modest linkage disequilibrium (p=0.04). The adiponectin levels, PWV, brachial SBP, PP and aortic PP according to adiponectin haplotypes are shown in figure 6.1-figure 6.5. Even the brachial PP and aortic PP were not significant differences within the two polymorphisms. However, after the subjects were divided according to their haplotypes, both the brachial PP and aortic PP showed significant difference. Subjects with the TT haplotype had significantly lower PWV and systolic blood pressure than any of the other haplotypes containing one or more of the at-risk alleles (Figures 6.2&6.3)(p=0.02).

Figure 6.1 SBP according to adiponectin haplotypes

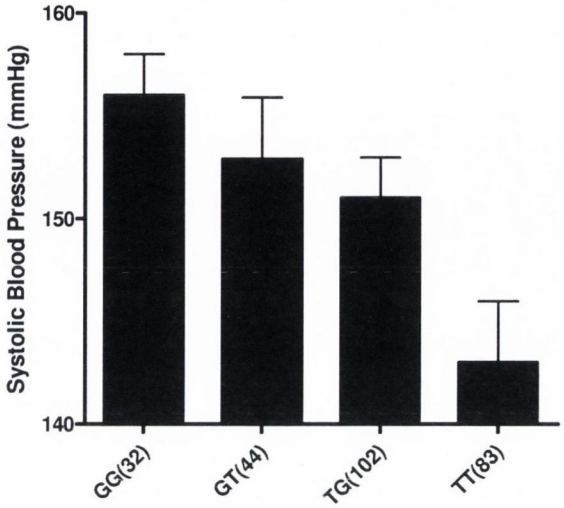


Figure 6.2 DBP according to adiponectin haplotypes

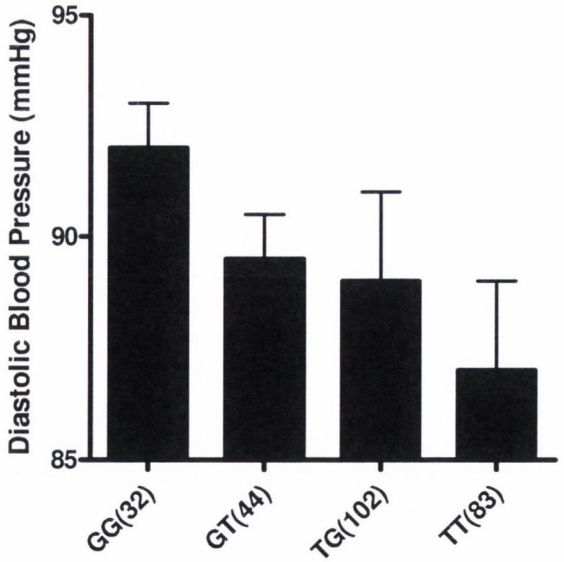


Figure 6.3 PWV according to adiponectin haplotypes

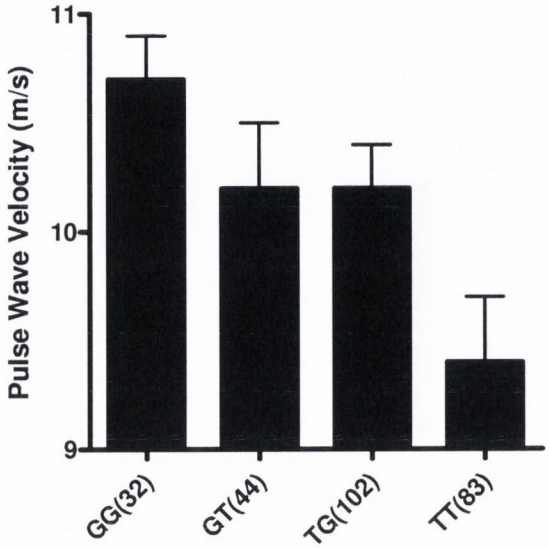


Figure 6.4 Brachial PP according to adiponectin haplotypes

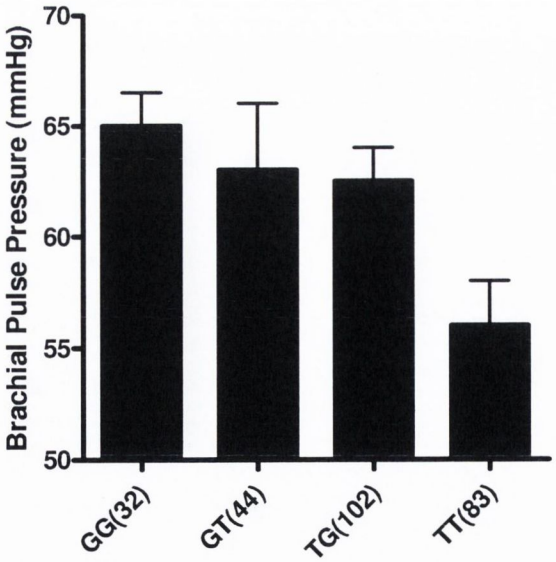
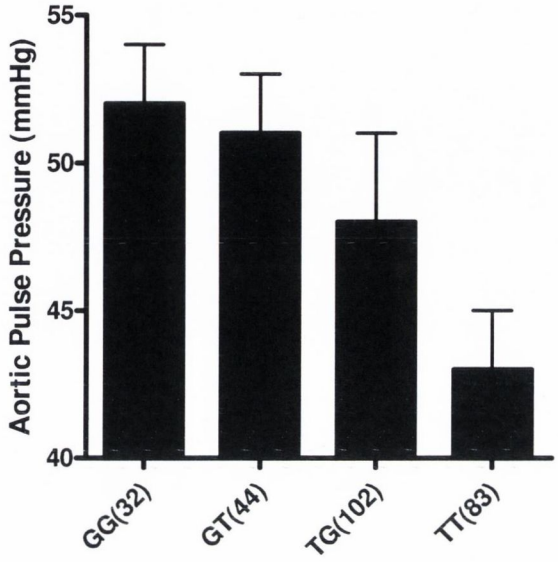


Figure 6.5 Aortic PP according to adiponectin haplotypes



6.4 Discussion

The present study is the first to show the relationship between polymorphisms in the adiponectin gene (276G>T intron region and 45T>G coding region), blood pressure, arterial stiffness and adiponectin levels in never-treated hypertensive patients. Both polymorphisms were able to affect, at some level, the systolic and diastolic BP in these patients. Additive effects were seen for carriers of both at-risk alleles. Furthermore, both polymorphisms affected the insulin and glucose levels. The haplotype of the 2 polymorphisms were independent predictors of BPs and PWV. In addition, the 276G>T polymorphism was an independent determinant of serum adiponectin levels in my study population, with higher adiponectin levels seen in the 276T allele carriers.

Adiponectin is an adipose tissue–specific protein composed of 247 amino acids that shares significant similarity with collagens VIII and X and complement protein C1q [253-256]. Adiponectin modulates endothelial adhesion molecules [280] and has been found in the subendothelial space of catheter injured carotid arteries and in atherosclerotic lesions within the endothelium [290]. It may prevent vascular restenosis. [421] Circulating adiponectin concentrations are protective against CAD [423] and also predictive of subsequent cardiovascular events in patients with ESRD. [440]

When single nucleotide polymorphisms of the adiponectin gene were genotyped in large datasets from various ethnic groups, two SNPs in the adiponectin gene, a T→G substitution in exon 2 (T45G) and G→T substitution in intron 2 (G276T), were significantly associated with Type 2 diabetes insulin resistence [429, 430]

Hypertension is a common disease that increases the risk for cardiovascular disease, and it is also a component of the metabolic syndrome, which is defined as the combination of obesity, insulin resistance, glucose intolerance and hyperlipidemia. Variable between hypertension and the plasma adiponectin concentration has been described. Mallamaci et al [441] reported increased plasma adiponectin levels in

hypertensive patients with renal dysfunction, while Adamczak et al [442] reported decreased adiponectin level in hypertensive subjects. Kazumi et al [268] reported that young Japanese men with high-normal blood pressure had lower adiponectin levels. Furuhashi et al [443] reported only hypertensive patients with insulin resistance showed lower adiponectin concentrations. In my research, I found that the 276 GG homozygote carriers have lower plasma adiponectin levels and even higher blood pressure than the T allele carriers. Also in the 45T>G polymorphism, the G allele carriers have somewhat higher blood pressure than the TT homozygotes.

There are three possible reasons for the negative relationship between hypertension and plasma adiponectin concentrations. First, as Ouchi et al [444] have reported that plasma adiponectin concentrations are independently correlated with the vasodilator response to reactive hyperemia, adiponectin concentrations could be an independent determinant of endothelial function. Endothelial dysfunction is an important feature of the early stage of atherosclerosis, which is related to pathogenic conditions including hypertension [445, 446]. Furthermore, in adiponectin-knockout mice, hypoadiponectinemia caused diet-induced hypertension. Secondly, an increase in sympathetic nerve activity, which is common in hypertensives [447], may inhibit adiponectin gene expression via β-adrenergic stimulation [448]. Thirdly, activation of the renin-angiotensin system may be induced in adipose tissue by hypoadiponectinemia, resulting in an increase in fat mass and blood pressure [449, 450]. However, further investigation is required to examine these hypotheses.

My results show that the G alleles of the 45T>G polymorphism and the GG homozygotes of 276G>T polymorphism have higher PWV than the other allele carriers. A significant inverse relationship between plasma adiponectin levels and PWV in hypertensive subjects has been noted previously [451]. The current finding of a negative relationship between the lower level of adiponectin and higher PWV is therefore in keeping with previous observations relating both PWV and adiponectin to possible cardiovascular risk. An increased PWV is associated with cardiovascular

events in the hypertensive [452] and diabetic [453] populations and in subjects with endstage renal disease (ESRD) [454]. Low plasma adiponectin concentrations may predict the risk of acute coronary syndrome, [455] but not of restenosis after coronary stenting [456]. How adiponectin and PWV are related is not immediately clear and may possibly be attributable to other metabolic and vasoactive factors not measured here. In the present study, the plasma adiponectin level was significantly correlated with the increased PWV in the 276G>T polymorphism GG group. On multiple regression analyses, the plasma adiponectin level was a significant independent determinant factor of PWV. These results suggest that the plasma adiponectin level probably contributes to arterial stiffness. This can be explained by two possible mechanisms linking hypoadiponectinemia and increased arterial stiffness. Firstly, adiponectin may be associated with arterial stiffness via insulin resistance. Plasma adiponectin was found to be closely correlated with whole-body insulin resistance [457]. Moreover, studies in different populations have suggested that low adiponectin levels are predictive of future development of insulin resistance and diabetes. [458-461]

In vivo, administration of adiponectin ameliorates insulin resistance by decreasing hepatic glucose production [462]. In my research, the 276G>T polymorphism GG group has a lower insulin level, lower HOMA-IR and higher PWV. The stiffness of the carotid and femoral arteries is determined at least in part by insulin resistance [463]. These findings together suggest that adiponectin probably contributes to arterial stiffness via insulin resistance. Secondly, adiponectin directly regulates the various alterations in the arterial wall, because adiponectin has an anti-inflammatory function. Adiponectin inhibits tumor necrosis factor α–induced expression of endothelial adhesion molecules in endothelial cells, reduces the atherogenic transformation of macrophages into foam cells by suppressing scavenger receptor expression [419, 464] and inhibits vascular smooth muscle proliferation [421].

Adiponectin has been shown to be a strong determinant of the plasma LDL level [465].

In macrophages, adiponectin inhibits the expression of LDL, resulting in markedly decreased LDL levels and inhibition of foam cell formation. [464]

In my study, while I did not find any difference between the both the brachial and the aortic PP and the 2 polymorphisms, the brachial and aortic PP were different according to the adiponectin haplotypes. Though the pulse pressure can be measured using a standard sphygmomanometer and is one of the simplest measures of arterial stiffness, specially considered in the clinical setting. Some oscillometric sphygmomanometers may be unreliable, particularly in older subjects [466]. Moreover, pulse pressure alone is inadequate to assess arterial stiffness accurately. Problems include the 'normal' amplification of the pressure wave as it travels from the aorta to the periphery, although this effect becomes less pronounced with increasing age. Thus, measurements of pulse pressure made in the periphery, for example in the upper arm, do not always accurately reflect the actual central pulse pressure [49]. Indeed, there can be differences of up to 20 mmHg between central pressures of patients with identical brachial blood pressure readings [14]. Interestingly, exaggerated amplification is thought to account for the phenomenon of pseudohypertension in the young [467]. Central pressure may, therefore, be a more accurate predictor of risk than peripheral blood pressure. Moreover, it is central pressure that contributes most to the development of left ventricular hypertrophy, itself an independent predictor of cardiovascular mortality and changes in left ventricular geometry [468]. Also, carotid intima medial thickness, a marker of cardiovascular risk, [469] is dependant on carotid but not brachial pulse pressure.[470]

In my research, I did not find any relationship between the AIx and adiponectin polymorphisms. The AIx, while considered by some to be a measure of systemic arterial stiffness, is not the same as large artery stiffness. It depends also on the amount of wave reflection from the periphery, which is influenced by the geometry of the arterial tree and small artery tone [5, 51, 65].

Haplotype association analysis has been suggested as a more powerful method for

identifying predisposing genes/alleles for complex conditions than individual SNPs [414, 471]. Haplotype analysis can improve the ability to detect an association with variants in the gene. In this study, the two polymorphisms were in linkage and both the genotypes influenced the BP and PWV. The combined effect of the two genotypes was additive with homozygotes for both the T and G alleles having the highest blood pressure and arterial stiffness. Although both genotypes were associated with increased blood pressure and PWV, higher levels were seen in the 276G>T GG homozygotes, indicating that, at a population level, the 276G>T T allele carriers are likely to be of greatest significance.

Although SNP 276 is located in an intronic region with no apparent biological function, this intronic SNP may affect the expression of the gene via an unknown mechanism. The association between the SNP 276 genotypes of the adiponectin gene and the circulating adiponectin concentrations was clearly established in the current study in untreated hypertensive subjects. In particular, the GG homozygote at SNP -276 was associated with lower plasma adiponectin. Carriers of the GG homozygote at position 276 have higher blood pressure and arterial stiffness. Therefore, this genetic marker may help in the identification of subjects who are at greater risk of hypertension and arterial stiffness. With regard to the molecular mechanisms associated with SNP+45 functional effects, some hypotheses can be proposed. The SNP+45 located in exon 2 results in a synonymous change (G15G), and is relatively close to the exon–intron boundary which may affect the splicing machinery. There is increasing evidence that even silent mutations in coding regions might modify RNA levels by affecting splicing and thus decreasing the expression of the gene [472].

A strong linkage between SNP45 and SNP 276 can be found in this research. SNP45/276 haplotypes showed an association with higher blood pressure and PWV although SNP 45 does not seem to affect the adiponectin level.

Chapter 7

RAGE haplotype could lead to higher blood pressure and greater arterial stiffness

7.1 Introduction

Hypertension is intimately related to both increased arterial stiffness and augmented wave reflection. Aging is a major contributor to cardiovascular disease and particularly aortic stiffness [473]. A consideration of factors associated with aging may provide another perspective on factors that influence arterial stiffness. AGEs have been identified in the aging process and earlier work in my department explored the positive relationship between AGEs and arterial stiffness [474] AGE accumulation within the human aorta has been studied. An immunohistochemical study of atherosclerotic aortas obtained at autopsy revealed intracellular AGE deposition in 2 of 12 specimes of aortic intima, while AGE deposition in the diffusely thickened intima occurred in 3 out of 22 cases [475]. Extracellular accumulation of AGE was demonstrated in 20 of 22 aortic tunica and media specimens while AGE deposition was not detected in aortic specimens from the 3 autopsy cases aged 10 years or less.

The receptor for advance glycation end products (RAGE) is a multi-ligand member of the immunoglobulin super-family of cell surface molecules [476]. Cloning of RAGE and subsequent homology analysis revealed it to be a member of the immunoglobulin superfamily of receptors, comprising an extracellular domain consisting of a single V-type immunoglobulin domain and two C-type immunoglobulin domains [303].

RAGE expression is shown to be up-regulated in atherosclerotic plaques of diabetic

animals [315]. Ligand-induced RAGE activation results in cellular signaling including induction of oxidative stress, increased expression of cytokines□adhesion molecules, activation of nuclear factor-κB (NF-κB) [477, 478]□apoptosis pathways [479], cell growth and motility pathways [480], and alteration of gene expression pathways [306].

A number of SNPs have been identified in regulatory and coding regions of the RAGE gene. Two functional -374T>A and -429T>C polymorphisms in the promoter region of the RAGE gene have been shown to exert significant effects on transcriptional activity [481]. We postulated that RAGE -374T>A and -429T>C polymorphisms may be related to homodynamic properties in hypertensive patients.

7.2 Methods

The population has been described previously in chapter 2. All the subjects were tested for their genotypes of -374T>A and –429T>C polymorphisms by the use of a polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), (see Chapter 2). The haemodynamic measurements are described in Chapter 2.

The 261 research subjects are divided into groups according to their genotypes of -374T>A and –429T>C polymorphisms. Data analysis is described in Chapter 2.

7.3 Results:

7.3.1 Distribution of –374T>A, –429T>C genotypes of the RAGE gene

These are tabulated in Table 7.1. The frequencies of the –374T>A genotypes were AA: 12.3%, AT: 36.1% and TT: 51.6%. Allele frequencies were A: 30% and T: 70%. The frequencies of the –429T>C genotypes were CC: 1.3%, CT: 26.8% and TT: 71.9%. Allele frequencies were C: 14.7% and A: 85.3%. Neither genotype distributions differed significantly from that predicted by the Hardy–Weinberg equilibrium. As the value of CC homozygote in the –429T>C is very limited, I combined the CC and CT together into the C allele carriers group. With the –374T>A polymorphism, I followed

the reported example that the AT and TT were combined together into the T allele carriers group. [482]

7.3.2 Clinical characteristics according to genotype

Demographic and clinical characteristics of the patient population classified according to the RAGE –374 T>A and –429T>C genotype are given in Table 7.1. There were no significant differences in age, gender, BMI, smoking, lipids or creatinine between the different genotypes.

Table 7.1 Clinical characteristics of the hypertensive patients according to the RAGE genotype at position -374 and -429 (n=261, mean±SEM)

		Age (years)	Gender, male (%)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	Smokers (%)	Total Cholesterol (mmol/l)	HDL (mmol/l)	Trigly (mmol/l)	Glucose (mmol/l)	Creatinine (umol/l)
SNP -374	AA (n=32)	47±1	50%	31±1	98.5±2	109±2	29%	5±0.1	1.3±0.04	1.5±0.1	5.7±0.	86±2
	T allele carriers (n=229)	46±1	52%	29±0.3	95±1	106±1	28%	5 ±0.1	1.3±0.04	1.6±0.1	5.4±0.	87±1
	p	0.78	0.78	0.05	0.12	0.1	0.90	0.25	0.81	0.72	0.34	0.34
SNP -429	TT (n=188)	47±1	52%	30±0.3	96±1	106±1	31%	5.2±0.1	1.3±0.02	1.6±0.0	5.4±0.	87±1
	C allele carriers (n=73)	45±1.5	51%	28.7±0	95±1	105±1	28%	5.1±0.1	1.4±0.04	1.7±0.1	5.4±0.	87±2
	P	0.34	0.84	0.09	0.58	0.20	0.62	0.48	0.89	0.94	0.67	0.58

7.3.3 Association between RAGE –374 T>A and –429T>C polymorphisms and blood pressure

For the –374 T>A polymorphism, systolic blood pressures and pulse pressure were significantly higher in the T allele carriers group compared with the AA homozygote Table 7.2 For the –429T>C polymorphism, only the systolic blood pressure was significantly higher in the TT homozygote carriers group compared with the C allele carriers. The effects of the T allele of the RAGE –374 T>A and the TT genotype of the RAGE –429 T>C polymorphisms on blood pressure were of a similar magnitude (11 and 6 mmHg differences between the two groups in systolic BP). To assess the relationship between the BP and the polymorphisms, we constructed stepwise regression models with known or likely confounders of BP. After age, the RAGE genotype was the major determinant of systolic BP. Triglycerides, body mass index and heart rate also emerged as significant predictors. Both polymorphisms were also independent determinants of diastolic BP but to a quantitatively lesser degree than for systolic BP.

7.3.4 RAGE –374 T>A and –429T>C polymorphisms and arterial stiffness

PWV was significantly higher in the T allele carriers group of the –374 T>A polymorphism (p=0.002) and the TT homozygote group of the –429T>C polymorphism (p=0.01) (Table 6.2). The effects of the T allele of the RAGE –374 T>A and the TT genotype of the RAGE –429 T>C polymorphisms on arterial stiffness were to produce differences of 1.2 and 0.5 m/sec in PWV magnitude. In a stepwise multiple regression model, with PWV as the dependent variable and the known or likely confounders of arterial stiffness as independent variables, age emerged as the major determinant of aortic PWV with a significant independent contribution from both the –374 T>A and –429T>C polymorphisms.

In contrast to PWV, there was no significant difference in AIx between the genotypes of the two polymorphisms (Table 7.2) and the results were unchanged when AIx was adjusted for major confounders such as age, gender, heart rate, height and smoking

status.

Table 7.2 Comparison of the BPs and arterial stiffness according to the genotypes of RAGE

		Brachial SBP (mmHg)	Brachial DBP (mmHg)	Aortic SBP (mmHg)	Aortic DBP (mmHg)	Brachial PP (mmHg)	Aortic PP (mmHg)	PWV (m/sec)	AIx (%)	Heart rate (min^{-1})
SNP -374	AA (n=32)	143±2	88.5±1	134±2.5	90±2	54±1	44.5±2	9.3±0.2	28±2	66±2
	T allele carriers (n=229)	154±1	90±1	141.5±1	91.5±1	60±1	50±1	10.5±0.2	28±1	70.5±1
	p	0.001	0.29	0.05	0.40	0.04	0.05	0.002	0.82	0.05
SNP -429	TT (n=188)	157±2	91±1	145±2	91±1	63±1.5	52±2	10.7±0.3	29±1	69±1
	C allele carriers (n=73)	151±1	90±1	139±1	92±1	60±1	49±1	10.2±0.2	28±1	70±1
	P	0.01	0.54	0.01	0.53	0.08	0.25	0.01	0.33	0.44

7.3.5 Rage haplotypes

The haplotype distribution was TT: 167 (64%), TC: 65 (25%), AT: 25 (9.5%) and AC: 4 (1.5%). The two SNPs were in modest linkage disequilibrium (p=0.05). According to the RAGE haplotypes, the brachial SBP, PWV, brachial and aortic PP are shown in figures 7.1-7.4. Subjects with the AC haplotype had significantly lower PWV and systolic blood pressure than any of the other haplotypes containing one or more of the at-risk alleles (Figures 7.1-7.4)(p=0.02).

Figure 7.1 SBP according to RAGE haplotypes

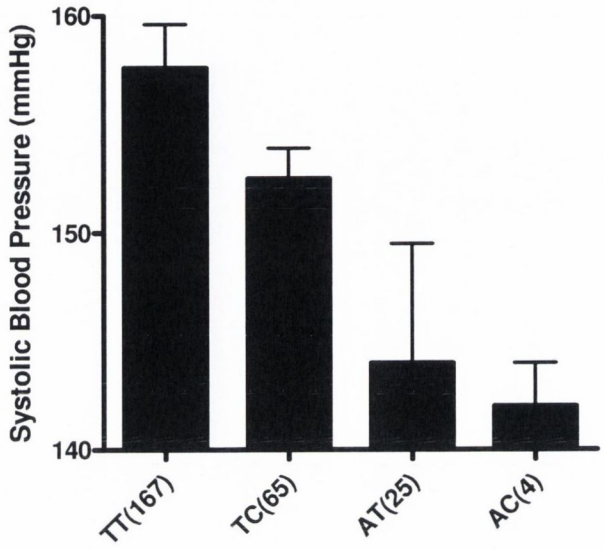


Figure 7.2 PWV according to RAGE haplotypes

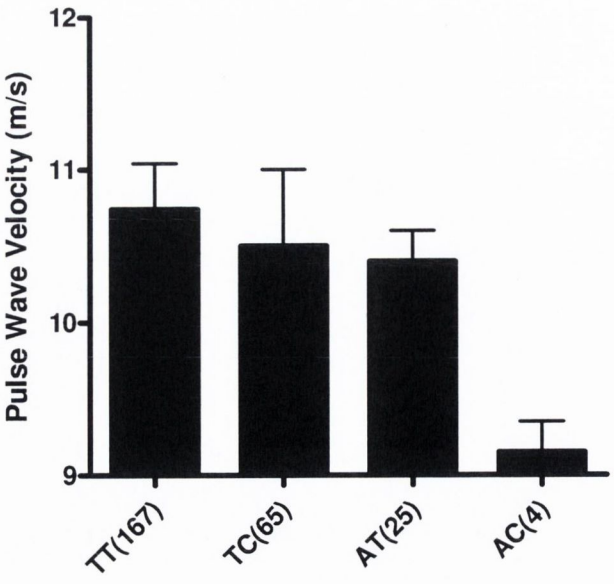


Figure 7.3 Brachial PP according to RAGE haplotypes

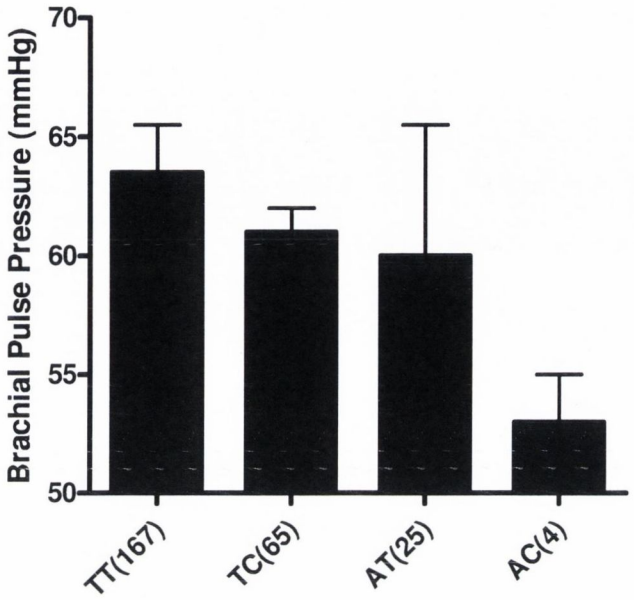
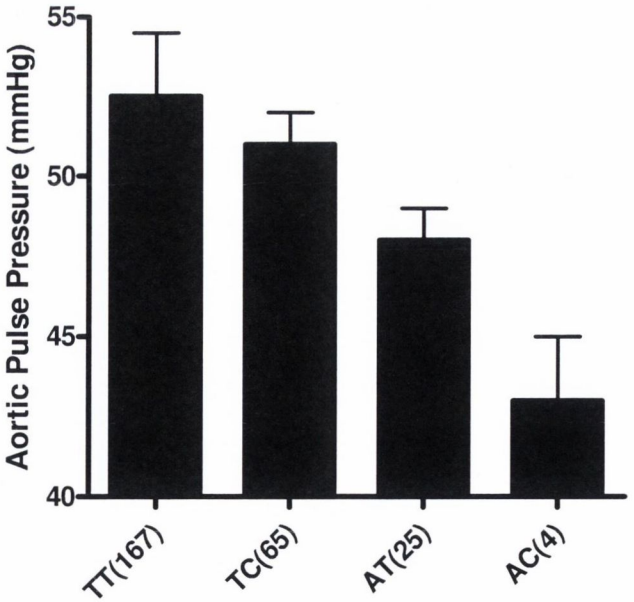


Figure 7.4 Aortic PP according to RAGE haplotypes



7.4 Discussion

The present study shows a relationship between polymorphisms in the RAGE gene (–374 T>A and –429T>C promoter region), blood pressure and arterial stiffness in never-treated hypertensive patients. Furthermore, both polymorphisms are independent predictors of both brachial, aortic systolic BP and arterial stiffness levels in these patients. In the –374 T>A polymorphism, the T allele carriers (AT & TT) and the TT homozygote of the –429T>C polymorphism have significantly higher blood pressure and arterial stiffness.

RAGE is a trans-membrane receptor of the immunoglobulin super-family that is hyper-expressed at sites of atherosclerosis [338]. Because AGEs can be produced by oxidant stress and inflammatory pathways, their consequence is likely to extend to euglycemic vascular disease [483].

Recently, Geroldi et al. found that soluble RAGE levels were significantly decreased in patients with essential hypertension and were inversely related to pulse pressure [484]. Furthermore, Falcone et al. reported that low levels of soluble RAGE in plasma are independently associated with the presence of coronary artery disease in non-diabetic men [485].

AGEs-RAGE interaction in the vessel wall may lead to inflammation, smooth muscle cell proliferation and extracellular matrix production. AGE-RAGE interaction in VSMC contributes to the stimulation of VSMC proliferation [486]. Thus, changes in the smooth muscle content, the extracellular matrix which contains both collagen and elastin and their structural arrangement are potential mechanisms for the relationship with arterial stiffness. It would have been interesting to see whether there was any difference in the AGEs concentration associated with individual genotypes as a previous study showed that the concentration of plasma AGEs is significantly higher in hypertensive compared to normotensive subjects and is related to aortic stiffness independent of age and blood pressure. [474]

Although first described as a receptor for AGEs, cloning of RAGE and subsequent homology analysis revealed it to be a member of the immunoglobulin superfamily of receptors. It binds to diverse ligands and consequently is implicated in a variety of distinct pathological processes [483]. The gene for RAGE is located on chromosome 6 in the major histocompatibility complex, a region of the genome containing a number of inflammatory genes and the denser region of genes in the genome. [487,488]

At the cellular level, RAGE is expressed by a variety of cell types, including endothelial, vascular smooth muscle, podocytes, and neuronal cells [305]. Various studies reveal a striking feature of RAGE expression under proinflammatory conditions. In a variety of diseases including diabetic vascular disease, RAGE expression is found to be highly up-regulated [305]. Recently, RAGE was shown to be involved in both microdiabetic [316] and macrodiabetic [317, 486] vascular complications [476]. This is perhaps not surprising as AGEs are commonly formed in the presence of raised glucose levels and have been implicated in diabetes associated pathology particularily in the eye and the kidney [489, 490]

Recent studies have highlighted the possibility that polymorphisms within key domains of RAGE may influence its function, such that under conditions of increased ligand accumulation, individuals may be predisposed to heightened inflammatory responses [491]. Hudson et al. identified polymorphic sites in the RAGE promoter region: the -429T >C and the -374T >A [481]. Because a functional -374T>A polymorphism in the promoter region of the RAGE gene has been shown to exert significant effects on transcriptional activity [481], investigation with this common variant revealed that the introduction of the -374A allele abolished a nuclear protein binding site, supporting the role of this polymorphism in affecting RAGE transcriptional repression [481]. This variant has become a topic of great interest. Although it has been shown that homozygosity for the minor A-allele is a possible risk factor for non-small cell lung cancer development [492], a recent report suggests that the -374 AA homozygous genotype may be a marker for less atherosclerosis in type 1

diabetic patients [493].

Although the precise mechanisms underlying this protective effect of RAGE are unclear, it is likely that the effect on CAD severity could be mediated by the lower RAGE expression in homozygous minor A allele carriers [475]. Indeed, RAGE over-expression has been related to adverse vascular effects in several previous investigations [494]. Thus, individuals who are homozygous for the AA homozygote of -374T>A and C allele carriers of –429T >C may prevent both the development and the progression of hypertension and arterial stiffness via down-regulation of RAGE gene expression.

Moreover, it has been shown that RAGE could play a relevant role in mediating intima formation after vascular injury [305]. Maybe the AA homozygote of -374T>A and C allele carriers of –429T >C could effect decreased expression of RAGE and thus could decrease the expression of RAGE thereby decreasing fibrotic organization in the arterial wall. Also the AA homozygote of -374T>A and C allele carriers of –429T >C with decreased expression of RAGE may introduce less migration and proliferation of smooth muscle cells (SMC) and mononuclear phagocytes (MPs) [318].

In my study, the two polymorphisms were in linkage and both the genotypes influenced the BP and PWV. The combined effect of the two genotypes was additive with the AA homozygote of -374T>A and the C allele carriers of –429T >C (for which the combined haplotype is AC) are related to the lowest blood pressure and arterial stiffness. The AC haplotype may confer a significant protective effect against the development of hypertension and arterial stiffness.

Questions have been raised as to the precision of the calculated AIx as a result of the variability of the transfer functions for each individual [495]. Measurement of AIx is dependent on higher-frequency signals than BP, and the transfer function may be less precise and may have greater variability at these high frequencies. This also results in underestimation of central systolic pressure and overestimation of central diastolic

pressure. In some studies, correlation of AIx and PWV is accordingly low, and the latter appears to be a more precise measure of the stiffness of central blood vessels. Some investigators suggest that AIx could be replaced by simple calculation of central aortic pressure, which may be more precise than the derived aortic pulse waveform used to calculate AIx, or even direct determination of AIx from the peripheral pulse [496].

Chapter 8

Influence of the G-protein beta-3 subunit gene C825 T polymorphism on the clinical phenotype and arterial stiffness in newly diagnosed essential hypertension

8.1 Introduction

The underlying cause of hypertension remains unknown in over 95% of cases. It is generally accepted that essential hypertension is a multifactorial, polygenic disorder, arising from the complex interaction between genotypes and the environment. To date, the genetic basis of essential hypertension remains poorly understood. However, a polymorphism in the heterotrimeric G protein (GNB3) gene has shown promise as a potential risk factor for essential hypertension. [497]

GNB3 is a member of the heterotrimeric G proteins, a family of multifunctional mediators of intracellular signal transduction. GNB3 plays a role in cell growth and apoptosis and may affect the activity of the sodium hydrogen exchanger 1 (NHE1), which regulates pH and sodium homeostasis [498]. A single-nucleotide polymorphism (C825T) in the GNB3 gene produces an alternative splice variant of the G protein beta3 subunit (Gbeta3). This results in a protein product, Gbeta3-s, in which 41 amino acids are deleted from Gbeta3 [499]. This splice variant has been associated with enhanced G-protein signaling in vitro. [499]

The T allele of the GNB3 825 polymorphism was found to be associated with

hypertension in a number of large, independent, case-controled and cross sectional Caucasian populations studies. However, the results of such studies are not entirely consistent. Odds ratios for the TT genotype are typically in the region of 1.3-1.8. [498]

More recent data suggestes that the GNB3 825 T allele may be associated with specific phenotypic characteristics, such as a low renin state [500], altered electrolyte homeostasis [501], obesity, insulin resistance and components of the metabolic syndrome. [497]

Thus, in cross sectional studies using conventional definitions for hypertension, the T allele of the GNB3 825 polymorphism appears to confer an increased risk of arterial hypertension. Whether this gene variant might be used in clinical practice to identify subjects with specific hypertension phenotypes is less clear.

The aim of this study was to genotype a population of newly diagnosed, never-treated hypertensive individuals and to determine whether the GNB3 polymorphism can predict earlier age of onset, more severe hypertension or specific endocrine/biochemical profiles. In addition, I sought to determine whether the GNB3 polymorphism was associated with an increased risk of target end-organ damage, as determined by measures of arterial stiffness (PWV and AIx) and the presence of microalbuminuria.

8.2 Methods

182 never treated Caucasian apparently healthy hypertensive subjects with a diagnosis of essential hypertension based on three outpatient measures of BP were studied, Clinical database was collected and all the subjects were tested for the 825C>T genotype (Chapter 2).

Data analysis is described in Chapter 2.

8.3 Results

The mean age (SD) of the study group was 48(12), 50% male. Allele frequencies were 0.61 for the C allele and 0.39 for the T allele. Genotype frequencies were CC (37%), CT (48%) and TT (15%) and were consistent with those predicted by the Hardy Weinberg equilibrium. The three genotype groups did not differ with regard to mean age at presentation, gender distribution or smoking status. (Table 8.1)

8.3.1 GNB3 polymorphism, blood pressure and target organ damage

There were no genotype differences in blood pressure severity at presentation, neither for systolic nor diastolic mean office nor for mean ambulatory blood pressure. (Table 8.1) Similarly, the genotype had no influence on either of the measures of arterial stiffness, namely PWV and AIx. (Table 8.1) Urinary microalbumin concentrations were also similar in the three genotype groups.

8.3.2 GNB3 polymorphism and the metabolic syndrome

37% of the overall study population fulfilled the ATPIII criteria for the metabolic syndrome. The TT genotype was associated with a lower prevalence of the metabolic syndrome. The prevalence for carriers of the CC genotype was 36%; 46% for CT and 15% for TT (p=0.02 for TT versus CT and CC). The lower prevalence appeared to be accounted for by smaller waist measurements and lower plasma triglyceride concentrations in TT carriers. (Table 8.1)

8.3.3 GNB3 polymorphism and endocrine/biochemical parameters

The plasma renin, aldosterone and aldosterone: renin ratio (ARR) was similar in all three groups. The T allele was associated with significantly lower plasma potassium levels (p=0.01), higher plasma bicarbonate (p=0.02) and higher fractional urinary potassium excretion (p=0.03). (Table 8.1) There was evidence of a gene-dose effect, with heterozygote exhibiting levels intermediate between those of CC and TT

homozygote.

Table 8.1 Characteristics of the study population according to GNB3 genotype

	Total	CC	CT	TT	p
N (%)	182 (100%)	68 (37%)	87 (48%)	27 (15%)	
Male gender n (%)	91 (50%)	35 (52%)	44 (51%)	12(44%)	0.82
Age years (SD)	48 (12)	49(13)	47 (12)	47 (13)	0.50
Ever smoker n (%)	82(51%)	35 (59%)	38 (49%)	9 (39%)	0.21
Blood Pressure					
Systolic BP (mmHg)	157(20)	155(21)	159(20)	155(19)	0.56
Diastolic BP (mmHg)	91(11)	90(11)	92(10)	90(9)	0.43
24hr systolic BP (mmHg)	139(14)	139(15)	141(14)	136(12)	0.28
24hr diastolic BP (mmHg)	84(9)	84(9)	86(10)	83(8)	0.16
Arterial stiffness					
Pulse wave velocity (m/s)	10.1(1.7)	10.2(1.9)	10.1(1.6)	10.2(2.2)	0.75
Augmentation index (%)	28.5(12.5)	28.7(11.1)	28.4(12.9)	28.1(12.5)	0.98
Metabolic syndrome					
BMI (Kg/m2)	30 (5)	30 (6)	30 (6)	28(4)	0.16
Waist measurement (cm)	95(16)	96(16)	97(17)	87(16)	0.04
Fasting glucose (mmol/L)	5.5(1.8)	5.8(2.4)	5.4(1.4)	5.3(0.8)	0.31
Total cholesterol (mmol/L)	5.1(0.95)	5.1(0.87)	5.2(1.01)	5.0(0.93)	0.65
HDL cholesterol (mmol/L)	1.38(0.43)	1.39(0.54)	1.37(0.35)	1.37(0.38)	0.93
Triglycerides (mmol/L)	1.50(1.0)	1.69(1.2)	1.90(1.3)	1.50(1.0)	0.03
Metabolic syndrome n (%)	68(37)	24(36)	40(46)	4(15)	0.02
Biochemical parameters					
Potassium (mmol/L)	4.0 (0.36)	4.08 (0.27)	3.95 (0.36)	3.87 (0.35)	0.01
Bicarbonate (mmol/L)	25.1(2.3)	24.6(2.3)	25.2(2.20	26.0(2.5)	0.02
Fractional K+ excretion (%)	16.1(9)	13.2(6)	17.4(9)	22.0(14)	0.03
Urine microalbumin (Mg/L)*	11.2(15)	8.9(17)	12.4(14)	10.7(13)	0.36
Albumin:creatinine ratio	2.2(4)	2.0(4)	2.4(4)	1.8(3.0)	0.80
Sodium (mmol/L)	139.8 (2.1)	139.7(2.47)	139.9(1.98)	140.4(1.50)	0.58
Renin (ng/ml/hr)*	1.5(1.3)	1.2(1.2)	1.6(1.6)	1.6(0.7)	0.65
Aldosterone (pmol/L)*	465(286)	451(284	453(262)	521(257)	0.53

* data expressed as median (interquartile range)

8.4 Discussion

The GNB3 polymorphism has been variously associated with hypertension [498], low-renin states [500] and components of the metabolic syndrome [497]. It is important to evaluate whether this gene variant influences the clinical presentation of hypertension. In this well characterised population of newly diagnosed, untreated hypertensive subjects, GNB3 genotype had no influence on the age at presentation with hypertension or the severity of hypertension as determined by ambulatory BP monitoring. Increased arterial stiffness, as measured by aortic pulse wave velocity (PWV) and augmentation index (AIx), is a significant independent predictor of adverse cardiovascular events in hypertensive populations [450, 451, 502]. Previous studies suggest a relationship between the T allele and increased arterial stiffness in young healthy males [503]. The other study also casts doubt on the clinical relevance of this finding, as the presence of end-organ damage at disease presentation, such as determined by arterial stiffness measurements and the presence of microalbuminuria, did not differ significantly according to genotype. No relationship was found with either the PWV or AIx in this hypertensive population. One explanation may be the absence of an effect of the genotype on BP or sodium concentrations.

With the relatively small sample size in this study, it is important to address the issue of statistical power. The study was sufficiently powered to detect clinically meaningful genotype differences, for example a difference of one grade of hypertension according to the WHO/ISH guidelines, [504] or an increased risk of cardiovascular events in the case of arterial stiffness measurements [361]. While smaller genotype effects cannot be ruled out, the data suggest that the GNB3 polymorphism does not influence the severity of hypertension or the presence of target organ damage at presentation in a clinically meaningful way.

Similarly there was no apparent relationship between the GNB3 genotype and renin-aldosterone axis activation. Data on the association between renin status and the

GNB3 polymorphism are conflicting [500, 501]. There were, however, significant genotype differences in electrolyte homeostasis. Specifically, the T allele was associated with significantly lower potassium levels, higher plasma bicarbonate and higher urinary potassium excretion. There was evidence of a gene-dose effect, with heterozygotes exhibiting levels intermediate between those of the CC and TT homozygote. This finding is consistent with the data of Martin et al, who also found a relationship between the T allele and lower potassium levels [501]. The differences in electrolytes are within the normal physiological range; therefore their clinical relevance remains uncertain. The findings do raise interesting hypotheses regarding the functional activity of the GNB3 mutation.

Overt disturbances of potassium and acid-base homeostasis with or without hypertension are characteristic features of primary hyperaldosteronism and a number of monogenic disorders including Liddle's syndrome, which results from mutations in the renal epithelial sodium channel (ENaC) and Gitelman's syndrome, caused by mutations in the renal thiazide-sensitive Na–Cl co-transporter [504]. Heterotrimeric G protein signaling has been shown to be an important mediator of vascular tone in these conditions [505]. The GNB3 variant may give rise to a *forme fruste* phenotype of these disorders. Additional studies of the biological function of the GNB3 variant in relation to potassium and acid-base homeostasis would be required to fully elucidate the nature of this relationship. The higher fractional urinary excretion of potassium suggests that the GNB3 variant may modulate potassium and hydrogen ion excretion at a normal level. Interestingly, a recent study found that subjects with the GNB3 TT polymorphism had a significantly better blood pressure lowering response to diuretic therapy than C allele carriers. [506]

The prevalence of the metabolic syndrome at presentation was significantly lower in subjects with the TT genotype, largely accounted for by smaller waist measurements and lower triglyceride levels. This may have been a chance finding, especially as there was no evidence of a gene-dose effect and the results are contrary to the published

literature suggesting increased risk for obesity with the T allele [497]. An alternative hypothesis is that the onset of hypertension in TT carriers is genetically driven and less influenced by environmental factors such as obesity, i.e. at the time of first presentation TT homozygote have a lesser load of environmental/lifestyle risk-factors compared with the less genetically susceptible C allele carriers.

In summary, the T allele of the GNB3-C825T did not influence the age at presentation, the severity of hypertension, the presence of target organ damage or arterial stiffness in this newly diagnosed hypertensive population.

Chapter 9

Conclusions

Aging, environmental and genetic factors are responsible for structural and functional changes of the arterial wall, leading to decreased elasticity and increased stiffness [5, 507, 508]. Increased arterial stiffness is responsible for a disproportionate increase in systolic blood pressure and a relative decrease in diastolic blood pressure, thus increasing pulse pressure at any given value of mean blood pressure [5].

The stability, resilience, and compliance of the vascular wall are dependent on the relative contribution of its 2 prominent scaffolding proteins: collagen and elastin. The relative content of these molecules is normally held stable by a slow, but dynamic, process of production and degradation. Dysregulation of this balance, mainly by stimulation of an inflammatory milieu, leads to overproduction of abnormal collagen and diminished quantities of normal elastin, which contribute to vascular stiffness [324]. Increased blood pressure, or hypertension, also stimulates excessive collagen production [149]. In gross pathologic vascular specimens, these molecular changes manifest as a doubling to tripling of intima-medial thickness between the ages of 20 to 90 [150, 151], as well as a hypertrophied vascular smooth muscle layer [153]. Histological examination of the intima of stiffened vessels reveals abnormal and disarrayed endothelial cells, increased collagen, frayed and broken elastin molecules, infiltration of vascular smooth muscle cells, macrophages and mononuclear cells, increased matrix metalloproteinases, transforming growth factor (TGF)-α, intracellular cell adhesion molecules, and cytokines [152]. In addition to vessel wall thickening, aging is associated with a gradual increase in central artery lumen diameter (9% per decade from 20 to 60 years in the ascending aorta), [166] although some recent studies have suggested this does not occur [20].

The extracellular matrix of the vessel wall is comprised of collagen, elastin, glycoproteins and proteoglycans. The first two provide structural integrity and elasticity, and are potently regulated by catabolic matrix metalloproteases (MMPs). Through their collagenolytic and elastinolytic effects, MMPs degrade the ECM by creating uncoiled, less effective collagen and broken and frayed elastin molecules, respectively. Vascular cells, as well as inflammatory cells such as macrophages and polymorphonuclear neutrophils, produce collagenases (MMP-1, MMP-8, and MMP-13) and elastases (MMP-7 and serine proteases) [381]. Further degradation of the basement membrane ECM and stimulation of chemotactic agents occur through gelatinase activation (MMP-2 and MMP-9) [509, 510]. Enzyme activity is regulated by augmented gene expression. Post-translational activation is augmented by cleavage of pro-MMP protein, by MMP–MMP interactions, and by plasmin, thrombin, and reactive oxygen species (ROS) [511-513]. Tissue inhibitors of MMPs counter this response, and the MMP–tissue inhibitors of MMPs balance are central in the controlling of remodeling [509]. Deposition of chondroitin sulfate, heparin sulfate, proteoglycans, and fibronectin can also thicken and stiffen the ECM of vessel walls. [514]

Collagen molecules provide the tensile strength of the vessel wall and are enzymatically cross-linked soon after their formation to render them insoluble to hydrolytic enzymes [515]. Breaks in the integrity of these intermolecular bonds cause unraveling of the collagen matrix. Moreover, because of their slow hydrolytic turnover rate, collagen is particularly susceptible to nonenzymatic glycation cross-linking. This leads to increased collagen content, often with a more unorganized and dysfunctional fiber distribution. Elastin molecules are also stabilized by cross-linking (by LOX) to form desmosine and isodesmosine. Disruption of these crosslinks contributes to weakening of the elastin array with predisposition to mineralization by calcium and phosphorous, together increasing arterial stiffness [166-168]. Moreover, activation of various serine and metalloproteases generate broken and frayed elastin molecules.

Alterations in elastin production and molecular repair mechanisms additionally contribute to the loss of vascular elasticity. [516-518]

Elastin molecules are susceptible to AGE crosslinking. This reduces the elastic matrix of the wall [332, 333]. AGE may also affect endothelial cell function by quenching nitric oxide and increasing the generation of oxidant species such as peroxinitrite [335]. Through their immunoglobulin superfamily receptors (RAGE), AGE stimulates stress signaling and inflammatory responses. It also increases oxidant radical formation, proinflammatory cytokines, growth factors and vascular adhesion molecules [336, 337]. Such mediators can increase vascular stiffness via MMPs, [519] contribute to endothelial dysfunction that elevates smooth muscle tone, depress endothelial flowmediated dilation, worsen the response to vascular injury, affect angiogenesis and promote atherosclerotic plaque formation [338, 520, 521]. A profibrotic response can also be triggered independently from a TGF-α pathway by the interaction of RAGE with AGE ligands. [522]

Genetic study of the processes involved in arterial stiffness has focused on genes involved in the renin-angiotensin-aldosterone system, which controls blood pressure, cell proliferation, matrix production, and vascular hypertrophy [523] In hypertensive patients, Benetos et al [524] found a positive association between PWV and both the angiotensin converting enzyme (ACE) I/D polymorphism and the A1166C polymorphism of the angiotensin II type 1 receptor. In a larger population, including hypertensive subjects who had never been treated and hypertensive subjects who had been treated, the same group showed that the A153G and A1166C polymorphisms could affect the increase of aortic stiffness with age, [74] while the ACE I/D and AGT T174 gene polymorphisms do not have the same effect [74]. The importance of these observations has not been explored to date. An important question is to what extent these polymorphisms constitute an increased or diminished cardiovascular risk and whether their presence influences the response to antihypertensive therapy, particularily that directed at the renin-angiotensin -aldosterone system. For this reason

I examined haplotypes of MMP-9 gene, adiponectin gene and RAGE gene.

Carotid artery stiffness was increased in never treated essential hypertensive patients with TT homozygous of the M235T polymorphism of the angiotensinogen gene [97]. In another study, with 77% hypertensive patients, carotid artery stiffness was associated with the ACE I/D polymorphism [525]. As the contribution of one gene polymorphism to the arterial stiffness is limited studies are therefore required to determine the interactions between aging, genetic variants, and arterial stiffness or between two or more gene polymorphisms [525]. For instance, femoral artery distensibility has been shown to be lower than the population mean in ACE DD subjects homozygous for α-adducin Gly460 [525].. Haplotype studies will assume greater importance in this area.

Polymorphism studies have also focused on matrix proteins, mainly elastin and collagens. An increase in carotid stiffness is reported in subjects carrying the A allele of the Ser422Gly polymorphism of the elastin gene [387]. In patients with coronary artery disease, genotypes 2 to 3 of the fibrillin-1 gene have been shown to be associated with a higher level of arterial stiffness and central pulse pressure than the other genotypes [526]. Matrix metalloproteinases (MMPs) are potential candidate proteins. A functional -1562 C/T polymorphism in the promoter region of MMP-9 has been shown to associate with large vessel stiffness in patients with coronary artery disease (CAD). [108]

I studied several polymorphism sites in different genes that are important in influencing arterial stiffness at a number of distinct sites. These include genes that can affect the structure of the arterial wall (elastin, collagen, MMP-9 and RAGE) and 'hormone' functional genes (adiponectin and GNB3). Their contributions to arterial stiffness are discussed in respective chapters. My studies demonstrate that even following adjustment for the ageing process the contribution of genetic polymorphisms persists well into older age. In time we may see genetic analysis in the healthy

population with those at risk for specific conditions being given individual advice. This work therefore provides a wide variety of potential new targets from alterations in gene expression and activity, alteration of the structural protein expression, the matrix degrading activity, modulation of endothelial adhesion molecules, inflammation, smooth muscle cell proliferation and extracellular matrix production.

Collagen type III is one of the key members of collagen family and is mainly responsible for tensile strength and elastic resilience [138]. Vascular remodeling tends to structural alterations in the vessel wall. This is not only associated with cardiovascular disease, but also with ageing. It is suggested that the amount of collagen decreases with age, while the increased concentration may be due to the loss of other components in the arterial wall, which lead to the increase of the arterial stiffness. The results in chapter 3 demonstrate that the AA homozygote of the COL3A1 2209G>A polymorphism has higher systolic, diastolic and pulse pressure. And the AA homozygote has higher levels of arterial stiffness. Moreover, there is an age-gene interaction between the AA homozygote and arterial stiffness. The explanation for this the mechanism could be that the 2209G>A polymorphism causes an amino acid substitution (from threonine to alanine) which may lead the collagen to be degraded with greater difficulty by the MMPs and FAP or other proteolytic attack. This may cause the relative increase of collagen in the amount.

Elastin is one of the main structural components within the arterial wall and is considered to be the major determinant of arterial stiffness. Indeed, disruption and fatigue fracture of elastin of the arterial wall resulting in a transfer of stress to the collagen fibers is likely to be responsible for age related arterial stiffening [383]. The absolute and relative amounts of elastin in the arterial wall are altered with ageing. Hypertension could aggravate this process, leading to elevated in arterial stiffening [384]. The study of the relationship of the ELN 549G>A polymorphism and arterial stiffness in Chapter 4 showed that the AA homozygote can affect the process of arterial

stiffness. Moreover, an age-gene interaction was found such that the AA homozygote of the ELN gene has more effect in accelerating arterial stiffness during ageing than the GA and GG genotype. The reason why the AA homozygote is associated with higher levels of arterial stiffness maybe due to the fact that the SNP 549G>A polymorphism of the ELN gene could change the amino acid from glycine to serine at position 422. This change, seen in those with the AA homozygote, makes elastin in the arterial wall much easier to degrade by elastases than the GA and GG genotype carriers. Also, the elastin in the arterial wall with the AA homozygote may be much more prone fracture under stress force than the GA and GG genotypes.

MMP-9, also known as gelatinase B or 92-kDa type IV collagenase, may be particularly relevant to large artery stiffening associated with vascular disease. MMP-9 expression is increased after vascular injury [527] and is particularly evident in inflammatory atherosclerotic lesions. High expression of MMP-9 has been associated with coronary plaque destabilization [221, 509, 528] and with outward aortic remodeling and aneurysm formation [529-531]. These phenomena presumably occur as a consequence of excessive degradation of extracellular matrix components. MMP-9 substrates include denatured collagens (gelatin), collagen types II, IV, and V, elastin, entactin, and vitronectin [186]. Excessive degradation of important matrix components may influence aortic stiffening.

In this study, I examined the relationship between two functional polymorphism sites in the MMP-9 gene and the blood pressure and arterial stiffness levels. The two polymorphisms include the -1562C>T polymorphism in the promoter region and the 836G>A polymorphism in the exon 6 which leads to the substitution from arginine to glutamine. The results in chapter 5 demonstrate that the TT homozygote of the -1562C>T polymorphism and the AA homozygote of the 836G>A polymorphism have higher blood pressure levels. And both the two homozygotes have higher arterial stiffness levels. The haplotype that combines the two polymorphism genotypes shows

that haplotype ATAT is associated with significantly higher blood pressure, arterial stiffness and MMP-9 plasma levels than the other haplotypes. The result is the TT homozygote of the -1562C>T polymorphism has higher transcript activity and can express more MMP-9 than the other genotypes. It could enlarge the MMP-9 biological activity that is degrading gelatine and lead to the relative increase of collagen levels in the arterial wall which in turn cause increased arterial stiffness. The A allele of the 836G>A polymorphism causes a substitution of a positively charged amino-acid (arginine) by an uncharged amino acid (glutamine). This substitution can enhance substrate-binding ability of MMP-9 [325, 532]; also, in turn it could increase the biological activity of MMP-9.

Adiponectin, predominantly synthesized in the adipose tissue, seems to play an important role in carbohydrate and lipid metabolism and vascular biology [418]. It has been found to be a major modulator of insulin action and resistance [457]. Furthermore, it seems to have substantial anti-inflammatory properties [418]. Adiponectin is also related to lipid metabolism, particularly higher levels of HDL cholesterol and lower levels of triglycerides [267]. These data suggest that high adiponectin levels may be related to lower risk for coronary heart disease (CHD).

In my study, I examined the relationship between two functional polymorphism sites in the adiponectin gene and the blood pressure and arterial stiffness levels. Two SNPs respectively located in the regulatory and coding regions were studied. Two single nucleotide polymorphisms (SNPs) in the adiponectin gene include a silent T to G substitution in exon 2 (45T>G) and a G to T substitution in intron 2 (276G>T). The results in chapter 6 demonstrate a protective role of adiponectin. The T allele of SNP 45 and T allele of SNP -276 were found to be associated with a lower level of blood pressure and to correlate with arterial stiffness. The T allele of SNP 276 is associated with higher adiponectin concentration. Combining the GG homozygote of SNP 45 and GG homozygote of SNP276, the haplotype of GG shows significantly higher levels of blood pressure and arterial stiffness and adiponectin concentration than the other

haplotypes. The mechanism maybe because even the SNP 276 G>T polymorphism is an intronic SNP, it may relate to the fact that it could still effect the expression of adiponectin and the more the adiponectin expression the stronger its function. With regards to the SNP 45 T>G polymorphism, it is located in exon 2 and results in a synonymous change (G15G), this location is relatively close to the exon–intron boundary which may affect the splicing machinery and then might modify RNA levels which in turn will affect the expression of adiponectin.

The receptor for advanced glycation end products (RAGE) can interact with advanced glycation end products (AGEs) in the vessel wall that may lead to changes in inflammation, smooth muscle cell proliferation and extracellular matrix production.

In this study, I examined the association of two polymorphism sites in the promoter region of the RAGE gene and the blood pressure and the degree of arterial stiffness. The two polymorphisms include the–374T>A polymorphism and the -429T>C polymorphism. Results in chapter 6 show that the AA homozygote of the -374T>A polymorphism and the C allele carriers of –429 >C polymorphism have lower blood pressure and lower arterial stiffness levels. The reason for this could be that the AA homozygote of the –374T>A polymorphism and the C allele carriers of –429T>C polymorphism have significant effects on transcriptional activity which could decrease the expression of RAGE. As the amount of RAGE decreases, the RAGE-AGEs could decrease which could result in less migration and proliferation of smooth muscle cells, less fibrotic organization and less inflammation in the arterial wall. The haplotype of AC combined as the AA homozygote of -374T>A polymorphism and the C allele carriers of –429T >C polymorphism confer a significant protective effect against the higher blood pressure and arterial stiffness.

Future work

Arterial stiffness is a polygenetic disease process, and not unexpectedly a large variety of polymorphisms of different genes are associated with arterial stiffness. In most cases, candidate genes are identified on the basis of their biomedical or physiological

functions that appear to affect arterial stiffness or blood pressure regulation. While arterial stiffness is determined by functional and structural components related to the intrinsic elastic properties of the artery, the central role of blood pressure should not be overlooked. As most drugs to date that reduce stiffness reduce blood pressure the focus should be on blood pressure independent mechanisms. This is particularly the case in systolic hypertension. Persistently elevated blood pressure (BP) accelerates arterial smooth muscle hyperplasia and hypertrophy, and collagen synthesis, thereby further increasing arterial stiffness. Candidate genes include those of the renin-angiotensin system, such as, the AGT gene, the ACE gene, the AT1R gene, the CYP11B2 gene; genes of the sympathetic nervous system include the α2AR gene, the β1AR gene, the β2AR gene; genes of the G protein signaling system (GKR4 gene), and vasoactive peptides including NOS3 gene, CPS1 gene, CYP 2C8 gene and the elastin gene. In addition to showing relationships with arterial stiffness an important and unanswered question is whether such polymorphisms influence the response to antihypertensive therapy.

The investigation of vascular remodeling in ageing and arterial stiffness using human vascular tissue could also allow for precise evaluation of specific changes within the vessel wall. While our department has studied the total collagen accumulation in the human aorta the sample studied was limited. Future studies need to relate in vitro changes in the vessel wall structure to in vivo measures of stiffness. This will help establish whether some of these polymorphisms are associated with structural changes in the arterial wall. Furthermore this field has been relatively neglected in terms of transgenic and knockout animal models.

As the epidemiological data and clinical studies, e.g. CAFÉ, confirm the biological and therapeutic importance of arterial stiffness, [533] a greater number of outcome studies need to incorporate PWV and AIx into these measures. Common polymorphisms, some described in this thesis, may impact on such outcomes. A number of the results in this thesis may point towards areas where the risk and outcome can be predicted with

greater confidence. It is also possible that new therapeutic targets may emerge from a greater understanding of the role of genetic polymorphisms in arterial stiffness.

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